

A CRISPR/dCas9 toolkit for functional analysis of maize genes

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Abstract

Background: The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system has become a powerful tool for functional genomics in plants. The RNA-guided nuclease can be used to not only generate precise genomic mutations, but also to manipulate gene expression when present as a deactivated protein (dCas9).

Results: In this study, we describe a vector toolkit for analyzing dCas9-mediated activation (CRISPRa) or inactivation (CRISPRi) of gene expression in maize protoplasts. An improved maize protoplast isolation and transfection method is presented, as well as a description of dCas9

vectors to enhance or repress maize gene expression. Additionally, we describe the utility of Foxtail Mosaic Virus (FoMV), a positive-sense RNA monocot virus, as a vector for delivering guide RNAs (gRNAs) to maize protoplasts in addition to whole plants.

Conclusions: We anticipate that this maize protoplast toolkit will streamline the analysis of gRNA candidates and facilitate genetic studies of important trait genes in this transformation-recalcitrant plant.

Keywords

Maize, protoplasts, CRISPR/Cas9, transcription activation, transcription suppression, Foxtail Mosaic Virus (FoMV)

Background

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system is the method of choice for plant genome editing projects, as it combines simplicity with efficiency and precision [1,2]. It relies on the nuclease activity of the Cas9 protein, a component of adaptive bacterial defense against bacteriophages or other nucleic acid threats [3]. The specificity of this system is conferred by the interaction of the Cas9 nuclease with an RNA composed of a scaffold RNA joined to a guide RNA (gRNA), which directs the protein to a specific target DNA sequence for cleavage [4]. The site of mutagenesis can therefore be programmed simply by adjusting the sequence of the gRNA, provided a protospacer adjacent motif (PAM) sequence is present in the target DNA [4,5]. The CRISPR/Cas9 system is also useful for other genetic manipulations beyond genomic sequence editing, such as regulation of gene expression and epigenetic modification [2,4]. CRISPR-mediated transcription inhibition

(CRISPRi) or activation (CRISPRa) is achieved by utilizing a nuclease-deactivated form of Cas9 (dCas9), a non-cutting variant which maintains its DNA-binding specificity [6,7]. While the interaction of dCas9 itself with a specific promoter can reduce gene expression levels, fusion with a repression domain can enhance this effect [6]. One such repressor used in plant studies is the 12 amino acid SRDX domain, also known as an ERF-associated amphiphilic repression (EAR)-motif found in some transcriptional repressors [8,9]. Conversely, dCas9 fused with an activation domain can be used to significantly elevate transcription from targeted native promoters [7,10]. Recently, the dCas9-VP64 and dCas9-TV systems, which are based on modular repeats of the herpes simplex activation domain, were described as strong dCas9 activators of plant gene expression [10,11].

The model species *Arabidopsis thaliana* can be easily transformed via the floral dip method, which can generate stable transgenics within 2-3 months [12]. In contrast, maize transformation methods are more laborious and time consuming, taking 6-9 months to generate stable mutants [13–16]. Consequently, alternative techniques to manipulate maize gene expression would be highly beneficial.

Protoplasts, live plant cells from which the cell wall has been removed, have proven to be a tractable system for a wide range of studies, including biochemistry, cell dedifferentiation, as well as genetic manipulation [17–20]. Methods for transient transgene expression in protoplasts are relatively quick and straightforward, making them a useful system when stable transgenic plants are unavailable or a high-throughput system is needed [20,21]. It has been reported that electroporation and polyethylene glycol (PEG)-mediated transfection can be used to introduce plasmid DNA into maize protoplasts [22–24]. Therefore, in this study we describe our

improvement of these published methods to generate high quality maize protoplasts suitable for gene expression analysis.

While online algorithms are available to aid in gRNA design, the list of top candidates provided nonetheless needs to be empirically verified for effective CRISPR/(d)Cas9 activity [25]. The CRISPRi and CRISPRa toolkits we describe offer a simple and time-efficient approach to facilitate screening gRNA candidates in maize protoplasts prior to the generation of stable transgenic lines, for example, which not only streamlines the gRNA selection process but also reduces the cost and time burdens associated with repeated maize transgenic production. Lastly, we describe our use of the Foxtail Mosaic Virus (FoMV) as a viral vector for delivering gRNAs to protoplasts transfected with dCas9 variant constructs. FoMV, a (+)-sense single-stranded RNA potexvirus, has been previously described as a useful vector for virus-induced gene silencing (VIGS) [26,27] and virus-mediated gene over-expression (VOX) [28–31] in addition to its gRNA delivery capabilities [28]. Its ease of infectivity through mechanical inoculation make it an ideal addition to the CRISPRa and CRISPRi toolkits, as it would enable future maize gene expression studies in dCas9-expressing plants as they become available.

Results

Improvement of protoplast transfection conditions

To develop a robust CRISPRa and CRISPRi system for maize protoplasts, we first analyzed the following protoplast transfection conditions. Protoplasts were isolated from two-week-old etiolated maize seedlings as described in Burdo et al. (2014). Next, we compared electroporation with PEG-mediated transfection, where 40% PEG was prepared in either 0.2 M or 0.4 M mannitol as described for *Arabidopsis* and rice protoplast transfection, respectively

[20,32]. After transfection with a construct that carried a GFP-expression cassette (pCXUN-HA-GFP, Additional File 2), we conducted western blot analysis. As shown in Figure 1A, maize protoplast transfection with 40% PEG in 0.4 M mannitol resulted in better expression of GFP compared to those with 0.2 M mannitol. As would be expected, an increase in incubation time resulted in higher GFP expression regardless of the concentration of mannitol.

Maize protoplasts were transfected with constructs carrying GFP driven by two promoters commonly used for high-level expression in maize: the cauliflower mosaic virus (CMV) 35S promoter or the maize ubiquitin promoter. According to microscopic observation (Figure 1B) and western blot analysis (Figure 1C), expression of GFP was stronger when driven by the maize ubiquitin promoter than that by the CMV 35S promoter. Further tests with protoplasts isolated from Early Sunglow and Silver Queen hybrids revealed their remarkable longevity after transfection with pCXUN-HA-GFP. As shown in Figure 1D, GFP expression was detected in both hybrids four days post transfection (dpt). Based on these observations, we adopted 0.4 M mannitol for protoplast transfection and utilized the ubiquitin promoter in subsequent CRISPRa and CRISPRi vector construction.

Expression of dCas9 variants *in planta*

To develop the maize CRISPRi and CRISPRa toolkit, we assembled the following series of constructs using a pTF101.1rev binary vector backbone: pDA2 (conferring dCas9 expression), pDA3 (dCas9-VP64, conferring dCas9-mediated expression activation), pDA4 (dCas9-SRDX, conferring dCas9-mediated expression repression), and pDA5 (dCas9-TV, conferring stronger dCas9-mediated expression activation). Each dCas9 derivative is N-terminally Flag-tagged and driven by the maize ubiquitin promoter (Additional File 2). Also included is a dual 35S-driven BAR cassette to confer glufosinate resistance. To confirm the expression of dCas9 variants *in*

planta within whole plants, we infiltrated agrobacteria strains carrying different pDA vectors into *Nicotiana benthamiana* leaves. Three days after infiltration, total protein was isolated and expression of dCas9 variants was detected at the expected size using an anti-Flag antibody (Figure 2). To confirm expression in maize plants by protoplast transient expression, we cloned the HindIII and SbfI fragment containing the dCas9 expression cassette from each pDA construct into the HindIII and PstI sites of pXUN, thereby decreasing the size of the construct by about 7 kb to increase the protoplast transfection rate. Total protein was isolated 16 h after maize protoplast transfection, and expression of the dCas9 variants were detected at the expected size by western blot with the anti-Flag antibody (Figure 2). We observed that pDA2 showed higher expression compared to the pDA3 and pDA4 dCas9 variants in both *N. benthamiana* plants and maize protoplasts. While the reason is not clear, we speculate that the addition of activation and repression domains to the dCas9 construct leads to this expression reduction. Although a direct comparison to dCas9 was not included, Li, et al. (2017) observed a similar trend with western blot analysis of dCas9-activation constructs in *Arabidopsis* protoplasts, where increases in the overall size of the activation domains correlated with reduced expression.

Testing the transcriptional changes of *ChlH* and *TrxH* using CRISPR/dCas9 constructs in protoplasts

To determine the effectiveness of the pDA vectors for either CRISPRa or CRISPRi, we designed gRNAs targeting the promoter of the maize Subunit H of magnesium chelatase gene (*ChlH*), a marker gene whose mutation in whole plants leads to yellowing seedling phenotype due to defects in chloroplast development [33]. Four gRNAs targeting different regions of the *ChlH* promoter (Additional File 1) were designed and co-expressed with pDA2 (dCas9) or pDA4

(dCas9-SRDX) in protoplasts. qRT-PCR analysis showed that gRNA1, gRNA2, and gRNA3 co-transfection with pDA2 resulted in an approximate 25% reduction in *ChlH* expression, while gRNA4 had no effect (Figure 3A). Co-expression of pDA4 with gRNA2 or gRNA4 resulted in nearly a 75% or 50% reduction in *ChlH* expression, respectively, compared to the negative control (Figure 3B). These data show that while dCas9 has some transcription repression activity, this can be enhanced with the addition of the SRDX suppressor. For CRISPRa, we analyzed *Thioredoxin H* (*TrxH*), a gene whose increased transcription in whole plants confers resistance to sugarcane mosaic virus (SCMV) (Liu et al., 2017). As with *ChlH*, we tested four gRNAs targeting the *TrxH* promoter (Additional File 1). When co-expressed with pDA3 (dCas9-VP64), gRNA2 or gRNA4 resulted in about a two-fold increase in *TrxH* transcripts (Figure 3C). These two analyses confirmed that our pDA3 and pDA4 vectors can be used for CRISPRa and CRISPRi approaches, respectively, and gRNAs for target genes can be tested in maize protoplasts for further experiments.

Testing the transcriptional changes of *PDS1* using three CRISPR/dCas9 constructs in maize protoplasts

To demonstrate CRISPRi with multiplexed constructs, gRNAs targeting the promoter of the maize *phytoene desaturase1* (*PDS1*) gene were designed. *PDS1* is a commonly used marker gene for virus-induced gene silencing (VIGS) as well as CRISPR/Cas9 genome editing analysis across a range of plant species, where silencing or mutation of the gene culminates in an easily observed photobleaching phenotype [26,34–37]. To test *PDS1* expression, we designed four gRNAs targeting the *PDS1* promoter (Additional File 1). Next, to multiplex two gRNAs in the same construct, we added three tRNAs to flank each side of the gRNA-scaffold sequences.

Multiple combinations of these four *PDS1* gRNAs were then co-transfected with either pDA2, pDA3, or pDA4 into maize protoplasts. We determined that a combination of gRNA2 and gRNA3 co-transfected with pDA4 showed a decrease of about 60% in *PDS1* transcription compared to the negative control, as measured by qRT-PCR (Figure 3D). We also tested whether the *PDS1* gRNAs could be used for transcription activation with pDA3 (dCas9-VP64) in maize protoplasts. With pDA3, a combination with gRNA2 and gRNA3 showed about 2.5 times of *PDS1* transcription activation (Figure 3E).

The dual-luciferase assay is rapid, sensitive, and reliable for analysis of transcriptional repressors, gene expression or functional interaction of signaling molecules [38]. The 35S-driven *Renilla* luciferase serves as an internal and transfection control, while Firefly luciferase is driven by a promoter of interest [39]. We tested whether this assay could be utilized to assess the repression or activation of *PDS1* using our CRISPR/dCas9 constructs. The vectors for this system were generated by first cloning a 1.4 kb promoter fragment of *PDS1* into the BamHI site of pGreenII-800-RNA1-Luc, resulting in the construct pGreenII-800-RNA1-*PDS1*:Luc (Additional File 3). Next, *PDS1* gRNAs were cloned into the BtgZI and BsaI sites with the different multiplex combinations as described above. After the co-transfection of *PDS1* gRNAs in pGreenII-800-RNA1-*PDS1*:Luc with CRISPR/dCas9 constructs, the protoplasts were lysed for Firefly and *Renilla* luciferase activity according to manufacturer's instructions (Promega). As shown in the preliminary results of Additional File 3, the relative expression patterns of *PDS1* measured by the dual-luciferase assay were similar to that detected by qRT-PCR (Figure 3D-E). Overall, a combination of gRNA2 and gRNA3 showed the best activation and suppression of *PDS1* with pDA3 and pDA4, respectively. As an additional control for the protoplast transfection rate, we modified the pGreenII-800-RNAi-Luc vector by inserting a GFP-expression

cassette. Prior to gRNA analysis via dual-luciferase or qRT-PCR, the transfection rate with GreenII-800-RNAI-GFP-Luc can be determined by western blot analysis (Additional File 3).

Transcriptional change of *PDS1* in protoplasts by gRNAs delivered via the FoMV viral vector.

We next tested the compatibility of virus-mediated gRNA delivery to maize protoplasts transfected with the CRISPRa/CRISPRi pDA vectors. Foxtail mosaic virus (FoMV) is a positive-sense RNA potexvirus containing three subgenomic (sg) RNAs, which enables expression of multiple inserted foreign RNAs without disturbing viral replication [26,40]. Mei, et al. utilized these multiple sgRNAs to produce infectious binary FoMV vectors containing two multiple cloning sites (MCS) for VIGS, protein expression, or gRNA delivery for plant genome editing [26,28]. As shown in Figure 4A, the MCSII site contains the duplicated coat protein promoter (DC) upstream of the cloning site and is suitable for protein expression and gRNA delivery, whereas the MCSI site has been successfully used for VIGS [26,28]. In our study, we tested the ability of these FoMV vectors to deliver single as well as multiple gRNAs for CRISPRa and CRISPRi in protoplasts (Figure 4B). In addition to inserting single gRNAs in MCSI or MCSII, we also multiplexed two gRNAs using tRNA processing. We also tested a FoMV construct in which we added the DC promoter upstream of MCSI to determine if that would enhance gRNA expression from that site. As shown in Figure 4C for *PDS1* suppression, maize protoplasts co-transfected with pDA4 and FoMV with a single gRNA2 cloned in MCSI or MCSII showed about 50% suppression of *PDS1* expression compared to the empty vector control. Interestingly, transfection of FoMV containing multiplexed gRNA2 and gRNA3 in either MCS did not show higher suppression compared to constructs with a single gRNA.

Unexpectedly, the addition of the DC promoter upstream of MCSI did not improve *PDS1* suppression.

Replication and movement *in planta* of FoMV carrying gRNAs within MCSI or MCSII

To verify whether FoMV carrying gRNAs in different MCSs can replicate in local (inoculated) leaves and whether it systemically moves in whole plants, *PDS1* gRNAs were cloned into the binary pCambia1308-FoMV-DC vector (Mei, et al., 2019) as either a single gRNA or multiplexed gRNAs. These constructs were then agro-infiltrated into *N. benthamiana* plants, and RT-PCR was conducted with total RNA extracted from the local leaves at 7 dpi and the systemic leaves at 14 dpi. As shown in Figure 5A, FoMV constructs with a single gRNA in MCSII replicated in both local and systemic leaves. However, for multiplexed gRNA constructs, amplified products corresponded to both the expected insert size as well as the empty vector control. Thus, in *N. benthamiana*, multiplexed *PDS1* gRNAs were less stable than single gRNA insertions in the FoMV MCSII. For MCSI insertions, however, FoMV with single or multiplexed gRNAs replicated in local leaves (Figure 5B). In systemic leaves, relative to the empty vector, FoMV with single gRNA inserts within MCSI replicated poorly. Addition of the DC upstream of MCSI did not improve FoMV replication with either single or multiplexed gRNAs. However, fusion of a single gRNA with a tRNA resulted in improved viral replication in both local and systemic leaves, similar to the empty vector in MCSI (Figure 5B).

After the confirmation of viral replication by RT-PCR, *N. benthamiana* local leaf tissues were ground with KP buffer and sap was used as an inoculum for rub inoculation of two-week-old B73 maize plants. About 10 days after the inoculation, the tiny yellow speckles characteristic of FoMV symptoms started to develop on the systemic leaves. Figure 6A shows symptom

development at 21 dpi, where FoMV-EV and FoMV-gRNA3_MCSII display the strongest symptoms compared to the FoMV multiplexed gRNA constructs. As shown in Figure 6B, FoMV could replicate with single gRNA inserts in either MCSI or MCSII, while multiplexed or tRNA-inserts did not. For multiplexed gRNAs in MCSII, the amplification product was smaller than wild type, suggesting the virus lost the insert and perhaps some genomic sequence as well.

Our observations that FoMV constructs containing single gRNAs replicate and spread to systemic leaves in maize suggest their potential use in transgenic maize plants expressing dCas9. Just as Mei et al. (2019) reported successful genomic editing in transgenic maize plants expressing active Cas9 via FoMV-delivered gRNAs, we anticipate similar success for our FoMV CRISPRa and CRISPRi constructs in dCas9-expressing pDA2/3/4/5 transgenic maize lines currently under development.

Discussion

Development of the CRISPRa and CRISPRi toolkit for maize

In this study, we described an efficient system for testing gRNAs targeting trait genes for CRISPRa and CRISPRi in maize protoplasts. To develop this system, we first modified maize protoplast transfection methods to robustly express the dCas9-variants for CRISPRa and CRISPRi. Specifically, we utilized the isolation solution described by Burdo, et al., (2014) that had been developed for electroporation, and instead paired this with a modified cost-effective PEG-mediated transfection described by Cao, et al. (2014) and Yoo, et al. (2007). Analysis of GFP-construct transfactions revealed these modifications produced robust GFP expression (Figure 1A-C). Additionally, we observed protoplasts remained viable and expressed GFP four

days after transfection, indicating this protoplast isolation/transfection method would be suitable for multi-day experiments such as those requiring virus replication (Figure 1D).

As with any CRISPR/(d)Cas9 system, it is important to empirically verify that the gRNA sequences predicted by design algorithms are indeed effective. Using our CRISPRa/CRISPRi protoplast system based on dCas9 (pDA2), dCas9-VP64 (pDA3) or dCas9-SRDX (pDA4) vectors, we tested the effectiveness of the top four gRNA candidates targeting maize promoters of *PDS1*, *ChlH*, or *TrxH*, respectively (Figures 2 and 3). Similar to other reports on dCas9 activity, we observed that pDA2 exhibited some suppression activity on *ChlH* expression (Figure 3A), although the magnitude of this suppression was greater with pDA4 for some gRNAs (Figure 3B). Tests with pDA3, the CRISPRa construct, revealed a two-fold increase in *TrxH* expression depending on which gRNA was co-transfected (Figure 3C). The varying effectiveness between gRNAs observed in this study reiterates the necessity of testing multiple gRNA candidates.

In addition to testing single gRNA constructs, we also examined the effectiveness of multiplexed gRNAs on *PDS1* expression in protoplasts co-transfected with pDA3 or pDA4. We hypothesized that expressing more than one gRNA could increase the extent of transcription activation or repression, respectively. However, as the results in Figure 3D-E revealed, the relative impact of multiple gRNAs on their target's expression remained similar in magnitude to what we observed for single gRNAs in Figure 3A-C. Further research is needed to assess if both gRNAs within the multiplexed construct were contributing to the observed changes in *PDS1* expression.

Our ultimate goal for this study was to produce the tools needed to modify agronomic traits such as disease resistance and abiotic stress tolerance by transiently changing the

expression of multiple trait genes without extra transformation steps to deliver gRNAs specific to each target gene. To achieve this goal, we used a viral vector, FoMV, to deliver gRNAs to the maize seedlings by the rub-inoculation method. Building upon FoMV's utility as a VIGS vector, Mei et al. (2019) developed FoMV constructs containing an additional multiple cloning site (named MCSII) preceded by a duplicated coat protein (DC) promoter (Figure 4A). From MCSII, this new construct (FoMV-DC) is suitable for protein expression as well as gRNA delivery for genome editing in maize and other hosts [28]. In our study, we examined FoMV-DC for delivery of CRISPRa/CRISPRi gRNAs from MCSII as well as MCSI. Our data indicate that single gRNA insertions in either MCS do not prevent viral replication in plants (Figures 5-6) and result in 50-60% reduction in *PDS1* expression in protoplasts transfected with pDA4 (Figure 4C). FoMV constructs containing multiplexed *PDS1* gRNAs, however, resulted in reduced virus replication as shown by semi-quantitative RT-PCR analysis (Figure 6B), although we did observe suppression of *PDS1* in protoplasts (Figure 4C). We hypothesize this reduction in FoMV replication is a consequence of tRNA cleavage within the multiplexed gRNA sequence (Figure 4B). We speculated that cleavage of tRNAs, and therefore the FoMV subgenomic RNA, is likely more detrimental at MCSII than at MCSI located within the viral 3' UTR. However, FoMV carrying multiplexed gRNAs within MCSI also did not replicate, suggesting the 3'UTR is still important for replication. It is intriguing, therefore, that the addition of a tRNA to a single gRNA within MCSI results in unperturbed FoMV replication in *N. benthamiana* (Figure 6B) but not in maize (Figure 7B).

Without tRNA processing, the FoMV-derived gRNA-scaffold complex is not a discreet gRNA unit (consisting of the 20 nucleotide (nt) gRNA and 76 nt of gRNA scaffold), but is rather an extension of the FoMV subgenomic RNA (sgRNA) transcript described in Mei, et al. (2016).

When cloned in MCSI, this sgRNA-gRNA transcript contains ORF5 and the 3' UTR and is over 800 bp. Therefore, the gRNA expressed in MCSI will be about 8 times larger than the actual mature gRNA. Interestingly, gRNA2 within FoMV MCSI showed *PDS1* repression when co-expressed with pDA4 vector in protoplasts (Figure 4C). To our knowledge, this is the first report for functional gRNA constructs over 300 nt. Recently, a study in rice described a functional chimeric gRNA transcript containing an additional 237 nt of template for homology-directed repair (HDR), indicating that gRNA transcripts can be longer than the standard 96 nt [41]. Considering our results with FoMV gRNA delivery, it will be interesting to see whether larger chimeric gRNAs can be used for genome editing using HDR in maize.

Application of toolkits for functional genomics

In this study, we generated CRISPRa and CRISPRi vectors for maize protoplast transfection. We also determined that FoMV could be utilized as a gRNA delivery vehicle in maize protoplasts. Based on our results, we believe that this toolkit will be very useful for characterizing genes of interest in maize, as this approach has multiple advantages over conventional screening methods such as VIGS. Mainly, the CRISPRa/CRISPRi protoplast system provides a scalable means for convenient and cost effective analysis of both gene activation and suppression. While many virus systems, including FoMV, have been developed to express plant proteins, insert stability as well as insert size restrictions can make it difficult for expressing large or multiple genes [28–30,42]. With the protoplast system, it is possible to multiplex at least two gRNAs within the same vector and obtain CRISPR-mediated gene activation or suppression using the pDA3 and pDA4 vectors (Figures 3 and 4C).

Recently, Mei et al. (2019) discussed their development of modified FoMV vectors for protein expression *in planta*, VIGS, and gRNA delivery for CRISPR/Cas9 genome editing.

They demonstrated CRISPR-mediated editing of plant genes using their FoMV-gRNA vectors in *N. benthamiana*, *Setaria viridis*, and maize plants expressing Cas9. In our study, we expand the utility of their FoMV-gRNA vectors to allow manipulation of maize gene expression in protoplast systems. In addition to streamlining gRNA selection using protoplast assays, our work also examines the feasibility and limitations of using FoMV to deliver single versus multiplexed CRISPRa and CRISPRi gRNAs in maize plants. Ultimately, our new CRISPRa and CRISPRi toolkits will provide the maize community with useful materials for gene expression studies.

Materials and Methods

Plant materials and growth conditions

Etiolated B73 maize (*Zea mays*) seedlings were prepared as follows. Kernels were imbibed for 24 h in room temperature water before being sown in moist ProMix (Sungro) soil. For three days, or until coleoptile emergence, plants were kept in a Conviron PGR15 growth chamber under a 12 h light (242 µmol)/12 h dark cycle, where temperatures and relative humidity were maintained at 26°C/20°C and 80%/60%, respectively. Plants were subsequently grown in complete darkness at 25°C and 60% relative humidity for up to two weeks or until full expansion of the second true leaves.

Maize protoplast isolation

Maize leaf protoplasts were prepared as described by Burdo, et al. (2014) with some modification. Briefly, etiolated B73 maize seedlings were cut into ~ 0.5 mm strips and placed in a flask containing 3% cellulase onozuka™ RS (Yakult Pharmaceutical Industry Co. Ltd., Japan), 0.7% macerozyme R10, 0.6 M mannitol, 10 mM KCl, 10 mM MES, 5 mM CaCl₂, 0.1 % (w/v)

BSA. This enzyme solution was vacuum infiltrated into the leaf tissue for 30 min at 20 mmHg and followed by agitation at 40-50 rpm for 2.5 – 3 h. To release the protoplasts, the flask was shaken at 90 rpm for 30 min. Protoplasts were filtered out of the enzyme solution through a 35 µm nylon mesh and collected by centrifugation at 300 x g for 2 min. The protoplast pellet was washed twice with W5 media (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 2 mM MES) prior to resuspension in MMG buffer (0.4 M mannitol, 15 mM MgCl₂ and 4 mM MES) for DNA transfection.

Protoplast transfection

Protoplast transfection was conducted based on the method described by Chen et al. (2006) with a few modifications. Briefly, 100 µL of protoplasts resuspended in MMG buffer were mixed with 10 µg of each plasmid followed by the addition of 110 µL freshly prepared 40% PEG-CaCl₂ solution (40% PEG-4000, 0.4 M mannitol and 0.1 M CaCl₂) and incubation for 5 min to 1 h at room temperature. After incubation, the protoplasts were washed with 1 mL of W5 buffer to remove PEG. After centrifugation at 300 x g for 3 min, the protoplasts were resuspended in 1 mL of W5 buffer and incubated for 16 h at room temperature.

Dual-luciferase assay

Protoplasts incubated for 16 h in darkness at room temperature were harvested by centrifugation at 300 x g for 3 min. The luciferase assay was conducted according to the manufacturer's instructions (Promega). Briefly, 50 µL of 1X Passive lysis buffer was added to the harvested protoplasts and mixed by pipetting 4-5 times. After centrifugation at 12000 x g for 5 min, 10 µL of supernatant was mixed with 40 µL of LARII buffer and firefly luciferase activity was measured for 1 min using a Glomax 20/20 luminometer. Next, 40 µL of Stop&Glo solution

was added to the sample to measure *Renilla* luciferase activity as an internal control. Three technical replicates were conducted for each sample.

Vector construction

pDA2-5 vectors were developed using pTF101.1.rev (kindly provided by Kan Wang, Iowa State University) as a backbone. Other components were cloned from vectors previously published; the ubiquitin promoter and nos terminator were cloned from pXUN [43], dCas9-VP64 and dCas-SRDX were cloned from pYPQ152 or pYPQ153, respectively [8], and dCas9-TV was cloned from pCambia-dCas9-TV [10]. Cloning was conducted using traditional restriction enzyme digestion and ligation followed by PCR screening and Sanger sequencing confirmation. For protoplast transient expression vectors, we cloned the HindIII and SbfI fragment from each pDA# construct into the HindIII and PstI sites of pXUN, which decreases the size of the construct but increases efficiency of protoplast transfection. To clone gRNAs in the expression vector, PstI and SalI fragment containing the gRNA expression cassette from pENTR-gRNA1 was cloned to the pGreenII-800-Luc vector [39], which was designated as pGreenII-800-RNA1-Luc.

Cloning of gRNAs in pGreenII-800-Luc vector

Four gRNAs targeting promoter regions of maize *PDS1* were designed using the CRISPR-P website (<http://crispr.hzau.edu.cn/CRISPR2/>), which is based on the B73 (AGPv.3.21) reference genome. *PDS1* gRNA3 was cloned in the BtgZI site and gRNA1, gRNA2 and gRNA4 were cloned in the BsaI site, resulting in two gRNAs per construct.

Furthermore, about 500 bp of the promoter region of *PDS1* were amplified by PCR, and the products were sequenced using templates from B74, B104 and A188 genomic DNA to identify any polymorphisms compared to the B73 reference genome assembly.

A single *PDS1* gRNA2 or gRNA3 was cloned either in MCSI using Bsu36I and PspOMI or MCSII of pFoMV-DC using XbaI and XhoI sites. To multiplex gRNAs using tRNA, gRNA scaffold and tRNA were amplified using pTGR as a template and ligated by GoldenGate assembly cloning as described by Xie et al. (2015) with a few modifications for the restriction enzyme sites in FoMV MCSI and MCSII. To clone gRNAs with DC in MCSI, gRNAs cloned in MCSII were amplified with DC and they were cloned in MCSII using XbaI and XhoI. For agroinfiltration, gRNAs cloned in pFoMV-DC were PCR-amplified and cloned into pCambia1380-FoMV vector.

Agroinfiltration of FoMV into *N. benthamiana* and RT-PCR

Agrobacteria (GV3101 strain) carrying FoMV with *PDS1* gRNAs were agroinfiltrated into *N. benthamiana* and agroinfiltrated local and systemic leaves were harvested 7 days and 14 days after the infiltration, respectively. Total RNA was isolated using Trizol (Invitrogen) and the first strand cDNA was synthesized using strand-specific primers according to manufacturer's instructions (Transbio).

Rub-inoculation of maize using sap from agroinfiltrated *N. benthamiana* leaves

Agroinfiltrated *N. benthamiana* local leaves were harvested at 7 days after infiltration and ground in KP inoculation buffer (50 mM potassium phosphate, pH 7.0) for use as an inoculum for rub inoculation described by [27]. Twenty-one days after the inoculation, systemic leaves were harvested for symptom analysis and total RNA extraction.

Western blot

Total protein from frozen plant tissue was isolated from mortar-ground leaves using a 100 mg tissue/400 µL ratio of extraction buffer (150 mM NaCl, 20 mM Tris, pH7.5, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1X protease inhibitor cocktail, and 5 µl/mL 1M DTT) as described in

Geng et al. 2016. Following a 5 min incubation on ice, the extracts were centrifuged at 16,000 x g for 10 min at 4°C. Supernatants were mixed with SDS loading dye and boiled for 5 min before being loaded on a 7.5% SDS-PAGE gel. Following membrane transfer, total protein was assessed with Ponceau staining. dCas9 proteins were detected with an anti-flag antibody and α-HSP90 was used as the loading control.

Figure Legends

Figure 1. Analysis of protoplast transfection methods. (A) Western blot analysis of maize protoplasts transfected with pCXUN-HA-GFP under different mannitol concentrations and incubation length, in addition to electroporation at 300V. GFP expression in *N. benthamiana* leaves is the positive control. (B) GFP expression driven by the 35S promoter (left) or the maize ubiquitin promoter (right). Exposure time was 1 second. (C) Western blot analysis of GFP expression in maize protoplasts driven by the 35S or ubiquitin promoters compared to the *N. benthamiana* positive control. (D) GFP expression in protoplasts from maize hybrids Early Sunglow (top) and Silver Queen (bottom) was observed at four days post transfection with pCXUN-HA-GFP.

Figure 2. Expression of dCas9 variants in planta. Western blot expression analysis of pDA2 (dCas9), pDA3 (dCas9-VP64), pDA4 (dCas9-SRDX) constructs in *N. benthamiana* leaves (left) and maize protoplasts (right).

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Figure 4. Cloning of PDS1 gRNAs in MCSI or MCSII FoMV. (A) Map of 35S-driven FoMV-DC vector (described in Mei, et al. (2019)) showing multiple cloning site 1 (MCSI) and MCSII locations for gRNA insertions. (B) gRNA insertion variants with or without tRNAs, as well as DC addition upstream of MCSI. (C) Dual luciferase assay results of *PDS1* gene expression in maize protoplasts after transfection with the pDA4 construct and the FoMV-gRNA constructs shown in (B) cloned in MCSI or MCSII. DC designates the duplicated coat protein promoter.

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Figure 6. Rub-inoculation of FoMV carrying *PDS1* gRNA in maize and RT-PCR. Leaves of two week-old B73 plants were rub-inoculated with sap from *N. benthamiana* agroinfiltrated with FoMV constructs carrying *PDS1* gRNAs in MCSI or MCSII, and systemic leaves were harvested 21 days after infiltration (dai) for symptom observation and RT-PCR. (A) Symptoms at 21 dai in plants inoculated with buffer (mock) or FoMV PDS1-gRNA constructs (where EV = empty vector, g2 = gRNA2, g3 = gRNA3, g32 = tRNA-gRNA3-tRNA-gRNA2-tRNA, and tg3 = tRNA-gRNA3). (B) Strand-specific RT-PCR indicate the FoMV-EV (black arrow), or constructs with

gRNA2 in MCSI (Red arrow) and gRNA3 (Blue arrow) in MCSII replicated in systemic leaves. FoMV with multiplexed gRNAs either in MCSI or MCSII did not replicate. PCR products were amplified with primers flanking the MCSI (upper panel) or the MCSII (lower panel).

Additional Files

Additional File 1 (.docx): gRNA sequences used in this study

Additional File 2 (PDF): Vector Maps. (A) pCXUN-HA-GFP used for testing protoplast transfection conditions. (B) Diagram of each pDA vector showing an ubiquitin-driven, Flag-tagged dCas9 with IV2 intron followed by transcription activators (VP64 and TAL-VP128) or suppressor (SRDX). A dual 35S promoter drives BAR for glufosinate resistance.

Additional File 3 (PDF): Dual luciferase assay components and preliminary results. (A) pGreenII-800-RNAI-PDS1-Luc dual luciferase construct, where Renilla luciferase provides an internal control for PDS1-driven Firefly luciferase. Dual luciferase assay using maize protoplasts co-transfected with indicated PDS1 gRNAs and dCas9-SRDX (B) or dCas9-VP64 (C). Data shown in B and C are from one biological replicate. An additional control construct was developed where GFP was expressed from the dual luciferase vector pGreenII-800-RNAI-Luc (D). Expression was detected by Western blot (E), which shows four separate maize protoplast transfection samples with this construct, compared to the no transfection, no DNA, and positive controls.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: G.L.W., P.B.K., M.G.R. designed the research. C.H.P., M.B., G.X., C.M, Q.Y., J.L, and I.N.G performed the research. T.L.S. and K.R.G. contributed to the protoplast method development. I.N.G., C.H.P. and G.L.W. analyzed the data and wrote the article.

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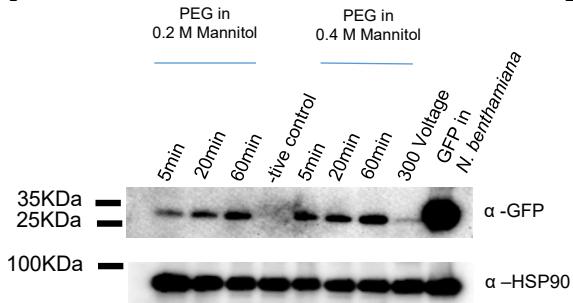
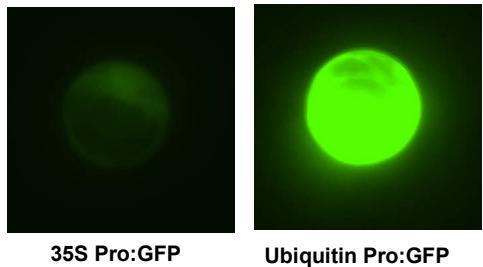
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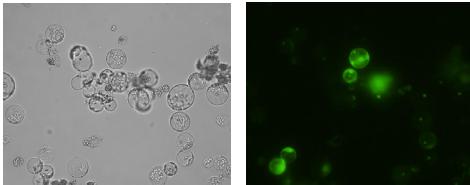
A**B****C**

α -GFP

α -HSP90

D

Early Sunglow



Silver Queen

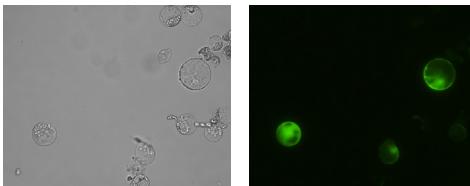


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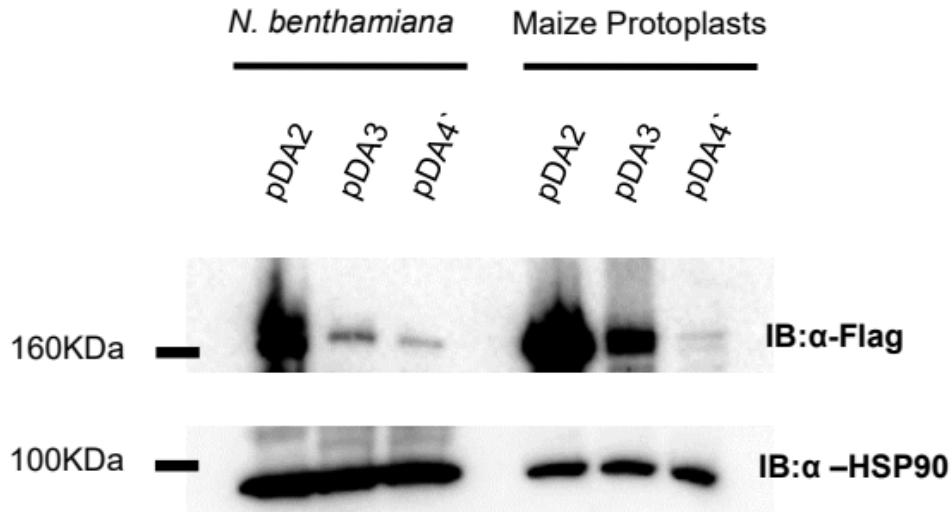


Figure 2. Expression of dCas9 variants *in planta*.
Western blot expression analysis of pDA2 (dCas9),
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(N. benthamiana leaves (left) and maize protoplasts
(right)).

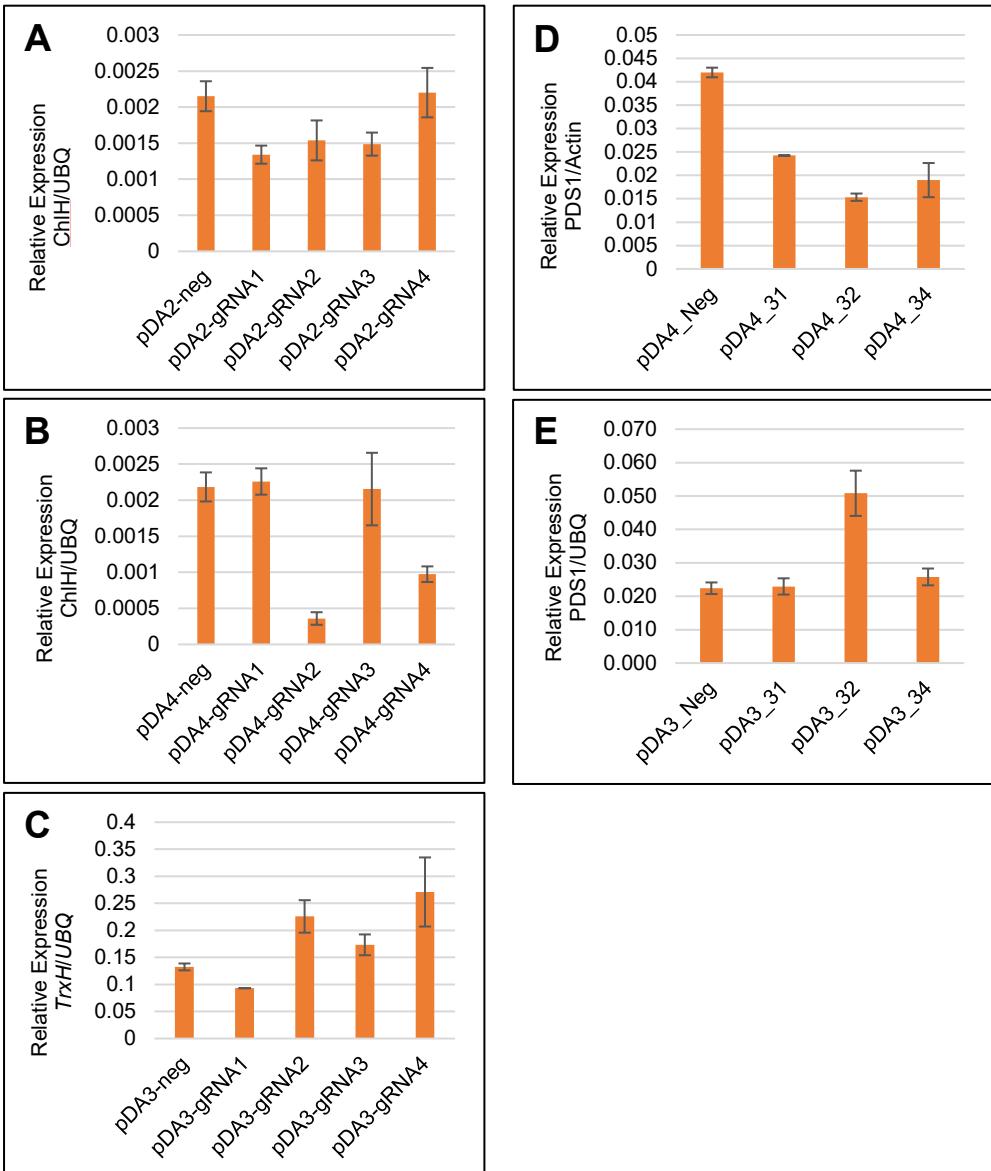
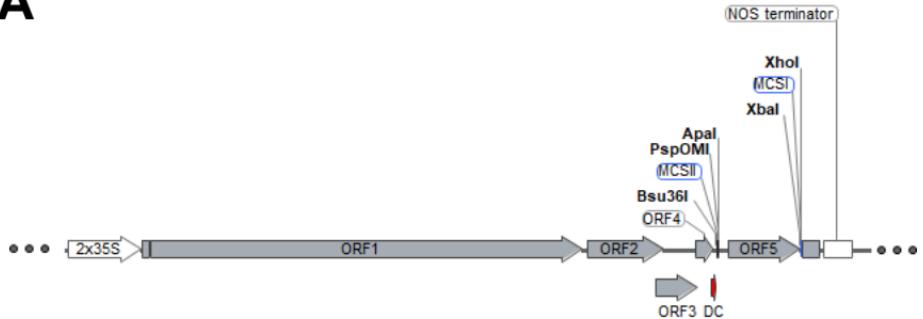
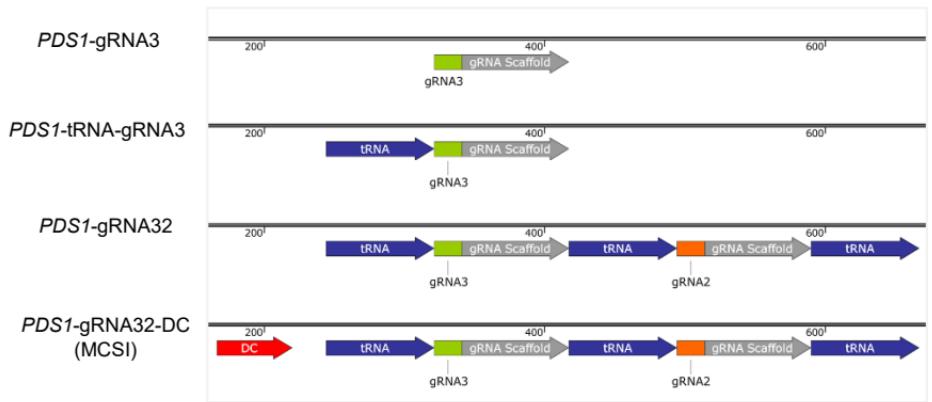


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A**B****C**

Relative expression of *PDS1* (Luc/Ren) -pDA4 (dCas9-SRDX)

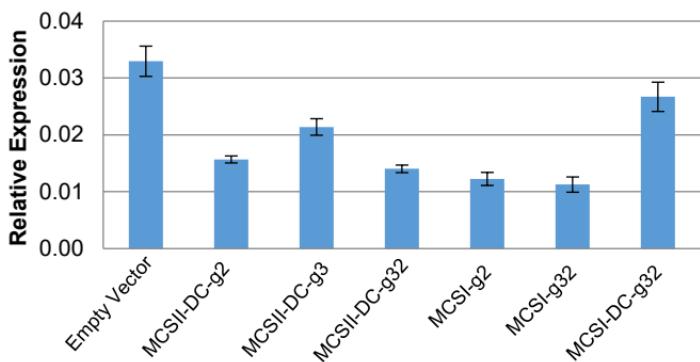
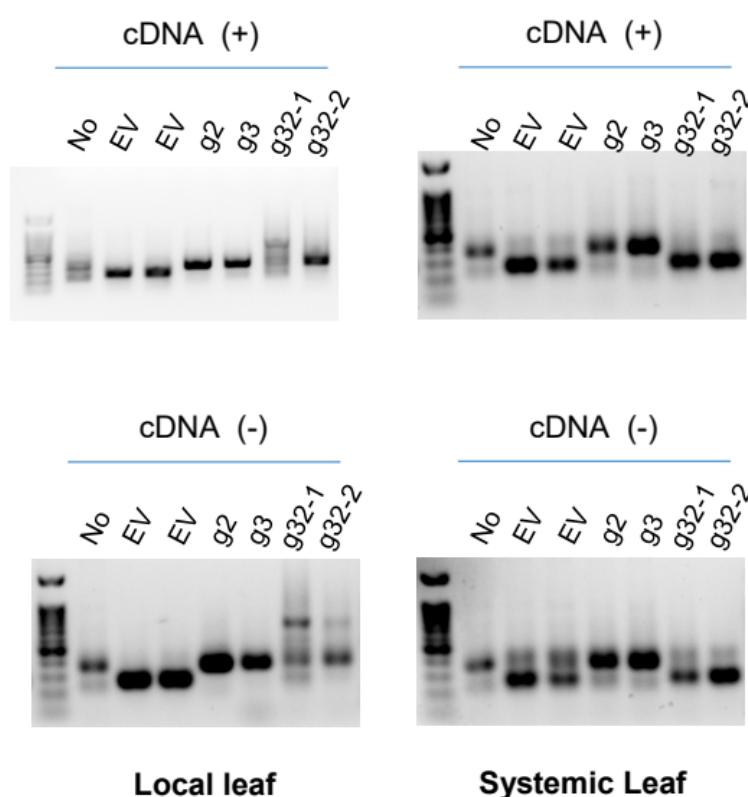


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A

MCSII

**B**

MCSI

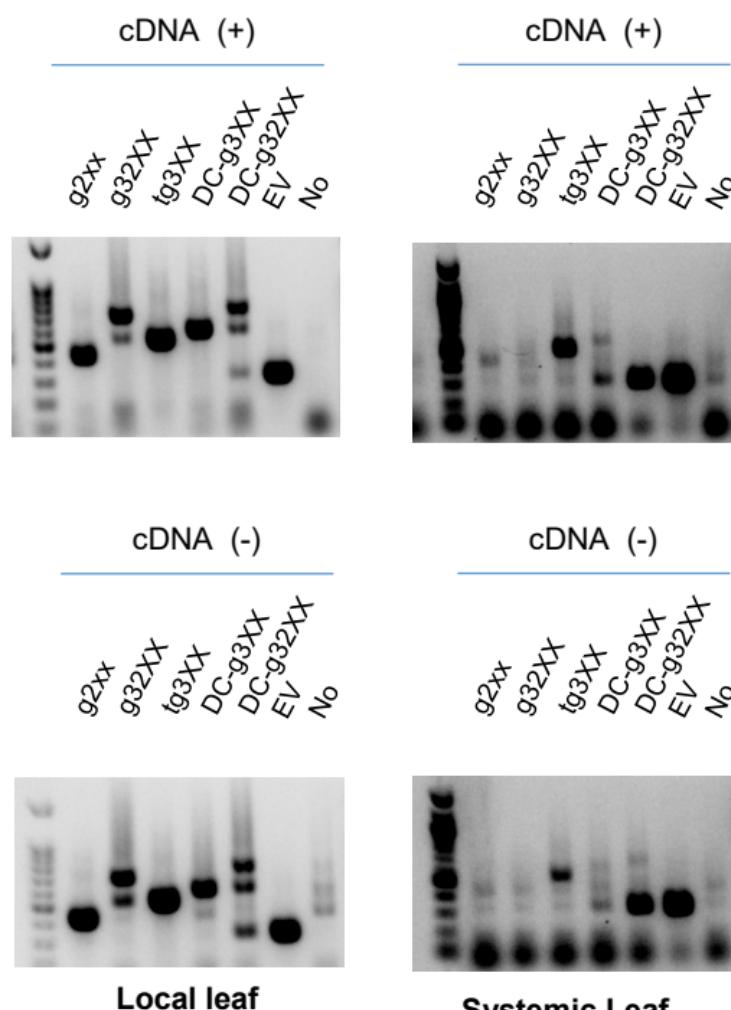


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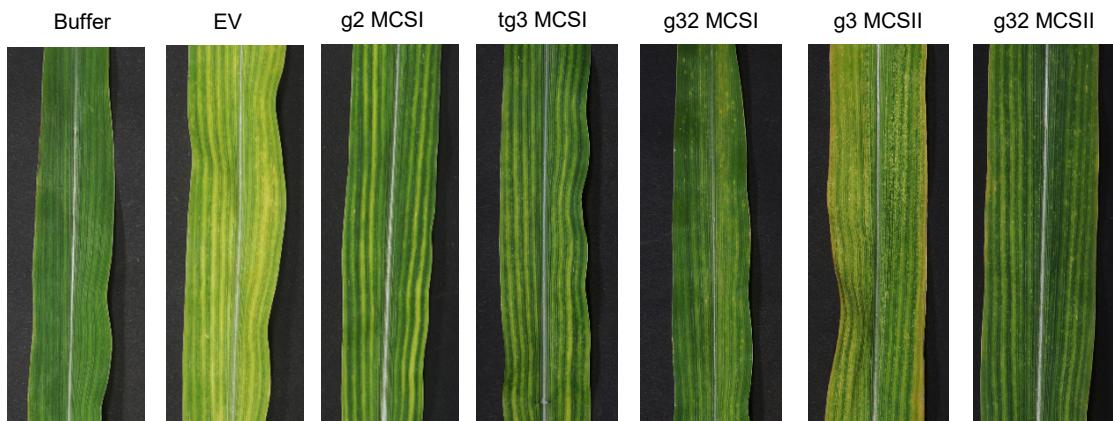
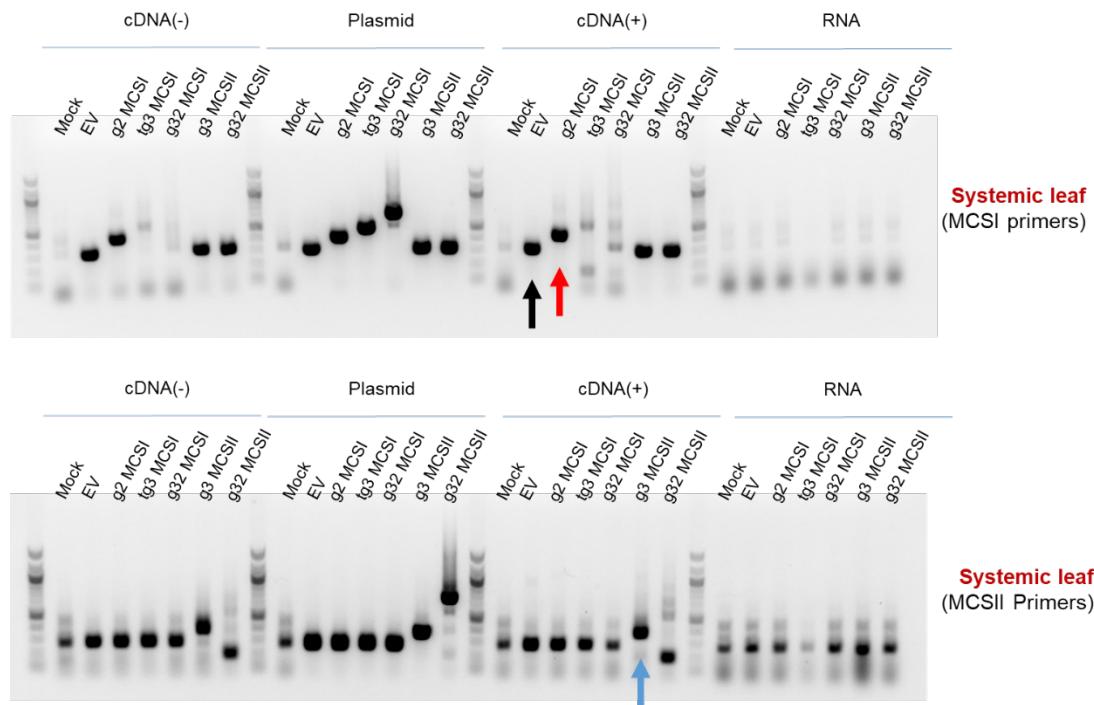
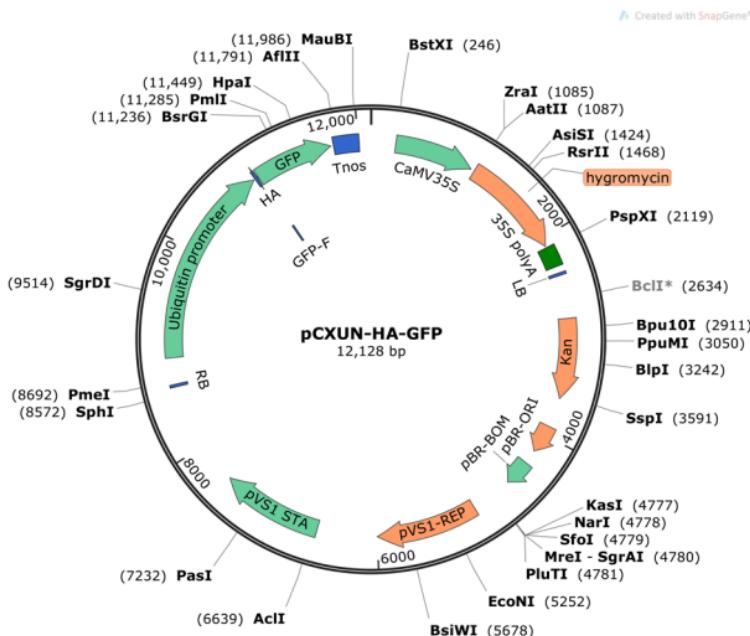
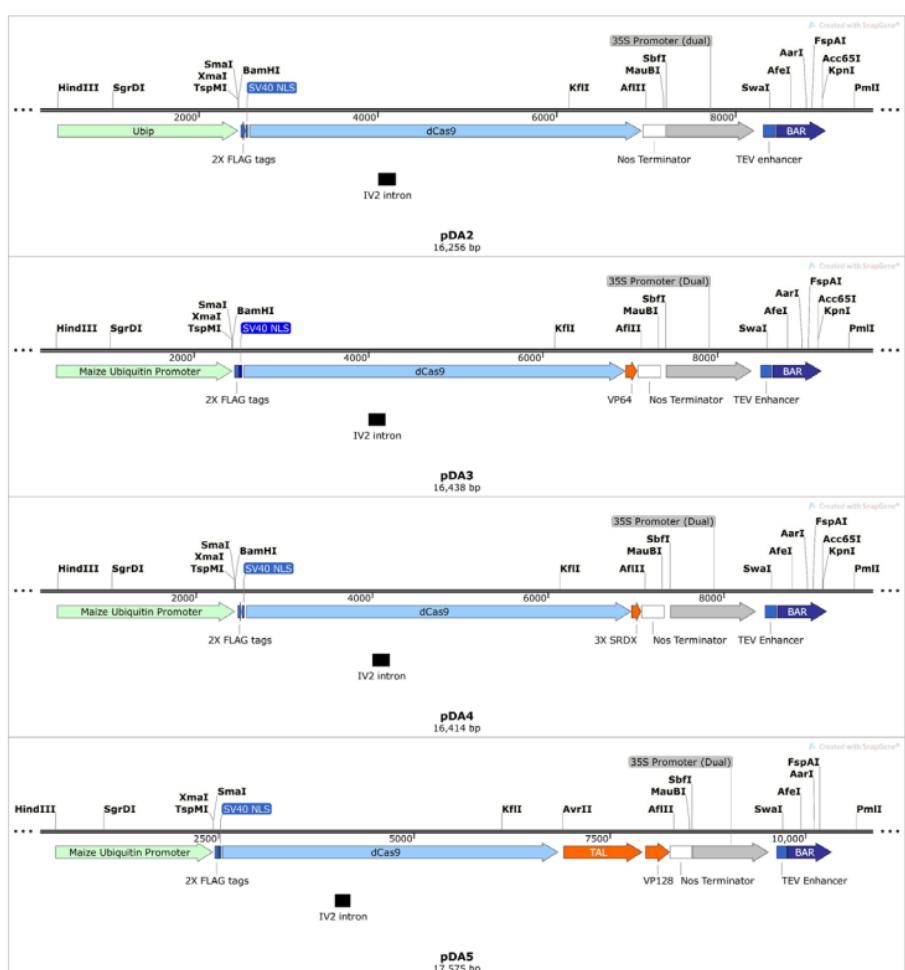
A**B**

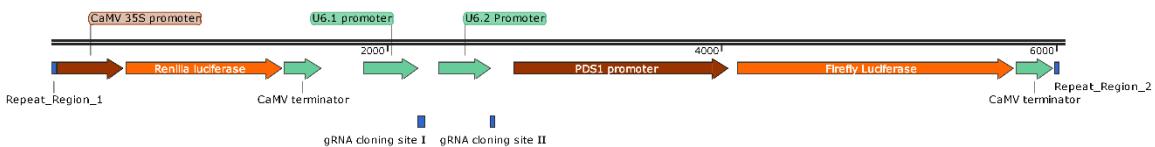
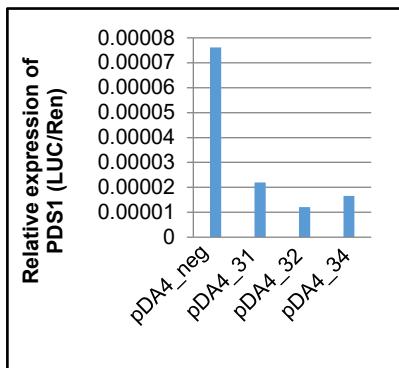
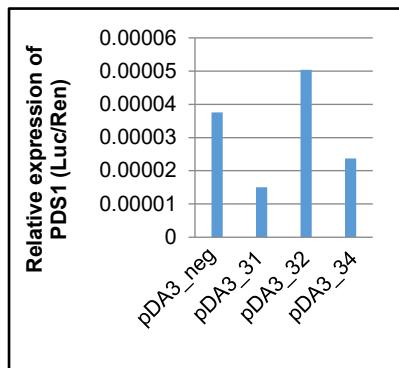
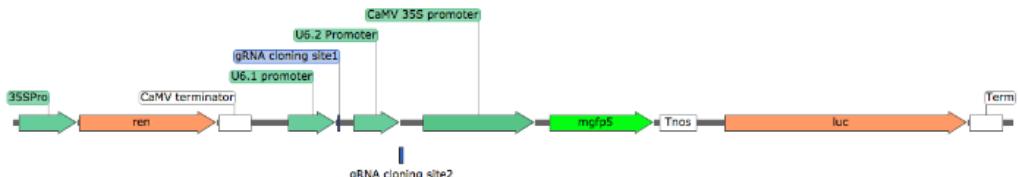
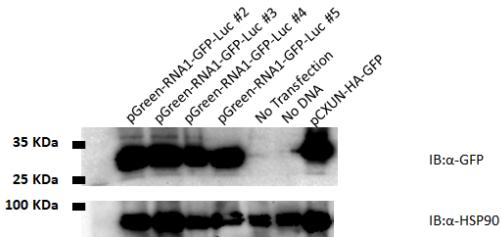
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Additional File 1. gRNA sequences used in this study

Maize gRNA targets	5' – 3' sequence	
<i>PDS1 promoter</i>	gRNA1 gRNA2 gRNA3 gRNA4	tctcgtaactatgtattccg cgggtagcggtatatacca tgtattccgcggcatcacac tctgttctaaggtaatatg
<i>ChlH promoter</i>	gRNA1 gRNA2 gRNA3 gRNA4	acactattataattatgcaa gatcaatcgacagtttaggg ccaaggcctctgccctcgacg tctcctccctccaggagacga
<i>TrxH promoter</i>	gRNA1 gRNA2 gRNA3 gRNA4	tgtcgcccagcctaaaaaa gtacgtacaagctcacaag cagctaggtaatgcaacca gcggatttatgtctcggtag
gRNA scaffold	gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaactgtaaaaagtggcaccgagtcggtgcc	
tRNA	aacaaaggcaccagtggctagtggtagaatagtagccctgccccgggttcattcccgctggtgca	

A**B**

Additional File 2: Vector Maps. (A) pCXUN-HA-GFP used for testing protoplast transfection conditions. (B) Diagram of each pDA vector showing an ubiquitin-driven, Flag-tagged dCas9 with IV2 intron followed by transcription activators (VP64 and TAL-VP128) or suppressor (SRDX). A dual 35S promoter drives BAR for glufosinate resistance.

A**B****C****D****E**

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