A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation[1[OPEN]]

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The relative ease, speed, and biological scope of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Protein9 (Cas9)-based reagents for genomic manipulations are revolutionizing virtually all areas of molecular biosciences, including functional genomics, genetics, applied biomedical research, and agricultural biotechnology. In plant systems, however, a number of hurdles currently exist that limit this technology from reaching its full potential. For example, significant plant molecular biology expertise and effort is still required to generate functional expression constructs that allow simultaneous editing, and especially transcriptional regulation, of multiple different genomic loci or multiplexing, which is a significant advantage of CRISPR/Cas9 versus other genome-editing systems. To streamline and facilitate rapid and wide-scale use of CRISPR/Cas9-based technologies for plant research, we developed and implemented a comprehensive molecular toolbox for multifaceted CRISPR/Cas9 applications in plants. This toolbox provides researchers with a protocol and reagents to quickly and efficiently assemble functional CRISPR/Cas9 transfer DNA constructs for monocots and dicots using Golden Gate and Gateway cloning methods. It comes with a full suite of capabilities, including multiplexed gene editing and transcriptional activation or repression of plant endogenous genes. We report the functionality and effectiveness of this toolbox in model plants such as tobacco (*Nicotiana benthamiana*), Arabidopsis (*Arabidopsis thaliana*), and rice (*Oryza sativa*), demonstrating its utility for basic and applied plant research.

Customizable sequence-specific nucleases (SSNs) are powerful tools for plant genome editing (Voytas, 2013; Carroll, 2014; Puchta and Fauser, 2014). SSNs can induce sequence-specific DNA double-strand breaks (DSBs), which are subsequently repaired by either nonhomologous end joining (NHEJ) or homologous recombination (Kanaar et al., 1998; Puchta, 2005). By directing DNA DSBs and harnessing DNA repair pathways, mutations or precise modifications can be introduced within a genome at desired loci. Historically, meganucleases or zinc finger nucleases (ZFNs) have been the SSNs of choice, but they are notoriously difficult to engineer and function inconsistently across different genetic loci (Carroll, 2011; Hafez and Hausner, 2012). As a result, such technologies have not been widely adopted within the plant research community (Puchta and Fauser, 2013).

Recent advances in SSN engineering and design have provided more viable options for plant genome editing. For ZFNs, new engineering methods have been developed, namely OPEN and CoDA (Maeder et al., 2008; Sander et al., 2011). We previously used OPEN- or CoDA-engineered ZFNs to successfully target endogenous plant genes, creating mutations, gene replacements, deletions, or inversions in Arabidopsis (*Arabidopsis thaliana*), tobacco (*Nicotiana benthamiana*), and soybean (*Glycine max*; Townsend et al., 2009; Zhang et al., 2010; Curtin et al., 2011; Qi et al., 2013b, 2014). However, ZFNs suffer from target site availability, activity, and occasionally toxicity (Carroll, 2011; Reyon et al., 2011; Sander et al., 2011). As ZFN limitations

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surfaced, a more versatile transcription activator-like effector nuclease (TALEN)-based SSN platform emerged (Christian et al., 2010; Li et al., 2011; Miller et al., 2011). Compared to ZFNs, TALENs possess a broader targeting range and are less difficult to engineer (Bogdanove and Voytas, 2011; Doyle et al., 2012). Moreover, TALENs appear to be more mutagenic than ZFNs (Chen et al., 2013) and are highly specific (Juillerat et al., 2014). To promote TALEN technology, we developed a streamlined TALEN assembly method (Cermak et al., 2011), which was later used for successful genome editing in many plant species (Christian et al., 2013; Shan et al., 2013a; Wendt et al., 2013; Zhang et al., 2013; Lor et al., 2014; Wang et al., 2014). Unfortunately, both ZFN and TALEN technologies require engineering of DNA-binding domains for individual targeting applications, demanding significant effort and expertise in molecular cloning.

Most recently, the *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Protein9 (Cas9) system has burst on the scene, after it was shown to effectively mediate RNA-guided DNA double-strand breaks in bacteria and mammalian cells (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013b). A distinct feature of CRISPR/Cas9 is that DNA cleavage sites are recognized through Watson-Crick base pairing by a guide RNA (gRNA)/Cas9 complex. This feature drastically simplifies DNA targeting. DNA cleavage by CRISPR/Cas9 requires three components: Cas9 protein, CRISPR RNA, and trans-activating CRISPR RNA (Jinek et al., 2012). However, the two RNA components have been reduced to a single gRNA that can be functionally expressed under small nuclear RNA promoters such as U6 or U3 (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013b). This improvement further simplifies the CRISPR/Cas9 system and enhances reagent delivery. After initial reports, many studies quickly announced successful and effective CRISPR/Cas9-mediated genome editing in plants (Feng et al., 2013, 2014; Jiang et al., 2013; Li et al., 2013; Mao et al., 2013; Miao et al., 2013; Nekrasov et al., 2013; Shan et al., 2013b; Xie and Yang, 2013; Fauser et al., 2014; Schiml et al., 2014; Zhou et al., 2014). Due to simplified engineering of target specificity and its dual-component nature, CRISPR/Cas9 allows for simultaneous targeting of multiple genomic loci. This multiplexing feature has been demonstrated in a number of the aforementioned studies where two gRNAs were simultaneously expressed along with Cas9 protein (Li et al., 2013; Schiml et al., 2014; Zhou et al., 2014).

Another advantage of CRISPR/Cas9 is that it can readily be converted to a reagent that creates single-strand breaks (a nickase). Zinc finger nickases and transcription activator-like effector (TALE) nickases have been made by generating a D450A mutation in the *Fok*I nuclease domain, but these nickases appear to have noticeable residual nuclease activity (Ramirez et al., 2012; Wang et al., 2012; Wu et al., 2015). On the contrary, either of the two endonuclease domains of Cas9, HNH, and RuvC can be mutated to form nickases (Gasiunas et al., 2012; Jinek et al., 2012). While CRISPR/Cas9 may pose a potential risk for off-target activity compared with ZFNs or TALENs (Fu et al., 2013), Cas9 nickases could greatly alleviate this problem. For example, mutations have been effectively generated at specific loci using paired Cas9 nickases with minimal off-target mutagenic effects in mice (Shen et al., 2014), human cells (Ran et al., 2013), *Drosophila* spp. (Ren et al., 2014), and Arabidopsis (Schiml et al., 2014). Implementation of paired nickases requires simultaneous expression of at least two gRNAs.

The CRISPR/Cas9 system can also be repurposed for transcriptional regulation. Fusion of a transcriptional activator domain such as VP16 or VP64 (Sadowski et al., 1988; Beerli et al., 1998) to a deactivated Cas9 (dCas9) lacking endonuclease activity can up-regulate endogenous gene expression in human cells (Cheng et al., 2013; Gilbert et al., 2013; Maeder et al., 2013; Mali et al., 2013a; Perez-Pinera et al., 2013). In these studies, single gRNA-mediated gene activation was generally not highly effective. However, when multiple gRNAs were coexpressed, a synergistic or additive transcriptional activation was observed. Repression of endogenous genes has also been demonstrated by expression of dCas9 or dCas9-repressor fusion proteins in human cells (Gilbert et al., 2013; Qi et al., 2013a) and tobacco (Piatek et al., 2014). Complex gene expression programs can be engineered with modified gRNA scaffolds that can simultaneously induce transcriptional activation or repression (Zalatan et al., 2015). This would be a potentially important tool for plant synthetic biology.

With the ability to genetically modify genes and regulate their transcription, the CRISPR/Cas9 system is clearly a powerful tool for basic and applied research in plants. To unleash the full potential of CRISPR/Cas9 for plant-based applications, an easy-to-use multiplexed assembly system is needed. Here, we developed a toolbox with a streamlined protocol for assembly of multifaceted multiplexed CRISPR/Cas9 reagents together into transfer DNA (T-DNA) vectors. The assembly is based on efficient Golden Gate cloning and Gateway recombination methods with no PCR required. By testing the toolbox in dicot and monocot plants, we demonstrated the flexibility of this toolbox for plant genome and transcriptional regulation.

RESULTS

An Efficient Assembly System for Diverse, Multiplexed CRISPR/Cas9 Applications

We sought to design a multifaceted and easy-to-use CRISPR/Cas9 system for the plant research community. As illustrated in Figure 1, potential applications include, but are not limited to (1) simultaneous targeted mutagenesis at multiple loci, (2) targeted chromosomal deletions, (3) synergistic or tunable activation of a gene of interest, (4) synergistic or tunable

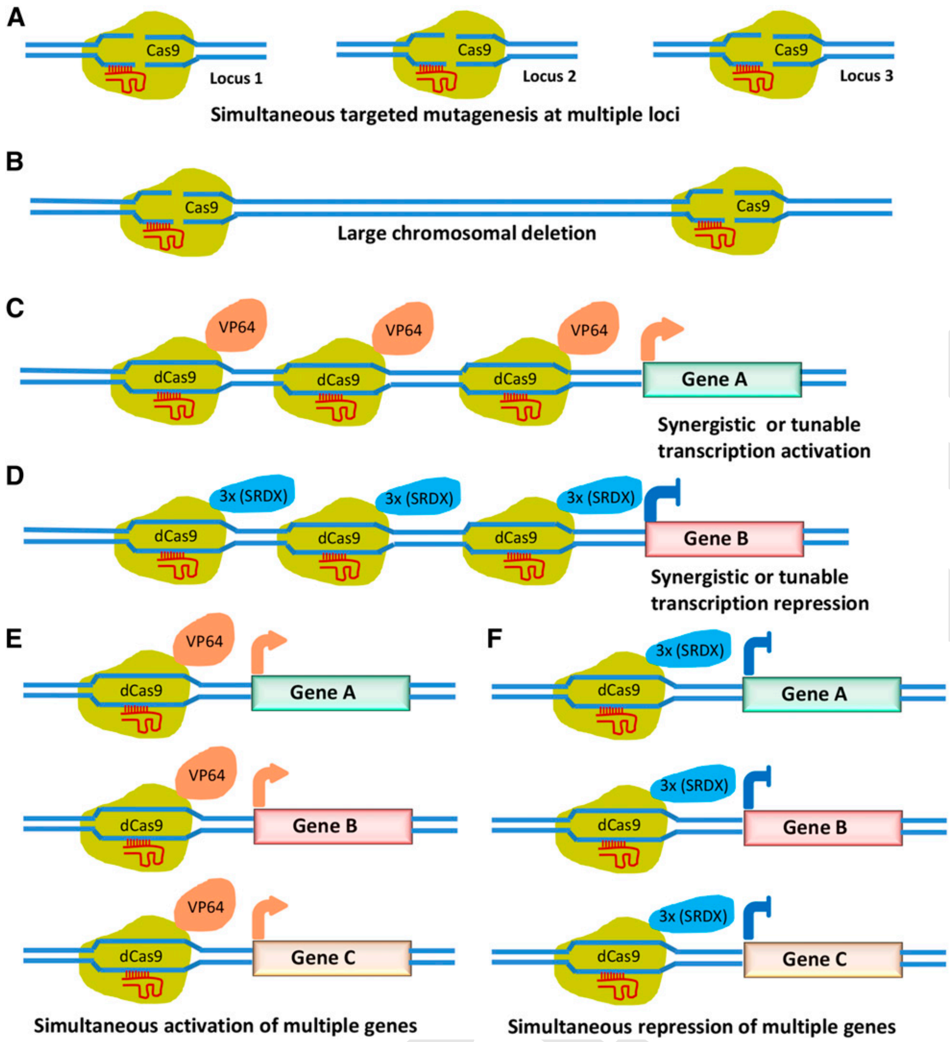

Figure 1. Applications of the multiplex CRISPR/Cas9 toolbox. The cartoon depicts various scenarios when applying different components of the toolbox. A, Simultaneous targeted mutagenesis at multiple loci in the same genes or in different genes. B, Chromosomal deletion. C, Synergistic or tunable transcription activation. D, Synergistic or tunable transcription repression. E, Simultaneous activation of multiple genes. F, Simultaneous repression of multiple genes.

repression of a gene of interest, (5) simultaneous activation of multiple genes, and (6) simultaneous repression of multiple genes. We chose to develop a CRISPR/Cas9 toolbox that allows for all of these applications in both monocot and dicot plants.

T-DNA-based transformation technology is fundamental to modern plant biotechnology, genetics, molecular biology, and physiology. As such, we developed a method for the assembly of Cas9 (wild type, nickase, or dCas9) and gRNA(s) into a T-DNA destination vector of interest. The assembly method is based on both Golden Gate assembly (Engler et al., 2008) and MultiSite Gateway recombination (Fig. 2A). Three modules are required for assembly. The first module is a Cas9 entry vector, which contains promoterless *Cas9* or its derivative genes flanked by attL1 and attR5 recombination sites. The second module is a gRNA entry vector that contains entry gRNA expression cassettes flanked by attL5 and attL2 sites. The third module includes attR1-attR2-containing destination T-DNA vectors that provide promoters of choice for Cas9 expression. Because such T-DNA destination vectors have been previously developed by others (Curtis and Grossniklaus, 2003; Earley et al., 2006), our work focuses on making entry clones for the first two modules (Fig. 2A).

Our Cas9 entry vector module contains eight plasmids (Table I; Supplemental Fig. S1), including three *Cas9* genes that have been previously used in higher plants. They are plant codon-optimized Cas9 (*pcoCas9*, pYPQ150; Li et al., 2013), Arabidopsis codon-optimized Cas9 (*AteCas9*, pYPQ154; Fauser et al., 2014; Schiml et al., 2014), human codon-optimized Cas9 (*hSp*Cas9, pYPQ158; Feng et al., 2013, 2014; Mao et al., 2013; Zhang et al., 2014), and a plant codon-optimized Cas9 harboring enriched guanine-cytosine content within key 5′ coding regions (Cas9p, pYPQ167; Ma et al., 2015). Also included within this module is the D10A nickase version of three out of these four *Cas9* genes (pYPQ151, pYPQ155, and pYPQ159), although testing these nickases is not our focus here. The last vectors in this module are pYPQ152 and pYPQ153, in which the deactivated pcoCas9 is fused with the transcriptional activator VP64 (Beerli et al., 1998) or

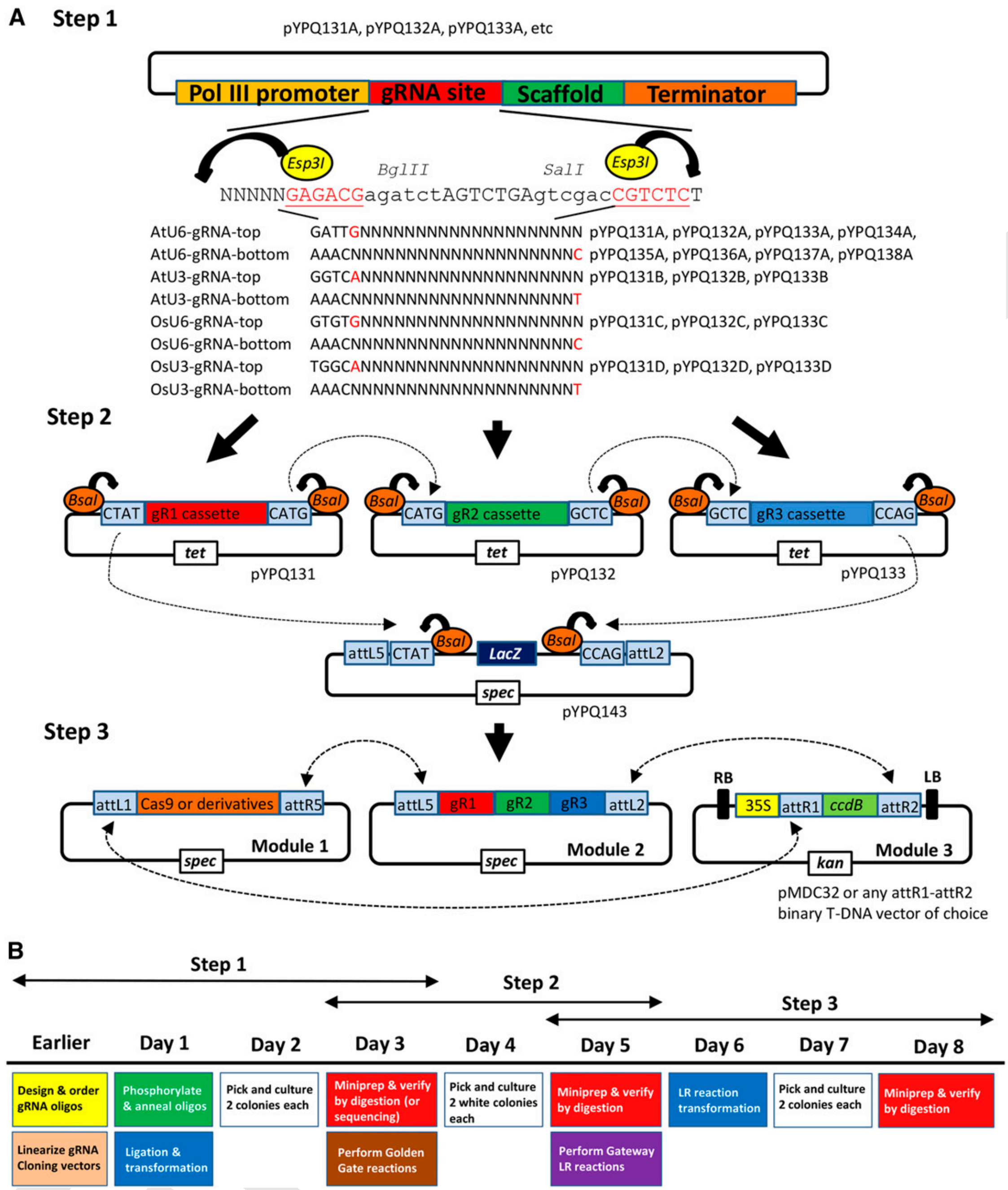

Figure 2. Streamlined assembly of multiplex CRISPR/Cas9 T-DNA vectors. The cartoon illustrates a three-step assembly procedure for construction of different multiplex CRISPR/Cas9 T-DNA vectors for different applications. A, Step 1 involves cloning individual gRNAs into a series of compatible Golden Gate entry vectors that contain different gRNA promoters. Step 2 involves assembly of multiple gRNA expression cassettes into one Golden Gate recipient vector, such as pYPQ143 (for three gRNA expression cassettes). Step 3 puts Cas9 clones, gRNA cassettes, and a promoter for Cas9 expression into a single T-DNA binary vector of choice through Gateway recombination. kan, Kanamycin resistance marker; LacZ, β-galactosidase gene; LB, left border region; Pol III, RNA polymerase III; RB, right border region; spec, spectinomycin resistance marker; tet, tetracycline resistance marker. B, A practical timeline for detailed tasks involved in the assembly from the beginning to the end. LR reaction, Invitrogen's LR Clonase II recombination reaction.

three copies of the transcriptional repressor domain SRDX (Hiratsu et al., 2003), respectively. We examine these vectors for transcriptional activation and repression of multiple endogenous genes to demonstrate unique applications and capabilities of our CRISPR/Cas9 toolbox.

The gRNA entry vector module contains two sets of plasmids that enable a two-step Golden Gate

Table I. *Vectors in the plant multiplex CRISPR/Cas9 toolbox*

Name	Vector Annotation	Addgene No.
pYPQ131A	Golden Gate entry vector; first gRNA under AtU6 promoter	69273
pYPQ132A	Golden Gate entry vector; second gRNA under AtU6 promoter	69274
pYPQ133A	Golden Gate entry vector; third gRNA under AtU6 promoter	69275
pYPQ134A	Golden Gate entry vector; fourth gRNA under AtU6 promoter	69276
pYPQ135A	Golden Gate entry vector; fifth gRNA under AtU6 promoter	69277
pYPQ136A	Golden Gate entry vector; sixth gRNA under AtU6 promoter	69278
pYPQ137A	Golden Gate entry vector; seventh gRNA under AtU6 promoter	69279
pYPQ138A	Golden Gate entry vector; eighth gRNA under AtU6 promoter	69280
pYPQ131B	Golden Gate entry vector; first gRNA under AtU3 promoter	69281
pYPQ132B	Golden Gate entry vector; second gRNA under AtU3 promoter	69282
pYPQ133B	Golden Gate entry vector; third gRNA under AtU3 promoter	69283
pYPQ131C	Golden Gate entry vector; first gRNA under OsU6 promoter	69284
pYPQ132C	Golden Gate entry vector; second gRNA under OsU6 promoter	69285
pYPQ133C	Golden Gate entry vector; third gRNA under OsU6 promoter	69286
pYPQ131D	Golden Gate entry vector; first gRNA under OsU3 promoter	69287
pYPQ132D	Golden Gate entry vector; second gRNA under OsU3 promoter	69288
pYPQ133D	Golden Gate entry vector; third gRNA under OsU3 promoter	69289
pYPQ141A	Gateway entry vector; single gRNA under AtU6 promoter	69290
pYPQ141B	Gateway entry vector; single gRNA under AtU3 promoter	69291
pYPQ141C	Gateway entry vector; single gRNA under OsU6 promoter	69292
pYPQ141D	Gateway entry vector; single gRNA under OsU3 promoter	69293
pYPQ142	Golden Gate recipient and Gateway entry vector; assembly of two gRNAs	69294
pYPQ143	Golden Gate recipient and Gateway entry vector; assembly of three gRNAs	69295
pYPQ144	Golden Gate recipient and Gateway entry vector; assembly of four gRNAs	69296
pYPQ145	Golden Gate recipient and Gateway entry vector; assembly of five gRNAs	69297
pYPQ146	Golden Gate recipient and Gateway entry vector; assembly of six gRNAs	69298
pYPQ147	Golden Gate recipient and Gateway entry vector; assembly of seven gRNAs	69299
pYPQ148	Golden Gate recipient and Gateway entry vector; assembly of eight gRNAs	69300
pYPQ150	Gateway entry vector with pcoCas9 (plant codon optimized)	69301
pYPQ151	Gateway entry vector with pcoCas9D10A	69302
pYPQ152	Gateway entry vector with pco-dCas9-VP64	69303
pYPQ153	Gateway entry vector with pco-dCas9-3X(SRDX)	69304
pYPQ154	Gateway entry vector with AteCas9 (Arabidopsis codon optimized)	69305
pYPQ155	Gateway entry vector with AteCas9D10A	69306
pYPQ158	Gateway entry vector with hSpCas9 (human codon optimized)	69307
pYPQ159	Gateway entry vector with hSpCas9D10A	69308
pYPQ167	Gateway entry vector with Cas9p (plant codon optimized; high guanine-cytosine content at 5′ region)	69309

assembly. This strategy of cloning relies on Type IIS restriction enzymes that cleave outside their respective recognition sequences (Fig. 2A; Engler et al., 2008). The first set of plasmids contain Golden Gate entry clones, each carrying a complete expression cassette for one gRNA under either the Arabidopsis U6 (AtU6) or AtU3 promoter (for dicot plants) or the rice (*Oryza sativa*) U6 (OsU6) or OsU3 promoter (for monocot plants; Fig. 2A; Table I; Fichtner et al., 2014). The first step is to clone individual gRNAs into these Golden Gate entry clones by a simple digestion (with Type IIS enzyme *Esp*3I or *Bsm*BI) and ligation step. This is a single tube reaction and only requires the end user to input an annealed oligonucleotide pair to serve as the gRNA molecule of choice (Fig. 2A, Step 1; for details, see Supplemental Materials and Methods S1). The second set contains Golden Gate recipient vectors in which a *LacZ* gene is readily replaced by gRNA expression cassettes via Golden Gate reactions (Fig. 2A; Table I). This is a similar strategy that we used to assemble TALE repeats (Cermak et al., 2011) using *Bsa*I as our Type IIS restriction endonuclease (Fig. 2A, Step 2). gRNA expression cassettes of this work, however, are much larger (820 bp for AtU6, 720 bp for AtU3, 500 bp for OsU6, and 600 bp for OsU3) than our previously cloned TAL repeat sequences (102 bp). Therefore, we needed to ascertain how many gRNA expression cassettes could be assembled during a single Golden Gate reaction. To this end, we constructed eight Golden Gate entry vectors harboring AtU6-based cassettes (pYPQ131A–pYPQ138A) and eight recipient vectors (pYPQ141–pYPQ148) for testing Golden Gate assembly for up to eight gRNA cassettes (Table I; Supplemental Fig. S2A). We found assembly of up to five gRNA cassettes was readily achieved (Supplemental Fig. S2B), and the efficiency for assembly of two or three gRNA cassettes was generally over 95% based on blue-white screen. However, assembly of six or more gRNA cassettes was far less efficient and often failed (Supplemental Fig. S2B). We reasoned that expression of three gRNAs should suffice for many applications. Thus, for AtU3, OsU6, and OsU3 promoters, we generated Golden Gate entry clones that allow assembly of up to three gRNAs (Fig. 1A; Table I). Subsequently, the following work mainly focuses on testing vectors expressing three gRNAs.

As illustrated in Figure 2, our assembly of a multiplex CRISPR/Cas9 T-DNA vector takes three steps and requires very basic molecular biology techniques; the assembly is readily carried out within 10 d. Importantly, the PCR is not used for cloning or validation throughout the procedure, which reduces the likelihood that mutations will occur within the CRISPR/Cas9 components. Having established the system, we next tested our reagents for genome editing and gene regulation in tobacco, rice (*Oryza sativa*), and Arabidopsis.

Targeted Chromosomal Deletions in Tobacco

We first tested our system for creating targeted chromosomal deletions in tobacco using an *Agrobacterium tumefaciens*-mediated transient expression system in which only a fraction of cells are transformed with our target constructs containing pcoCas9 (Li et al., 2013). Although this system limits our ability to assay genome-editing efficacy, it allows rapid testing of multiple assembled gRNAs. *Flagellin-Sensitive2* (*FLS3*) and *Brassinosteroid Kinase1* (*BAK1*) are important immune receptor or coreceptor genes in Arabidopsis (Boller and Felix, 2009; Boller and He, 2009). We identified their orthologs in tobacco, namely *NbFLS2* and *NbBAK1*. We then designed a total of 14 gRNAs that target the coding sequences of both genes for deletions (Supplemental Fig. S3, A and B). Multiple T-DNA expression vectors were constructed for the expression of either two or three gRNAs under expression of the AtU6 or AtU3 promoters. In the case of targeting *NbFLS2*, expression of two pairs of gRNAs (gR1 and gR2 or gR4 and gR5) both resulted in expected deletions, as detected by PCR (Supplemental Fig. S3C). Deletions created by gR1 and gR2 were further confirmed by DNA sequencing (Supplemental Fig. S3G). Our data also suggested that the expression of these two gRNAs was not impacted by having a third gRNA cassette (NbBAK1-gR3) behind them in the construct (Supplemental Fig. S3C) or between them (Supplemental Fig. S3D). We further verified the deletions by sequencing (Supplemental Fig. S3, H and I). These gRNAs were expressed under an AtU6 promoter. We also tested expression of gRNAs (gR7 and gR9) under an AtU3 promoter (Supplemental Fig. S3A) and observed these to effectively target chromosomal deletions (Supplemental Fig. S3, E and J). Similarly, targeted deletions could be created by expressing multiple gRNAs at a time in *NbBAK1* (Supplemental Fig. S3F), and the deletions of more than 2 kb were confirmed by sequencing (Supplemental Fig. S3, K and L). Thus, our multiplex CRISPR/Cas9 system allows effective expression of at least three gRNAs.

Targeted Deletion and Simultaneous Mutagenesis in Rice

Having shown our multiplex Cas9 system works in dicot species, we next tested the system in a monocot, namely rice. We aimed to evaluate our vector system for genome-editing efficacy as well as to compare the OsU6 and OsU3 promoters. For this purpose, we generated transgenic protoplasts as well as stable transgenic plant lines. We chose three target sites in two genes (*Rice Young Seedling Albino* [*OsYSA*] and *Rice Outermost Cell-specific Gene5* [*OsROC5*]) that have been previously targeted for mutagenesis (Fig. 3A; Feng et al., 2013). We assembled two T-DNA constructs that contain the *pcoCas9* gene and expression cassettes for three gRNAs. In both vectors, each gRNA was arranged in a fixed order (YSA-gR1 at position no. 1, ROC5-gR1 at position no. 2, and YSA-gR2 at position no. 3), with the only difference being their promoters, either OsU6 or OsU3 (Fig. 3B). These two T-DNA constructs were used to transform rice protoplasts or calli for analysis and comparison.

We first tested both T-DNA constructs in a rice protoplast system. Targeted deletions at the *OsYSA* locus were generated using two gRNAs, YSA-gR1 and YSA-gR2. Both OsU6 and OsU3 promoters effectively yielded targeted deletions of approximately 200 bp, which were clearly detected by PCR (Fig. 3D) and further confirmed by DNA sequencing (Fig. 3, F and G). Estimated deletion frequencies in all samples were as high as approximately 10% (Fig. 4D), indicating comparable efficiency for both OsU6 and OsU3. Next, targeted mutagenesis at all three independent target sites was determined. Target site flanking regions were PCR amplified and followed by restriction enzyme digestion using *Sfi*I (for the YSA-gR1 site), *Eco*NI (for the YSA-gR2 site), and *Ahd*I (for the ROC5-gR1 site). Mutagenesis at these target sites inhibits PCR product digestion when using these nucleases, whereas intact, nonmutagenized target sites are cleaved. Based on this analysis, we found high mutagenesis frequencies at all three sites with either OsU6 or OsU3 promoters (Fig. 3E). Although transformation efficiency may not reach 100% in our protoplast system, the mutation frequencies in harvested protoplasts still ranged from 42% to 67%, suggesting that multiple target loci could have been simultaneously modified in any given cell. Furthermore, we validated mutations at *OsROC5* by DNA sequence analysis (Fig. 3, H and I). After obtaining the high-frequency genome-editing data in rice protoplasts, we pursued stable transgenic rice by using the same T-DNA constructs (Fig. 3C). Although the mutagenesis frequency in calli is slightly lower than in protoplasts, mutants for *ysa* (albino seedling phenotype; Fig. 3J) and *roc5* (curly leaf phenotype; Fig. 3K) were readily

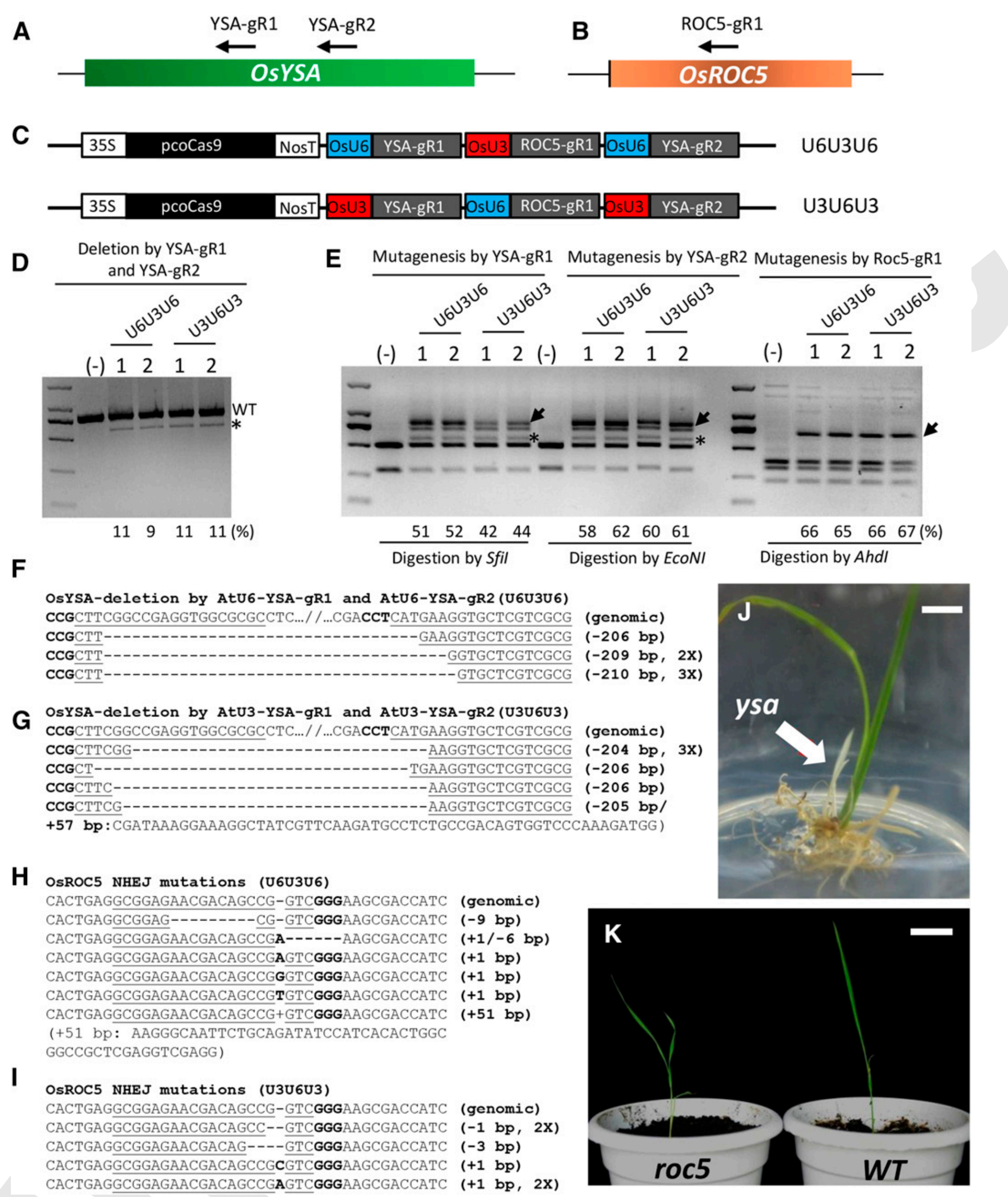

Figure 3. Targeted deletion and simultaneous mutagenesis in rice. A and B, Illustrations of target genes and relative positions of gRNA binding sites in *OsYSA* and *OsROC5*, respectively. C, An illustration of core components of two T-DNA vectors under study. Note the fixed positions for three gRNAs with different promoters. NosT, *A. tumefaciens* nopaline synthase transcriptional terminator sequence. D, PCR-based detection of deletions at *OsYSA*. The larger bands are the wild type (WT), and the small bands indicate deletions (marked by asterisks). The deletion frequencies were calculated and listed underneath the gel picture. E, NHEJ mutations induced by three different gRNAs in *OsYSA* and *OsROC5*. The bands representing mutated DNA are indicated by arrows, while deletion bands at *OsYSA* are indicated by asterisks. The NHEJ mutagenesis frequencies at all three sites were calculated and listed underneath the gel picture. F to I, Sequence confirmation of deletions at *OsYSA* and NHEJ mutations at *OsROC5* with either of the two T-DNA constructs. J, Phenotype of an *ysa* mutant (albino seedling) that was regenerated from transformed rice calli harboring the U3-U6-U3 construct. K, Phenotype of a *roc5* mutant (curly leaves) that was regenerated from transformed rice calli harboring the U3-U6-U3 construct. Bars = 1 cm (J) and 2 cm (K).

regenerated from transformed rice calli. By surveying regenerated T0 plants from both constructs (Fig. 3C), we found the mutation frequencies at individual target sites ranged from 33.3% to 53.3% and mutations are predominantly biallelic (Supplemental Table S1). Taken together, the data suggest that our multiplex CRISPR/Cas9 system is highly active in monocot rice.

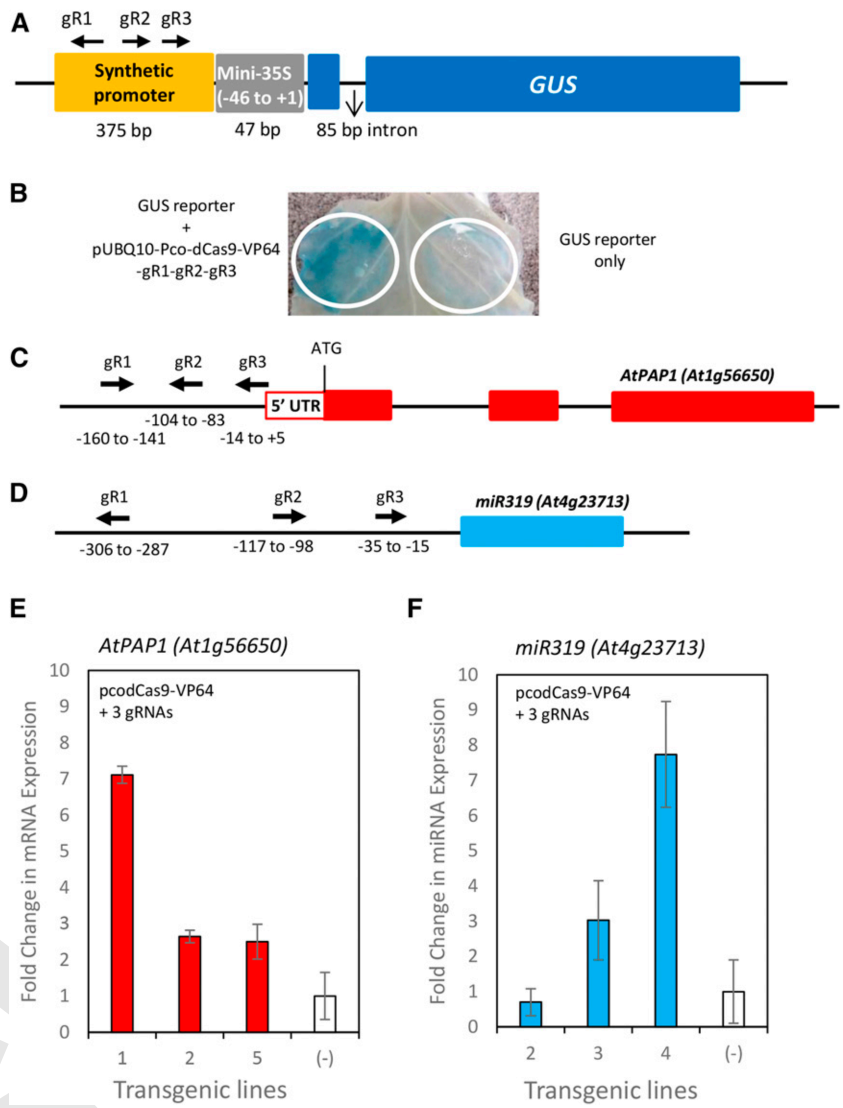

Figure 4. Activation of coding and noncoding genes by dCas9-VP64 and multiplexed gRNAs. A, Diagram showing the intron-containing *GUS* reporter gene is under a synthetic minimal promoter containing all three target sites from the promoter of miR319. B, Activation of the *GUS* reporter gene by pco-dCas9-VP64 coupled with three gRNAs that target the synthetic promoter. C and D, These two diagrams depict gene structure and target sites of gRNAs for *AtPAP1* and *miR319*, respectively. E and F, qRT-PCR analysis of target gene transcript levels. For each experiment, three independent transgenic lines were randomly chosen for analysis. Transgenic plants that express Cas9 nuclease with a similar T-DNA vector backbone were used as negative control (–). Error bars represent standard deviations of technical replicates (n = 3). UTR, Untranslated region.

Targeted Transcriptional Activation of Protein-Coding and Noncoding Genes by dCas9-VP64 with Multiplexed gRNAs

A primary design goal for our vector toolbox was to enable RNA-guided multiplex transcriptional regulation in plants. This capability represents an important and promising application of the CRISPR/Cas9 system, and we know of no current toolkits that facilitate this function in plants. To obtain a Cas9-based transcriptional activator, we fused a VP64 transcriptional activation domain to the C terminus of deactivated pcoCas9 (Fig. 1C; Supplemental Fig. S1; Table I). We reasoned that upon coexpression of this transcriptional activator and multiple gRNAs targeting a gene promoter of interest, we would be able to increase corresponding gene transcription. To test this strategy in vivo, we first generated a reporter T-DNA construct with an intron-containing *GUS* gene fused to a minimal synthetic promoter. The minimal synthetic promoter contains multiple gRNA binding sites and was designed to test the functionality of our pco-dCas9-VP64 transcriptional activator fusion without the confounding variables of endogenous gene promoter regulatory elements (Fig. 4A). We then assembled a T-DNA construct that contains the pco-dCas9-VP64 under the Arabidopsis ubiquitin10 promoter (pUBQ10) and three gRNAs under the AtU6 promoter (Fig. 4A). These two T-DNA constructs were used for *A. tumefaciens*-mediated transient expression in tobacco leaves. We found that coexpression of both constructs resulted in strong activation of GUS expression, whereas the *GUS* reporter construct alone showed little GUS activity above background (Fig. 4B). This data suggests our synthetic dCas9-based transcriptional activator functions in a transient expression system.

We next explored transcriptional activation in Arabidopsis. To determine the efficiency of the system on both protein-coding and non-protein-coding genes, we chose to target *Arabidopsis Production of Anthocyanin Pigment1* (*AtPAP1*; encodes a transcription factor;

Borevitz et al., 2000) and *miR319* (a microRNA; Weigel et al., 2000; Palatnik et al., 2003). To promote efficient transcriptional activation, three gRNAs were designed to target the promoter of each gene, at sites ranging from +5 to –306 relative to the transcriptional start site (Fig. 4, C and D). Two T-DNA constructs were made with each containing pco-dCas9-VP64 under pUBQ10 control and three gRNAs under the AtU6 promoter. Transgenic plants were obtained, and three random lines for each construct were chosen for quantitative real-time (qRT)-PCR analysis. For *AtPAP1*, all three lines displayed transcriptional activation of 2- to 7-fold when compared with the control plant (Fig. 4E). For *miR319*, two of three lines showed gene activation at 3- and 7.5-fold (Fig. 4F). Collectively, our data demonstrate that Cas9-based transcriptional activator systems can activate expression levels of both protein-coding and non-protein-coding genes in plants. Overexpression of *AtPAP1* or *miR319* could potentially lead to changes in leaf color (Borevitz et al., 2000) or leaf morphology (Palatnik et al., 2003). However, we didn't observe such phenotypes in the lines we analyzed (Fig. 4), which suggests that higher fold activation may be required for phenotypic observation. It is possible to further improve our dCas9-based transcriptional system by recruiting more transcriptional activators, as was recently demonstrated in mammalian cells (Konermann et al., 2015; Zalatan et al., 2015).

Targeting a Methylated Promoter to Activate an Imprinted Gene in Arabidopsis

DNA methylation is a prevalent epigenetic modification in plant genomes and commonly methylated cytosine sites include CpG, CpHpG, and CpHpH (Law and Jacobsen, 2010). Recent analysis indicates that about 14% of cytosines are methylated in Arabidopsis (Capuano et al., 2014). Methylated cytosines in principle should restrict DNA targeting with TALEN and ZFN. By contrast, such modifications should not affect the Cas9-based DNA targeting system, as recognition is based on RNA-DNA base pairing. In fact, it was shown that Cas9-mediated DNA cleavage is unaffected by DNA methylation in human cells (Hsu et al., 2013). DNA methylation is a common mechanism used by plants to turn off transposons and imprinted genes (Gehring, 2013; Mirouze and Vitte, 2014). We wanted to test if a Cas9-based transcriptional activator could be used to reverse methylation-based silencing on plant gene promoters. It would be a highly valuable tool for studying and modifying silenced or imprinted genes in plants.

To investigate if a Cas9-based transcriptional activator can reverse methylation-induced gene silencing, we targeted an Arabidopsis imprinted gene, *AtFIS2* (Luo et al., 2000; Jullien et al., 2006). *Arabidopsis Fertilization-Independent Seed2* (*AtFIS2*) is silenced in vegetative tissues, which is likely due to active DNA methylation within its promoter (Fig. 5A; Jullien et al., 2006; Hsieh et al., 2009; Zemach et al., 2013). Three gRNAs were used to target the methylated CpG island within the *AtFIS2* gene promoter. We anticipated that gRNAs can bind to methylated cytosines and recruit dCas9-VP64 to activate the transcription of *AtFIS2* (Fig. 5B). After analyzing transgenic plants expressing dCas9-VP64 and target gRNAs, we found significant activation of *AtFIS2* transcription in Arabidopsis

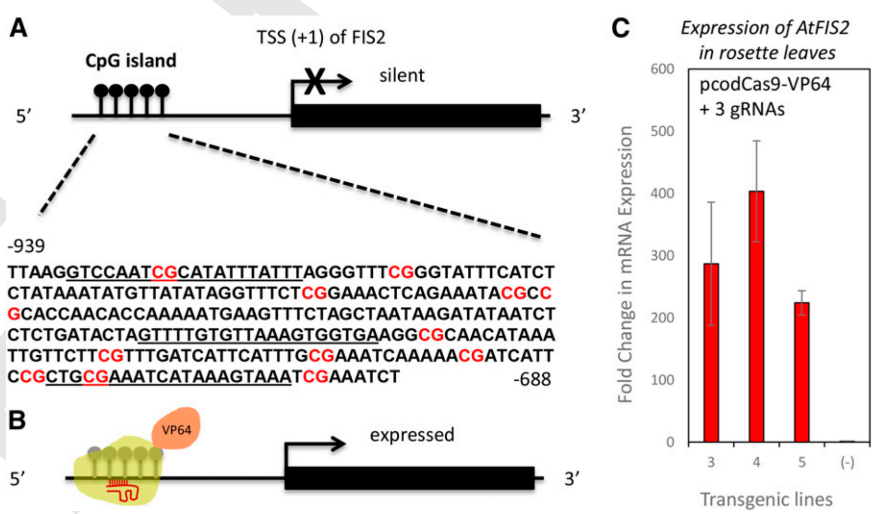

Figure 5. Activation of a silenced gene by RNA-guided targeting of promoter methylation sites. A, Diagram showing *AtFIS2* and its promoter. A CpG DNA methylation island is indicated using balls and sticks. Partial DNA sequence of the methylation island is shown. Within the methylation sequence, three gRNA targeting sites are underlined, and CpG methylation sites are marked in red. *AtFIS2* is silenced in rosette leaves due to DNA methylation within the promoter. TSS, Transcription start site. B, Model diagram showing dCas9-VP64 binding to the DNA methylation island within the promoter and activating *AtFIS2* gene expression. Note only one dCas9-VP64 is depicted. C, Activation of *AtFIS2* in rosette leaves of transgenic plants as examined by qRT-PCR analysis. Three independent transgenic lines were randomly chosen for this analysis. Transgenic plants that express Cas9 nuclease with a similar T-DNA vector backbone were used as negative controls (–). Error bars represent standard deviations of technical replicates ($n = 3$).

rosette leaves (Fig. 5C). All analyzed lines showed significant dCas9-VP64-based gene activation, with transgenic line number 4 showing about a 400-fold increase in mRNA expression. These data show that our Cas9-based transcriptional activator system can recognize methylated DNA and significantly activate silenced genes in plants.

Multiplexed and Simultaneous Gene Repression in Arabidopsis by dCas9-3X(SRDX)

Our toolbox also enables transcriptional repression in plants with a synthetic pco-dCas9-3X(SRDX) transcriptional repressor (Fig. 1D; Supplemental Fig. S1; Table I). We tested this synthetic repressor system on both protein-coding and noncoding genes in Arabidopsis. The *Arabidopsis Cleavage Stimulating Factor64* (*AtCSTF64*) gene encodes an RNA processing factor (Liu et al., 2010), and we designed three gRNAs targeting its promoter (Fig. 6A). For testing repression on non-protein-coding genes, we picked two homologous and functionally redundant microRNA genes, *miR159A* and *miR159B* (Fig. 6, B and C; Palatnik et al., 2003). We designed one gRNA targeting *miR159A* and two gRNAs targeting *mi159B*. All three gRNAs were assembled into a single T-DNA vector harboring pco-dCas9-3X(SRDX) under pUBQ10 control. Using these constructs, we evaluated the system for simultaneous multigene repression.

Both constructs were used to obtain transgenic plants, and gene expression analysis was carried out using qRT-PCR. Among three independent transgenic lines harboring the *AtCSTF64* targeting construct, we detected consistent gene repression, with transcript levels reduced by approximately 60% compared with the control (Fig. 6D). Similarly, all three independent transgenic lines expressing the *miR159A*/*miR159B* targeting construct showed significant reduction of transcript levels for both microRNAs, with transcript levels being reduced to one-half or even lower (Fig. 6E). Interestingly, repression of *miR159A* by only one gRNA worked well when compared with *miR159B*, which was targeted by two gRNAs (Fig. 6E). In one line (no. 2), the repression of *miR159A* was much stronger than that of *miR159B*. We noticed that the gRNA we designed for *miR159A* targets a more proximal DNA region relative to the transcriptional initiation site (Fig. 6B). Target proximity relative to the transcriptional start site likely explains the enhanced transcriptional repression we observed using only a single gRNA targeting miR159A compared with the two gRNAs targeting miR159B, which were more distal to the transcriptional start site. In sum, our data show that multiplex Cas9 systems can be used for transcriptional repression of both coding and noncoding genes in plants. Importantly, we show that multiple genes can be repressed simultaneously by multiplexed gRNAs.

DISCUSSION

A critical aspect of designing multiplex CRISPR/Cas9 experiments is deciding which strategy to use for expressing multiple gRNAs simultaneously. Different strategies have been explored, such as a self-processing ribozyme system (Gao and Zhao, 2014), a transfer RNA-processing system (Xie et al., 2015), or the Csy4 ribonuclease system (Haurwitz et al., 2010; Tsai et al., 2014). However, the most popular approach uses small RNA promoters such as U6 or U3. In our study, we show the use of small RNA promoters works very well

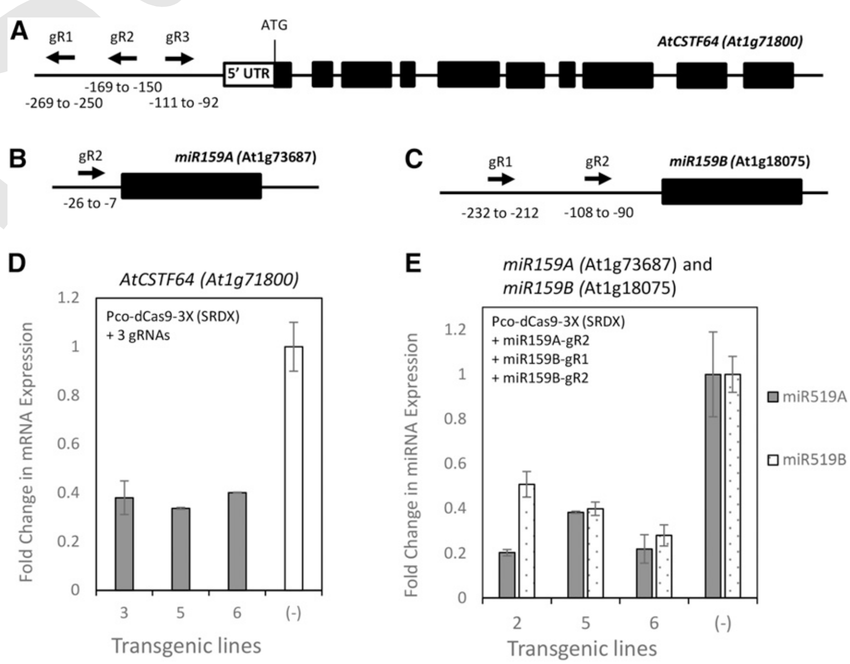

Figure 6. Multiplex and simultaneous gene repression in Arabidopsis by dCas9-3X(SRDX). A to C, These three cartoons depict gene structure and target sites of gRNAs for *AtCSTF64*, *miR159A*, and *miR159B*, respectively. D, Targeted repression of *AtCSTF64* by pco-dCas9-3X(SRDX) with coexpression of three multiplex gRNAs. E, Targeted simultaneous repression of two redundant microRNA genes by pco-dCas9-3X(SRDX) with coexpression of three gRNAs. For each experiment, three independent transgenic lines were randomly chosen for analysis. Transgenic plants expressing only Cas9 nuclease within a similar T-DNA vector backbone were used as negative control (–). Error bars represent standard deviations of technical replicates (*n* = 3). UTR, Untranslated region.

in our system. Although we have focused on applications with simultaneous expression of multiple gRNAs, we also include in the kit a set of four vectors (pYPQ141A–pYPQ141D) for expression of only a single gRNA for applications in monocot and dicot plants (Table I). When using these vectors, one must only follow a simplified procedure, because the Golden Gate-based multivector assembly step (Fig. 2A, Step 2) is no longer required.

An important issue for targeted mutagenesis using SSNs is effectively screening for germinal mutations, which are typically small insertions and deletions. Generating larger deletions by simultaneous expression of two gRNA targeting sequences in close proximity makes such screens more feasible. In fact, simultaneous targeting of genes using multiple gRNAs has been demonstrated repeatedly in plants (Li et al., 2013; Mao et al., 2013; Upadhyay et al., 2013; Brooks et al., 2014; Gao et al., 2015). In our study, we achieved high-frequency deletions of approximately 200 bp in rice (Fig. 3D). An alternative approach to achieving relatively large deletions is by using paired Cas9 nickases (Ran et al., 2013; Ren et al., 2014; Schiml et al., 2014; Shen et al., 2014). As the functional core of our toolkit has been validated by experimentation, we opted not to demonstrate paired nickase activity, but we note that our toolbox includes Cas9 nickase capability, should others want to implement this strategy for their own studies (Table I).

Deletion of very large chromosomal regions containing multiple genes can be a useful tool for researchers, especially for reducing unwanted or confounding genetic redundancy. Previously, we made large chromosomal deletions in Arabidopsis with ZFNs and TALENs (Christian et al., 2013; Qi et al., 2013a). To avoid engineering and simultaneous delivery of multiple ZFNs and TALENs, we used a single pair of ZFNs or TALENs to target conserved sequences among gene clusters. This strategy is, however, somewhat cumbersome and has been rendered obsolete by multiplex CRISPR/Cas9 systems. For example, large chromosomal deletions have recently been demonstrated effectively in rice using CRISPR/Cas9 when two gRNAs were coexpressed (Zhou et al., 2014). Here, we used this approach to generate deletions ranging from approximately 300 bp to over 2 kb in tobacco by coexpression of multiple gRNAs targeting *NbFLS2* and *NbBAK1* (Supplemental Fig. S3). Thus, our multiplex CRISPR/Cas9 system is useful for generating large deletions in plants.

While we were preparing our article, Xing et al. (2014) and Ma et al. (2015) reported a CRISPR/Cas9 toolkit for targeted mutagenesis in plants. They showed simultaneous knockout of multiple genes in Arabidopsis and rice, which, again, demonstrates the versatility and power of a multiplex CRISPR/Cas9 genome-editing system. Compared with their toolkits, ours differs significantly in many aspects. First, our comprehensive toolbox is designed for both genome editing and transcriptional regulation. To achieve this, we included four well-characterized *Cas9* genes, including the one described by Ma et al. (2015), all of which show high activity in plants (Feng et al., 2013; Li et al., 2013; Fauser et al., 2014). Aside from codon optimization, these Cas9 homologs differ from each in epitope tag fusions (Supplemental Fig. S1). Homologous Cas9 clones differing in codon optimization and epitope tag fusions are thought to demonstrate variable activity in plant cells and have different cytotoxic effects in bacteria (Johnson et al., 2015). Therefore, having multiple *Cas9* homologs to choose from should prove advantageous and will provide enhanced kit flexibility. Second, we made Cas9D10A nickase versions of three *Cas9* clones, which allow for paired nicking and homologous recombination while minimizing potential off-target mutagenic effects. Third, we have included a synthetic transcriptional activator pco-dCas9-VP64 and a synthetic transcriptional repressor pco-dCas9-3X(SRDX). Fourth, our assembly strategy positions Cas9 or its variants, gRNA expression cassettes, and Cas9 promoters into three separate Gateway-compatible cloning modules. This provides enhanced flexibility to the system, as each module can be independently changed or upgraded while still maintaining compatibility with other modules. Finally, our assembly protocol is completely PCR independent, which ensures high fidelity and efficiency.

In addition to reporting the toolbox and demonstrating its use in plant genome editing, an important focus in our study is to demonstrate RNA-guided transcriptional activation and repression in stable transgenic plants. Very recently, dCas9-based activators and a repressor were tested using transient expression in tobacco, where different components were codelivered by multiple T-DNAs (Piatek et al., 2014). Our work differs significantly from this work because we have developed an efficient way to construct all components (promoters, dCas9-based synthetic transcriptional regulators, and multiple gRNA expression cassettes) into a single T-DNA for easy delivery (Fig. 2). By focusing on stable transgenic plants, we showed that both protein-coding and noncoding genes can be effectively activated (Fig. 4). Further, we successfully targeted a methylated promoter to activate an imprinted gene (Fig. 5). Our data thus suggests that CRISPR/Cas9-mediated genome editing and transcriptional regulation is unaffected by DNA methylation in plants. Our synthetic repressor also worked at repressing protein-coding or noncoding genes (Fig. 6). Importantly, we could simultaneously repress two microRNAs (*miR159A* and *miR159B*), even though one microRNA was targeted by a single gRNA. The scope of gene activation and repression in our CRISPR/Cas9 system is similar to those reported in mammalian systems (Gilbert et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013a). Taken together, we have shown that all types of plant endogenous genes are amenable to transcriptional perturbation using our CRISPR/Cas9 toolbox and the straightforward module assembly method.

CONCLUSION

In this study, we developed and tested a multifaceted multiplex CRISPR/Cas9 toolbox that consists of 37 Golden Gate- and Gateway-compatible vectors (Table I). Based on these vectors, we assembled 29 gRNAs into 14 T-DNA constructs for genome editing and transcriptional regulation of endogenous genes in tobacco, rice, and Arabidopsis. To demonstrate multiplexing, we expressed three independent gRNAs simultaneously, an approach that has not yet been explored much in plants. For, to our knowledge, the first time, we successfully activated and repressed transcription of both protein-coding and noncoding genes and imprinted genes in Arabidopsis, demonstrating another very important use of CRISPR/Cas9 in plant research. To this end, we have successfully demonstrated most applications offered by this versatile toolbox (Fig. 1). We believe this toolbox will be very useful in plant basic research and plant synthetic biology. Its modularity and flexibility will allow for easy and continuous improvement in the future.

MATERIALS AND METHODS

Plant Material and Growth

The wild-type Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia), tobacco (*Nicotiana benthamiana*), and *japonica* rice (*Oryza sativa*) 'Nipponbare' were used in this study. Plants were grown in BM2 soil (Berger) in a growth room or growth chambers at 25°C under a long-day setting (16 h under the light and 8 h in the dark).

Transient Expression in Tobacco

Agrobacterium tumefaciens strain GV3101/pVP90 carrying different expression T-DNA vectors were cultured at 28°C in Luria-Bertani liquid medium supplemented with 50 μg mL^{-1} Gentamycin and 50 μg mL^{-1} Kanamycin. *A. tumefaciens* cells were collected by spin at 8,000*g* for 2 min. The pelleted cells were suspended with MES buffer (10 mM MES-KOH, 10 mM $MgCl_2$, and 150 mM acetosyringone, pH 5.6) to a final optical density at 600 nm of 0.2. The bacterial suspension was further primed by shaking at 150 rpm at 28°C for 2 h, and then it was used for infiltration of 3- to 4-week-old leaves of tobacco with a 1-mL needleless syringe. The leaf tissue was collected for DNA extraction or GUS staining 4 d after infiltration.

Arabidopsis Transformation and Screen

Arabidopsis ecotype Columbia plants were transformed with T-DNA vectors carried by *A. tumefaciens* GV3101/pVP90 by following the floral dip protocol (Clough and Bent, 1998). To screen transgenic plants, T1 seeds were surface sterilized with diluted bleach and kept in 0.1% (w/v) agar in a dark cold room for 6 d. Then, the seeds were plated onto Murashige and Skoog (MS) medium, which contains 0.8% (w/v) agar, one-half-strength MS with macro- and micronutrients and vitamins (Caisson Labs), 1% (w/v) Suc, 20 μg mL^{-1} Hygromycin B, and 100 μg mL^{-1} Timentin (Gold Biotechnology). One-week-old transgenic plants were transferred to clean MS plates for growth for another week before being transplanted into soil.

Rice Protoplast Isolation and Transformation

Rice seeds of *japonica* 'Nipponbare' were sterilized and germinated in one-half-strength MS solid medium in a plastic container and grown at 28°C in the dark in a growth chamber for 8 to 10 d. Healthy rice stem and sheath tissues from 30 to 40 rice seedlings were cut into approximately 0.5- to 1-mm strips. The strips were transferred into a 90-mm plate and digested in 8 to 10 mL of enzyme solution (1.5% [w/v] Cellulase R10, 0.75% [w/v] Macerozyme R10, 0.6 M mannitol, 10 mM MES, pH 5.7, 10 mM $CaCl_2$, and 0.1% [w/v] bovine serum albumin), followed by vacuum infiltration for 30 min in the dark using a vacuum pump at –15 to –20 (in Hg). After a 5- to 6-h digestion with gentle shaking (60–80 rpm), protoplasts were filtered through 40-μm nylon meshes into another 90-mm plate and further transferred into a 50-mL sterile tube. The pellets were collected by centrifugation at 100*g* for 5 min and suspended with 10 mL of W5 buffer for washing. Then, the pellets were collected again by centrifugation at 100*g* for 2 min and resuspended in Mg-mannitol solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.7). Approximately 5×10^6 cells were used per transformed experiment. The protoplast transformation was carried out in polyethylene glycol (PEG) solution (40% [w/v] PEG 4000, 0.2 M mannitol, and 0.1 M $CaCl_2$). The transformation system (30 μg of plasmid DNA mixed with 200 μL of protoplasts and 230 μL of PEG solution) was gently mixed. After 20-min incubation at room temperature in the dark, 900 mL of W5 buffer was added to stop transformation. The cells were centrifuged and resuspended in 1 mL of washing/incubating solution (0.5 M mannitol, 20 mM KCl, and 4 mM MES, pH 5.7) and cultured in six-well plates in the dark at room temperature for 48 h before harvesting.

A. tumefaciens-Mediated Transformation of Rice

Our method is modified from previously published protocols (Nishimura et al., 2006; Hiei and Komari, 2008). The expression vector (Fig. 3C) was transformed into *A. tumefaciens* strain EHA105. Mature seeds of *japonica* rice 'Nipponbare' were used for stable transformation. First, dehusked seeds were sterilized with 70% (v/v) ethanol for 1 min and washed five times with the sterile water. Then, these seeds were transferred into 2.5% (v/v) sodium hypochlorite, which contained a drop of Tween 20, to be further sterilized for 15 min. After being washed five times, these seeds were sterilized in 2.5% (v/v) sodium hypochlorite again without Tween 20 for 15 min. The seeds were then rinsed five times with sterile water. Finally, these seeds were dried on a sterilized filter paper and cultured on solid medium at 28°C under the dark in the growth chamber for 2 to 3 weeks. Actively growing calli were collected for subculture for 1 to 2 weeks at 28°C under the dark. Cultured *A. tumefaciens* cells were collected and resuspended in liquid medium containing 100 μM acetosyringone (optical density at 600 nm = 0.06–0.1). Rice calli were immersed in the *A. tumefaciens* suspension for 30 min. They were then dried on a sterilized filter paper and cocultured on solid medium at 25°C under the dark in the growth chamber for 3 d. The infected calli were moved into a sterile plastic bottle and washed five times with sterile water to removed excessive *A. tumefaciens*. After being dried on sterilized filter paper, the calli were transferred onto screening medium at 28°C under the dark in the growth chamber for 5 weeks. During the screening stage, infected calli were transferred onto fresh screening medium every 2 weeks. After the screening stage, actively growing calli were moved onto regenerative medium for regeneration at 28°C with a 16-h-light/8-h-dark cycle. After 3 to 4 weeks for regeneration, transgenic seedlings were transferred into sterile plastic bottles containing fresh solid medium for growing for 2 to 3 weeks before being transferred into soil.

Assays for CRISPR/Cas9 Activity in Vivo

For CRISPR/Cas9-mediated deletions in tobacco, an enrichment PCR procedure was applied. Briefly, plant genomic DNA was extracted from transiently transformed tobacco leaves with the *Hexadecyltrimethylammonium bromide* (cetyl-trimethyl-ammonium bromide) DNA extraction method (Stewart and Via, 1993). The extracted DNA was digested with *Eco*RI (for deletions by gR1 and gR2 at *NbFLS2*), *Eco*RI and *Mfe*I (for deletions by gR1 and gR3, by gR4 and gR6, or by gR7 and gR9 at *NbFLS2*), and *Bam*HI and *Hin*dIII (for deletions by gR1 and gR3 or by gR4 and gR6 at *NbBAK1*). Then, PCR reactions were conducted using digested genomic DNA as templates with Taq polymerase (New England Biolabs) and corresponding primers listed in Supplemental Materials and Methods S1. The PCR products were resolved in 1.5% (w/v) agarose gel. The bands corresponding to deletions were excised and cloned into pcr2.1 vector with the TA Cloning Kit (Life Technologies). Positive clones were picked for sequencing analysis.

For CRISPR/Cas9-mediated deletions and NHEJ mutations in rice, protoplasts transformed with the T-DNA vectors were collected for DNA extraction with the cetyl-trimethyl-ammonium bromide method. The genomic DNA was used for PCR with KOD FX DNA polymerase (TOYOBO) using primers YSA-F/R for the *OsYSA* gene and primers ROC5-F/R for the *OsROC5* gene. *OsYSA* PCR amplicons were digested with *Sfi*I or *Eco*NI, and *OsROC5* PCR amplicons were digested with *Ahd*I. PCR and digested products were resolved in 2% (w/v) agarose gel. To calculate deletion and NHEJ frequencies, signal intensity of each band from nonsaturated gel pictures were measured by ImageJ (http://imagej.nih.gov/ij/).

The corresponding deletion or NHEJ mutation bands were also cut and purified with AxyPrep DNA Gel Extraction Kit (Fisher Scientific). Recovered DNA fragments were cloned into cloning vector pMD19-T with the pMD19-T Vector Kit (TaKaRa). Positive clones were picked for sequencing analysis.

GUS Staining

The GUS staining was done by following the procedure described previously (Baltes et al., 2014).

RNA Extraction and cDNA Synthesis

Fifty to one hundred milligrams of Arabidopsis rosette leaf tissue was collected from either 2- or 3-week-old hygromycin-resistant seedlings cultured on MS plates or hygromycin-resistant plants growing on soil. Total RNA was extracted using TRIzol Reagent (Invitrogen), and homogenization was carried out using a hand drill and micropestle on dry ice. RNA was isolated and precipitated according to the TRIzol Reagent product recommendations, with the exception that 1.2 M NaCl and 0.8 M trisodium citrate (in diethylpyrocarbonate water) and one-half volume of isopropanol were used to precipitate RNA. Directly following RNA extraction, contaminating genomic DNA was degraded using DNase I (New England BioLabs) as recommended by the manufacturer. Complementary DNA (cDNA) was synthesized from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamers.

Quantitative PCR and Data Analysis

Quantitative PCR was carried out using Applied Biosystems SYBR Green Master Mix (Invitrogen) and cDNA templates (described above) on an Applied Biosystems 7300 Real-Time PCR System. Disassociation curves of SYBR green wells were cross checked to eliminate nonspecific, false-positive amplification. Data are normalized to Actin2 mRNA expression (internal control), and fold changes are displayed relative to control plant lines using the comparative threshold cycle method. Error bars represent standard deviations of technical replicates ($n = 3$). Refer to Supplemental Materials and Methods S1 for gene-specific qRT-PCR primers.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Architecture of Cas9 proteins in the toolbox.

Supplemental Figure S2. Golden Gate assembly of up to 8 gRNA expression cassettes.

Supplemental Figure S3. Targeted chromosomal deletions at NbFLS2 and NbBAK1 in *N. benthamiana*.

Supplemental Table S1. Genotyping summary of T0 plants.

Supplemental Materials and Methods S1.

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