Plant-based biosensors for detecting CRISPR-mediated genome engineering

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Running title: Biosensors for detecting CRISPR systems

Abstract

CRISPR/Cas has recently emerged as the most reliable system for genome engineering in various species. However, concerns about risks associated with CRISPR/Cas9 technology are increasing on potential unintended DNA changes that might accidentally arise from CRISPR gene editing. Developing a system that can detect and report the presence of active CRISPR/Cas tools in biological systems is therefore very necessary. Here, we developed the real-time detection systems that can spontaneously indicate CRISPR-Cas tools for genome editing and gene regulation including CRISPR/Cas9 nuclease, base editing, prime editing and CRISPRa in plants. Using the fluorescence-based molecular biosensors, we demonstrated that the activities of CRISPR/Cas9 nuclease, base editing, prime editing and CRISPRa can be effectively detected in transient expression via protoplast transformation and leaf infiltration (in *Arabidopsis*, poplar, and tobacco) and stable transformation in *Arabidopsis*.

Keywords: CRISPR, genome editing, biosensor, detection, transient gene expression.

Introduction

Different CRISPR/Cas-based genome engineering tools, such as base editors (BEs), prime editors (PEs), CRISPR activation (CRISPRa) and interference, have been developed in life sciences.^{1, 2} Although CRISPR/Cas is a revolutionary technology, it poses potential biosecurity risks, increasing ethical and safety concerns.^{3, 4} Therefore, it is necessary to develop a system that can detect the presence of active CRISPR/Cas tools in biological systems. Multiple CRISPR/Cas technologies have been tested in multiple plant species.⁵ However, methods for detecting CRISPR systems have not yet been reported in plants. Here, we developed fluorescence-based biosensors to detect in planta activities of CRISPR/Cas9, BEs, PEs, and CRISPRa.

Results and Discussion

To detect active CRISPR/Cas9 nucleases, we created two variants of biosensor 1 (BS1), BS1-1 and BS1-2, which contain different frameshift GFP mutants (*gfp1a* or *gfp1b*), and single guide RNAs (sgRNAs) targeting *gfp1a* and *gfp1b*, respectively (Figure 1a). T-DNA vector p201N-Cas9⁶ was co-expressed with BS1-1/BS1-2 to detect CRISPR/Cas9-mediated gene editing, which generates functional GFP through insertion/deletions in *gfp1a* or *gfp1b*, in *Arabidopsis thaliana* protoplasts and *Nicotiana benthamiana* (tobacco) leaves. Bright green fluorescence was observed in both the protoplasts transformed with 35Sp:GFP (positive control) and those co-transformed with p201N-Cas9 and BS1-1/BS1-2, but not in the protoplasts transformed with BS1-1/BS1-2 only (negative control) (Figure 1b). Furthermore, ~34% and ~15% of the cells exhibited GFP signals in the positive control and the samples with Cas9-BS1 co-transformation, respectively (Figure 1c). Similar results were obtained through *Agrobacterium*-mediated leaf infiltration in tobacco (Figure 1b). These results indicate that BS1 can be used as an efficient biosensor for detecting active Cas9 in plant systems.

To detect active adenine base editors (ABEs) and PEs, we generated BS2 and BS3 by integrating a dark-GFP mutant (*gfp2*) harboring a premature termination codon (PTC) with corresponding sgRNA and pegRNA (Figure 1d and e). As such, an active ABE or PE will^{7, 8} generate BS3 to generate a functional GFP protein. Two BS2 variants, BS2-1 (*gfp2a*, Q69>TAG) and BS2-2 (*gfp2b*, Q80>TAG), were tested by co-expressing with a plant adenine base editor PABE-7⁹ using protoplast transformation in *Arabidopsis*, poplar ‘717’ (*Populus tremula x alba* INRA ‘717-1B4’), poplar ‘WV94’ (*Populus deltoides* ‘WV94’), and tobacco leaf infiltration. In *Arabidopsis*, ‘717-1B4’ and ‘WV94’, strong GFP fluorescence was observed in both the positive control (35Sp:GFP) and the protoplasts co-transformed with PABE-7 and BS2-1/BS2-2 but not those transformed with BS2-1/BS2-2 alone (Figure 1f), indicating that base editing mediated by PABE-7 successfully rescued the *gfp2* mutants. In *Arabidopsis*, ~64% and ~45% of the cells exhibited GFP fluorescence in the positive control and the samples with BS2-PABE-7 co-transformation, respectively (Figure 1g). In poplar ‘717-1B4’, GFP fluorescence was detected in ~71%, ~45% and 35% of the cells in the positive control, the samples co-transformed with PABE-7 and BS2-1, and the samples co-transformed with PABE-7 and BS2-2, respectively (Figure 1h). In poplar ‘WV94’, GFP fluorescence was detected in ~73% and 38% of the cells in the positive control and the protoplasts co-transformed with PABE-7 and BS2-1/BS2-2, respectively (Figure 1i). These results indicate that both BS2-1 and BS2-2 are relatively efficient for detecting base editing in protoplasts derived

from herbaceous and woody plants. Similar results were also observed in the tobacco leaf (Figure 1f). Furthermore, after co-transformation of the BS2-1 and PABE-7 using floral dip, GFP signals were observed in the primary root of *Arabidopsis* transgenic lines (Figure 1j). Taken together, results from both transient gene expression and stable transformation demonstrate that BS2 is a reliable and robust system for the detection of ABE-mediated gene editing in plants.

BS3 containing the *gfp2b* mutant and a pegRNA was tested by co-expressing with a plant prime editor PPE2¹⁰ in *Arabidopsis* protoplasts. GFP signals were detected in the protoplasts with the positive control (35Sp:GFP) and those co-transformed with BS3 and PPE2, but not in the protoplasts transformed with BS3 alone (Figure 1k). This result indicates that the prime editing successfully rescued the *gfp2b* mutant, producing functional GFP proteins. Notably, a relatively low percentage (5%) of protoplasts displayed GFP signals in the co-transformation of BS3 and PPE2 in comparison with that (63%) in the positive control (Figure 1l). This could be explained by the lower efficiencies of the current generation of PEs in plants.² Therefore, while BS3 can be used to detect active prime editing through protoplast transient expression, the efficiency needs to be improved in the future.

To detect active CRISPRa, we generated BS4 by incorporating the *ProOsTPR-like*:mCherry and a sgRNA targeting *ProOsTPR-like*¹¹ (Figure 1m). As such, mCherry signals are expected to be much stronger in the presence of a CRISPRa system. The efficacy of BS4 was tested by co-expressing BS4 and a plant CRISPRa system, CRISPR-Act3.0¹¹ in *Arabidopsis* protoplasts. Strong red fluorescence was observed in 75% of protoplasts in the positive control (35Sp:mCherry) and 32% of those in the samples co-transformed with BS4 and CRISPR-Act3.0 whereas no red fluorescence signal was detected in the protoplasts transformed with BS4 alone (Figure 1n and o). This result indicates that BS4 is a highly efficient biosensor for detecting CRISPRa activity in plants.

In summary, we developed efficient biosensors for detecting four different CRISPR tools, providing new opportunities to monitor and evaluate the efficiency of different CRISPR/Cas-based genome engineering tools in real-time and may serve as an early-detection system for unwanted genome engineering.

Materials and methods

Construction of vector

To build the BS1, the mutant *gfp1a* and *gfp1b* were created by inserting a gblock containing frame shift mutation sequences at the 5' end of GFP coding sequence of pGFPGUSPlus (Plasmid #64401 in addgene) using the NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs, Catalog #E5520S). Then, a gblock harboring a U6 promoter, sgRNA and terminator was assembled into the mutant *gfp1a* and *gfp1b* through NEBuilder HiFi DNA Assembly. To build the BS2 and BS3, the mutant *gfp2a* and *gfp2b* were created by replacing the GFP coding sequence of pGFPGUSPlus with a gblock containing PTC mutation. BS2 was then assembled by inserting a gblock harboring a U6 promoter, sgRNA and terminator into the mutant *gfp2a* and *gfp2b* through NEBuilder HiFi DNA Assembly. BS3 was assembled by inserting a gblock harboring a U6 promoter, pegRNA and terminator into the mutant *gfp2b*. To build the BS4, a gblock harboring a

U3 promoter, sgRNA and terminator was inserted into the vector *ProOsTPR-like:mCherry*¹¹ using NEBuilder HiFi DNA Assembly. All gblocks were synthesized from Integrated DNA Technologies. All plasmids were confirmed by Sanger sequencing.

Protoplast transformation

The isolation and transformation of *Arabidopsis* and poplar protoplast were performed as described previously.¹²

Arabidopsis transformation

The floral dip method of transformation was performed by immersion of *Arabidopsis* wild type ‘Col-0’ inflorescences in a suspension of *Agrobacterium* ‘GV3101’ with modification as described by Yuan et al.¹³

Tobacco leaf infiltration

N. benthamiana leaves were infiltrated using a syringe without a needle as described by Li.¹⁴

Author contributions

G.Y., P.A. and X.Y conceived the research. G.Y., T.Y. H.L. and M.H. conducted the experiments. G.Y. wrote the paper. M.H., H.L., T.Y., M.V., J. L., W.M., J.-G.C., G.T., P.A. and X.Y. revised the manuscript. C.P. and Y.Q. provided CRISPRa reagents and revised the manuscript.

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Competing interests

The authors declare no conflict of interests.

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Figure 1. Detection of CRISPR tools for genome editing and gene regulation in plants.

(a) BS1 for detection of the CRISPR/Cas9 nuclease. (b) Detection of CRISPR/Cas9 by BS1 through *Arabidopsis* protoplast transformation and tobacco leaf infiltration. (c) Statistical analysis of GFP-positive cells with and without the CRISPR/Cas9 in *Arabidopsis*. (d) BS2 for detection of the adenine base editor (ABE). (e) BS3 for detection of the prime editor. (f) Detection of an ABE by BS2 through protoplast transformation in *Arabidopsis* and poplar, and tobacco leaf infiltration. (g-i) Statistical analysis of GFP-positive cells with and without ABE in *Arabidopsis*, poplar '717', and poplar 'WV94'. (j) Detection of an ABE by BS2 through stable transformation in *Arabidopsis*.

(k) Detection of prime editor PPE2 by BS3 through *Arabidopsis* protoplast transformation. (l) Statistical analysis of GFP-positive cells with and without PPE2. (m) BS4 for detection of CRISPRa. (n) Detection of CRISPRa by BS4 through *Arabidopsis* protoplast transformation. (o) Statistical analysis of mCherry-positive cells with and without the CRISPR–Act3.0 activation system. Scale bar, 100μm. All data are presented as the mean ± SE. (n = 5 independent scopes).