CRISPR/Cas9-mediated targeted mutagenesis for functional genomics research of crassulacean acid metabolism plants

Degao Liu1,2,*, Mei Chen1,3,*, Brian Mendoza4, Hua Cheng1, Rongbin Hu1, Linling Li1, Cong T. Trinh2,4, Gerald A. Tuskan1,2 and Xiaohan Yang1,2,†

1 Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
2 DOE-Center for Bioenergy Innovation (CBI), Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
3 School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan 621010, China
4 Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, TN 37996, USA

* These authors contributed equally to this work.
† Correspondence: yangx@ornl.gov

Received 25 February 2019; Editorial decision 28 August 2019; Accepted 29 August 2019

Editor: Kevin Hultine, Desert Botanical Garden, USA

Abstract

Crassulacean acid metabolism (CAM) is an important photosynthetic pathway in diverse lineages of plants featuring high water-use efficiency and drought tolerance. A big challenge facing the CAM research community is to understand the function of the annotated genes in CAM plant genomes. Recently, a new genome editing technology using CRISPR/Cas9 has become a more precise and powerful tool than traditional approaches for functional genomics research in C3 and C4 plants. In this study, we explore the potential of CRISPR/Cas9 to characterize the function of CAM-related genes in the model CAM species Kalanchoë fedtschenkoi. We demonstrate that CRISPR/Cas9 is effective in creating biallelic indel mutagenesis to reveal previously unknown roles of blue light receptor phototropin 2 (KfePHOT2) in the CAM pathway. Knocking out KfePHOT2 reduced stomatal conductance and CO2 fixation in late afternoon and increased stomatal conductance and CO2 fixation during the night, indicating that blue light signaling plays an important role in the CAM pathway. Lastly, we provide a genome-wide guide RNA database targeting 45 183 protein-coding transcripts annotated in the K. fedtschenkoi genome.

Keywords: Crassulacean acid metabolism, CRISPR/Cas9, functional genomics, genome editing, phototropin 2.

Introduction

Crassulacean acid metabolism (CAM) is a specialized photosynthetic CO2-concentrating mechanism that enhances plant water-use efficiency and associated drought tolerance by shifting all or part of the CO2 uptake to the night-time, when evapotranspiration rates are reduced compared with the daytime (Borland et al., 2014; Yang et al., 2015, 2017). Recently, interest in the CAM pathway has increased markedly due to public concerns about the decreasing supply of clean water and adverse effects of increasing heat and drought stress on plant growth (Yang et al., 2015, 2017; Abraham et al., 2016; Liu et al., 2018). Significant progress has been achieved in both basic and applied research on CAM plants (Borland et al., 2014, 2016, 2018; Liu et al., 2018; Winter, 2019). However, functional genomics research in CAM plants is still lagging behind the achievements made in C3 and C4 photosynthesis plants.

Loss-of-function analysis in CAM plants has relied on the characterization of mutants created through chemical and physical mutagens (Cushman et al., 2008), or RNAi (Dever
et al., 2015; Boxall et al., 2017). The mutations induced by the chemical and physical treatments are random, not suitable for site-directed mutagenesis. The RNAi approach has several limitations, such as incomplete loss of function and extensive off-target activities (Xu et al., 2006; Liu et al., 2016). Recently, a new genome editing technology using type II clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR–associated protein 9 (Cas9) from Streptococcus pyogenes has become a more precise and powerful tool than traditional approaches for functional genomics research in C₃ and C₄ plants (Feng et al., 2013; Shan et al., 2013; Xie et al., 2015; Liu et al., 2016; Shimatani et al., 2017; Kang et al., 2018; Salesse-Smith et al., 2018). The CRISPR/Cas9-guided by an engineered 20-nucleotide RNA sequence, can cleave chromosomal DNA in a target manner, producing double strand breaks (DSBs), which are repaired by non-homologous end-joining (NHEJ) events that give rise to random insertions and deletions (indels) of variable lengths at target sites. The CRISPR/Cas9-mediated indel mutagenesis can cause frameshift mutations in protein-coding sequences or disruption of the cis-regulatory elements in promoters or enhancers, resulting in knockout mutants for loss-of-function analysis (Belhaj et al., 2015; Liu et al., 2016; Ma et al., 2016; Puchta, 2017). Thus, developing and implementing an advanced toolbox for CRISPR/Cas9 applications in Kalancheë fedtschenkoi, an emerging model for CAM functional genomics research due to its small genome and amenability to efficient genetic modification (Boxall et al., 2017; Yang et al., 2017), could facilitate functional genomics research of CAM plants.

Phototropins (PHOTs) are blue light receptors that are involved in many biological processes, such as phototropism (Harmer and Brooks, 2018), root growth (Galen et al., 2007), stomatal movement (Horrer et al., 2016), chloroplast relocation (Jarillo et al., 2001), palisade cell development (Kozuka et al., 2011), anthocyanin accumulation (Kadomura-Ishikawa et al., 2013), and circadian oscillation of PSII operating efficiency (Littauer et al., 2015). Different members of phototropins may have overlapping or specific roles in such processes that are related to CAM. Recently, our comparative genomic analyses revealed that the timing of peak transcript abundance of PHOT2 in the CAM species K. fedtschenkoi (Kaladp0033s0113, KfePHOT2) and Ananas comosus (Aco014242) showed 11 h (Kalanchoë) and 9 h (pineapple) phase shifts, respectively, relative to that of the PHOT2 gene in the C₃ species Arabidopsis thaliana (AT5G58140) (Yang et al., 2017). This change in diel transcript abundance pattern suggests that the PHOT2 gene might be involved in the CAM pathway (Yang et al., 2017).

In this study, we developed and implemented a toolbox for CRISPR/Cas9 application in the model CAM species K. fedtschenkoi and tested the capability of the CRISPR/Cas9 system to characterize the function of CAM-related genes, and to link gene function with CAM physiology. We (i) demonstrate efficient generation of biallelic indel mutations in phototropin 2 by a dual-small guide RNA (sgRNA) strategy in K. fedtschenkoi; (ii) show novel roles of phototropin 2 in the CAM pathway; and (iii) provide a genome-wide sgRNA database of K. fedtschenkoi.

Materials and methods

Plant materials

Kalanchoë fedtschenkoi ‘M2’ plants were propagated clonally from stem cuttings and leaf margin adventitious plantlets using the same clonal material as that for genome sequencing (Yang et al., 2017).

Genome-wide sgRNA design

The genome sequence and gene annotation of K. fedtschenkoi v1.1 (Yang et al., 2017) were downloaded from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). The CASPER software package (CRISPR Associated Software for Pathway Engineering and Research) (Mendoza and Truth, 2018) was used to identify all potential sgRNA target (protospacer) sequences in the K. fedtschenkoi genome utilizing the Streptococcus pyogenes Cas9 endonuclease and the protospacer adjacent motif (PAM) ‘NGG’. The sgRNAs used in the experiments (see Supplementary Table S1 at [XBR online]) were firstly designed by using the software sgRNAcas9 (Xie et al., 2014) and then validated by the software CASPER for genome-wide analysis. Genome-wide off-target analysis performed by CASPER used a cut-off score of 0.05, which is half of the highest theoretical score given by the algorithm to a completely mismatched 12 bp tail sequence following a perfectly matched 8 bp seed.

Construction of CRISPR/Cas9 binary vectors

To generate the CRISPR/Cas9 vectors, the Arabidopsis U6 gene promoter and terminator, along with all the sgRNAs, were synthesized by the Integrated DNA Technology (Coralville, IA, USA) and assembled into the plant binary vector pKSE401 (Addgene plasmid #62202) (Xie et al., 2014) using the Gibson assembly method (Gibson et al., 2009). The recombinant vectors were transformed into the Agrobacterium tumefaciens strain GV3101 for plant transformation.

Plant transformation

Agrobacterium tumefaciens-mediated stable transformation of K. fedtschenkoi ‘M2’ was carried out according to Dever et al. (2015) with modifications. Specifically, to generate sterile explants, mature leaves of K. fedtschenkoi were washed in 70% (v/v) ethanol for 30 s and then surface sterilized in 25% bleach solution for 15 min, followed by rinsing four times with sterile water. The leaves were placed on Murashige and Skoog 30 (MS30) medium (Murashige and Skoog medium with Gamborg vitamins; PhytoTechnology Laboratories, Lenexa, KS, USA), 3% (w/v) sucrose, pH 5.8, 0.7% (w/v) Phytogar containing 1 mg l⁻¹ thidiazuron (TDZ), and 0.2 mg l⁻¹ indole-3-acetic acid (IAA). The plantlets developed on the leaf margin were employed for transformation. The Agrobacterium suspension was prepared for the inoculation as described by Liu et al. (2014). Leaf discs were excised from plantlets and infected for 1 h with the bacteria on a reciprocal shaker at room temperature, blotted on sterile filter paper, and then placed in a Petri dish containing MS30 medium with 1 mg l⁻¹ TDZ, 0.2 mg l⁻¹ IAA, and 100 μM l⁻¹ acetosyringone. The co-cultivation was conducted for 4 d in the dark at 25±1 °C. After the co-cultivation, the leaf discs were washed twice with liquid MS30 medium containing 300 mg l⁻¹ timentin, and then cultured on solid MS30 medium supplemented with 1 mg l⁻¹ TDZ, 0.2 mg l⁻¹ IAA, 300 mg l⁻¹ timentin, and 150 mg l⁻¹ kanamycin under 16 h of light (~55 μmol m⁻² s⁻¹) and 8 h of dark. The leaf discs were transferred to fresh medium every 2 weeks. Six weeks after selection, the obtained kanamycin-resistant calli were transferred to solid MS30 medium with 1 mg l⁻¹ benzylaminopurine (BP), 0.2 mg l⁻¹ IAA, 300 mg l⁻¹ timentin, and 150 mg l⁻¹ kanamycin. The regenerated shoots were further transferred to solid MS30 medium with 300 mg l⁻¹ timentin and 150 mg l⁻¹ kanamycin for root induction.

Detection of mutations

Genomic DNA was extracted from the leaves of transgenic plants and wild-type plants using the GenElute™ Plant Genomic DNA Miniprep.
Fig. 1. Target selection and vector construction. (A) Target site selection in the KfePHOT2 gene. The two sgRNA target sites (KfePHOT2-T1 and KfePHOT2-T2) were located in the fifth and 12th exon of KfePHOT2, respectively. (B) Schematic representation of the CRISPR/Cas9 system binary vector used in this study. Two Arabidopsis thaliana promoters, U6-26 and U6-29, were used to drive the two sgRNAs targeting KfePHOT2-T1 and KfePHOT2-T2, respectively. The two sgRNA expression cassettes were inserted into the binary vector pKSE401 (Xie et al., 2014). ‘LB’ and ‘RB’ are the left and right borders of T-DNA, respectively. KanR, kanamycin resistance gene as a selection marker for screening transgenic plants; 35S and rbcS-E9, 35S promoter and rbcS-E9 terminator, respectively.

Fig. 2. Targeted mutagenesis of Kalanchoë fedtschenkoi PHOTOTROPIN 2 (KfePHOT2) by a dual-sgRNA CRISPR/Cas9 system. Sequences from selected biallelic mutant lines kfephot2_L5 (A) and kfephot2_L10 (B) containing site-specific mutations. Indels are shown in red rectangles, in which deletions are shown as dashes, and insertions are denoted with letters. Sequences between two target sites are indicated by black dots. PAM, protospacer adjacent motif; sgKfePHOT2-T1 and T2, sgRNAs targeting KfePHOT2-T1 and KfePHOT2-T2, respectively; MT, mutation type.

Table 1. Off-target analysis of sgRNAs in the mutant lines

| sgRNAs | Putative off-target sequences (genomic region) | Putative off-target gene | Putative off-target location | Lines examined | Lines with off-targets |
|--------|--------------------------------------------|--------------------------|-----------------------------|----------------|-----------------------|
| KfePHOT2_T1 | AtgTGACTgAGCcACCTCCTTG (Scaffold_37: +1183708) | Kaladp0037s0155 | Exon | 2 | 0 |
| KfePHOT2_T2 | CCGcTgGGAATggTGATACCTG (Scaffold_18: ±1311969) | Kaladp0018s0188 | Exon | 2 | 0 |
| KfePHOT2_T2 | CCGcTgGGAATggTGATACCTG (Scaffold_55: -568324) | Kaladp0055s0063 | Exon | 2 | 0 |

a KfePHOT2: phototropin 2 in Kalanchoë fedtschenkoi. T1 and T2 are the targeted sites in the corresponding gene.
b Underlining indicates mismatched bases.
c Two independent lines (kfephot2-L5 and kfephot2-L10) having different mutations were used for off-target analysis.
Kit (Sigma-Aldrich, St. Louis, MO, USA). A PCR screening was performed to verify the insertion of the CRISPR transgene with primers specific to the sequence of Cas9/sgRNA vector before further characterization.

The potential edited regions of targeted genes were amplified using gene-specific primers (Supplementary Table S2) with Q5® High-Fidelity DNA Polymerases (New England Biolabs, Ipswich, MA, USA). PCR amplifications were performed as described by Xing et al. (2014). The PCR products were purified using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, USA) and then either subjected to direct Sanger sequencing or inserted into the pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA) followed by the Sanger sequencing service of Eurofins Genomics LLC (Louisville, KY, USA). Sequence results were aligned against the sequence of the untransformed control to identify mutations using SnapGene software (GSL Biotech; https://www.snapgene.com/).

Gas exchange analysis

Two-month-old plants were grown in 18-cm diameter pots containing Fafard 3B soil mix (Sun Gro Horticulture, Agawam, MA) in 12 h of light (250 μmol m–2 s–1), 25 ºC, 60% humidity, and 12 h of dark, 18 ºC, 70% humidity in a walk-in controlled-environment chamber (Conviron model BDW80, Winnipeg, Canada). Leaf pair 6 (LP6; counting from the top of the plants) was used for gas exchange analysis. The gas exchange measurements were performed at a CO2 concentration of 400 μmol mol–1, 60% relative humidity, 500 μmol s–1 air flow, and a photon flux density of 250 μmol m–2 s–1 using a LI-6400-XT Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA). The leaf was continuously clamped into the leaf cuvette. All experiments were repeated at least three times using intact LP6 (Supplementary Fig. S1) from three separate clonal plants.

Statistical analysis

All quantification experiments were repeated three times and the data, presented as the mean ±SD, were analyzed by a two-tailed Student’s t-test. A P-value of <0.05 or <0.01 was considered statistically significant.

Results

Target selection and vector construction

To explore the capability of CRISPR/Cas9 systems based on dual-sgRNAs to induce the targeted indel mutagenesis in the CAM plant K. fedtschenkoi, phototropin 2 (Kaladp0033s0113, KfePHOT2), the ortholog of a blue light photoreceptor involved in blue light regulation of stomatal opening in Arabidopsis (AT5G58140), was chosen as a target gene. Recently, we found that the timing of peak transcript abundance of PHOT2 shifted from dawn in Arabidopsis (AT5G58140) to dusk in the CAM species K. fedtschenkoi (Kaladp0033s0113, KfePHOT2) and A. comosus (Aco014242) (Yang et al., 2017), leading to a hypothesis that PHOT2 might be involved in the CAM pathway. Here we also sought to utilize the CRISPR/Cas9-mediated biallelic indel mutagenesis to test the hypothesis through loss-of-function analysis.

Two target sites (designated as KfePHOT2-T1 and KfePHOT2-T2) within two different exon regions were selected for sgRNA design (Fig. 1A; Supplementary Table S1). Promoters U6-26 and U6-29 were used to drive expression of the two gRNAs targeting KfePHOT2-T1 and KfePHOT2-T2, respectively (Fig. 1B). The expression cassettes, including the two targets, were cloned into the binary vector pKSE401 (Xie et al., 2014). The assembled construct was transferred into K. fedtschenkoi through Agrobacterium-mediated transformation.

Characterization of mutations in mutant lines in the first generation

CRISPR/Cas9-induced mutations in the transgenic plants were identified by Sanger sequencing (Feng et al., 2018). The allelic status of transmontants was divided into two categories (Char et al., 2017): (i) monoallelic (MA) mutants having a mutation in one allele of the target gene (or locus) regardless of the site of the allele with the other allele unmutated; and (ii) diallelic/biallelic (DA) mutants having mutations (the same mutation or different mutations) in both homologous copies of the gene. The assembled construct was transferred into K. fedtschenkoi through Agrobacterium-mediated transformation.

Characterization of mutations in mutant lines in the first generation

CRISPR/Cas9-induced mutations in the transgenic plants were identified by Sanger sequencing (Feng et al., 2018). The allelic status of transformants was divided into two categories (Char et al., 2017): (i) monoallelic (MA) mutants having a mutation in one allele of the target gene (or locus) regardless of the site of the allele with the other allele unmutated; and (ii) diallelic/biallelic (DA) mutants having mutations (the same mutation or different mutations) in both homologous copies of the gene.
target gene (or locus). Our sequencing analysis detected mutations at both target sites of KfePHOT2. We determined that 12 of 13 randomly selected transgenic lines (92.3% efficiency) had mutations in KfePHOT2. Among the 12 mutant lines, two lines were MA mutants and the other 10 lines were DA mutants, suggesting that the CRISPR/Cas9 system based on dual-sgRNAs is highly efficient in creating biallelic indel mutagenesis. Two homogeneous DA mutants (kfephot2-L5 and kfephot2-L10), in which the indel mutations lead to disruption of protein-coding sequences, were used for phenotype characterization (Fig. 2).

**Off-target analysis**

To evaluate the precision of CRISPR/Cas9, potential off-target loci following PAM sequences that are highly homologous to the sgRNAs targeting KfePHOT2 were predicted and analyzed for potential cleavage by CRISPR/Cas9 (Table 1). Sequencing of the PCR products showed that no mutations were found in these putative off-target sites (Table 1), indicating that this dual-sgRNA CRISPR/Cas9 system is highly efficient and precise.

![Fig. 4. Physiological characterization of the kfephot2 mutant. (A) CO2 fixation and (B) stomatal conductance for leaf pair 6 (counting from the top of the plants) using plants pre-entrained for 7 d under 12 h light/12 h dark cycles. The black and white bars indicate night-time and daytime, respectively. Data are presented as mean ±SD (n=3 different leaves). * and ** indicate a significant difference from that of the WT at P<0.05 and P<0.01, respectively, as determined by two-tailed Student’s t-test.](image-url)
Morphological and physiological characterization of the kfephot2 mutant

Growth performance of the kfephot2 mutants under well-watered conditions was evaluated. Although all plants grew well, the kfephot2 mutants had significantly lower fresh and dry weight of the shoots and roots than the wild type (WT) (Fig. 3). To investigate the impact of the kfephot2 mutations on CAM physiology, we investigated the 24 h pattern of stomatal conductance and CO₂ exchange for full-CAM leaves (LP6) from two DA mutants (kfephot2-L5 and kfephot2-L10), and the WT plants that had been entrained in 12 h light/12 h dark cycles under well-watered conditions (Fig. 4). Both the WT and the kfephot2 mutants fixed the majority of their CO₂ at night (phase I of CAM: stomata open, primary atmospheric CO₂ fixation via phosphoenolpyruvate carboxylase) (Fig. 4A).

In comparison with WT plants, the kfephot2 plants showed reduced stomatal conductance and CO₂ fixation in the late afternoon (phase IV of CAM: stomata re-open to allow net CO₂ uptake via Rubisco) and increased stomatal conductance and CO₂ fixation during the night (phase I of CAM) (Fig. 4A, B). No differences in dawn (phase II of CAM: stomata remain open briefly, CO₂ fixation via phosphoenolpyruvate carboxylase and Rubisco) or daytime (phase III of CAM: stomata are closed, Rubisco refixation of CO₂ released from malate decarboxylation) were observed. These results demonstrated that the biallelic indel mutations of KfePHOT2 generated by the CRISPR/Cas9 system confirmed the predicted function of KfePHOT2 relevant to CAM physiology, suggesting that the CRISPR/Cas9 system has great potential for the rapid genotype–phenotype mapping in plants.

Discussion

Roles of Phototropin 2 in the CAM pathway

The role of blue light in the CAM pathway has not been fully resolved yet (Males and Griffiths, 2017). Some studies show the involvement of blue light signaling in the induction of CAM in Clusia minor (Grams and Thiel, 2002) and in the regulation of stomatal conductance in CAM bromeliads (Ceusters et al., 2014). However, two other studies have found that facultative CAM plants display blue light regulation of stomatal conductance only when these plants are in C₃ mode, but not in the CAM mode of photosynthesis (Lee and Assmann, 1992; Tallman et al., 1997). Recently, we hypothesized that the blue light receptor gene KfePHOT2 was involved in the CAM pathway (Yang et al., 2017). More recently, Gotoh et al. (2019) showed that blue light-dependent stomatal opening occurs in the obligate CAM plants Kalanchoë pinnata and K. daigremontiana independent of photosynthetic CO₂ assimilation mode. In this study, we found that knocking out of
$KfePHOT2$ reduced stomatal conductance and CO$_2$ fixation in the late afternoon (phase IV of CAM: stomata re-open to allow net CO$_2$ uptake via Rubisco) and increased stomatal conductance and CO$_2$ fixation during the night (phase I of CAM) (Fig. 4). These results support the hypothesis that blue light plays an important role in the CAM pathway.
Application of CRISPR/Cas9 systems to functional genomics research of CAM plants

In this study, we demonstrate for the first time that CRISPR/Cas9 technology enables efficient targeted indel mutagenesis for rapid and efficient generation of desirable mutants in the model obligate CAM species \textit{K. fedtschenkoi}. As proof-of-concept, we found novel insights into the roles of \textit{KfePHOT2} in the CAM pathway. Our results showed that the \textit{KfePHOT2} gene was involved in CO\textsubscript{2} fixation and stomatal regulation in \textit{K. fedtschenkoi} plants. Phototropin-dependent blue light signaling plays a key role in the regulation of stomatal opening by inducing a distinct starch degradation pathway in guard cells (Horrer et al., 2016), and maintaining circadian oscillation of PSII operating efficiency (Lithauer et al., 2015) in the C\textsubscript{3} plant \textit{Arabidopsis}. Therefore, it would be interesting to investigate the impact of \textit{KfePHOT2} knockout on the stomatal opening, starch degradation, and circadian rhythm in the mutants in the future. Also, we observed that the \textit{kfephot2} mutant plants grew more slowly than the WT, but had higher diel carbon gain. We hypothesize that the \textit{KfePHOT2} gene may be involved in other biological processes, such as phototropism (Harmer and Brooks, 2018), root growth (Galen et al., 2007), chloroplast relocation (Jarillo et al., 2001), palisade cell development (Kozuka et al., 2011), anthocyanin accumulation (Kadomura-Ishikawa et al., 2013), and so on, as demonstrated in other plant species. This hypothesis can be tested through comprehensive phenotypic, transcriptomic, and metabolomic analyses of gain-of-function and loss-of-function mutants of \textit{KfePHOT2} in the future. At this point, we do not know why mutants grew more slowly than the WT in spite of greater carbon gain of LP6. Future study could be required to test whether the difference in growth rate is caused by the difference in leaf area ratio (LAR; the ratio of total leaf area per plant to total dry mass per plant). Genome-wide sgRNA design was performed with CASPER (Mendoza and Trinh, 2018) to streamline and facilitate rapid and wide-scale use of CRISPR/Cas9-based technologies for \textit{K. fedtschenkoi}. A total of 3,270,513 sgRNAs targeting 45,183 protein-coding transcripts were identified (File 1 at Dryad). A pipeline for streamlined application of the CRISPR/Cas9 system to the functional genomics research of CAM plants is illustrated in Fig. 6. The entire experimental cycle was ~22–25 weeks (Fig. 6), counting from the target design to the phenotyping analysis. The confidence in target region and potential off-target regions.

The genome-wide sgRNA database of \textit{K. fedtschenkoi} (File 1) and off-targets information (File 2) were deposited at the Dryad Digital Repository (https://datadryad.org/stash/dataset/doi:10.5061/dryad.3qk1303). The off-target score in the genome-wide sgRNA database (File 1) is presented as the average score. The off-target score in the genome-wide off-targets database (File 2) reports scores of individual off-targets above the cut-off value of 0.05.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Gas exchange assay.

Table S1. Summary of the target sequences of sgRNAs.

Table S2. Primer sequences used for PCR amplification of sgRNA target regions and potential off-target regions.

Acknowledgements

This research is supported by the Department of Energy (DOE), Office of Science, Genomic Science Program under Award Number DE-SC0008834, and the Center for Bioenergy Innovation (CBI), which is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. We thank Timothy J. Tschaplinski for critical review and comments on the manuscript. Oak Ridge National Laboratory is managed by UT-Battelle, LLC for the US DOE under contract no. DE-AC05-00OR22725. CTT would also like to acknowledge financial support through the DARPA YFA award #D17AP00023. The views, opinions, and/or findings contained in this article are those of the authors and should not be interpreted as representing the official views or policies, either expressed or implied, of the funding agencies. The authors declare no conflicts of interest.

Author contributions

XY conceived, designed, and supervised the project, and reviewed and edited the manuscript; DL conceived, designed, and performed the experiments, and wrote the manuscript; MC, HC, and RH, performed the experiments; LL interpreted the data; BM and CT performed sgRNA design, and wrote the manuscript; and GT reviewed and edited the manuscript.
References

Abraham PE, Yin H, Borland AM, et al. 2016. Transcript, protein and metabolite temporal dynamics in the CAM plant Agave. Nature Plants 2, 16178.

Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. 2015. Editing plant genomes with CRISPR/Cas9. Current Opinion in Biotechnology 32, 76–84.

Borland AM, Guo HB, Yang X, Cushman JC. 2016. Orchestration of carbohydrate processing for crassulacean acid metabolism. Current Opinion in Plant Biology 31, 118–124.

Borland AM, Hartwell J, Weston DJ, Schlauch KA, Tschapliniski TJ, Tuskan GA, Yang X, Cushman JC. 2014. Engineering crassulacean acid metabolism to improve water-use efficiency. Trends in Plant Science 19, 327–338.

Borland AM, Leverett A, Hurtado-Castano N, Hu R, Yang X. 2018. Functional anatomical traits of the photosynthetic organs of plants with crassulacean acid metabolism. In: Adams WW III, Terashima I, eds. The leaf: a platform for performing photosynthesis. Cham: Springer, 281–305.

Boxall SF, Dever LV, Knerová J, Gould PD, Hartwell J. 2017. Phosphorylation of phosphoenolpyruvate carboxylase is essential for maximal and sustained dark CO2 fixation and core circadian clock operation in Arabidopsis. The Plant Cell 23, 3684–3695.

Lee DM, Assmann SM. 1992. Stomatal responses to light in the facultative crassulacean acid metabolism species, Portulacaria afra. Physiologia Plantarum 85, 35–42.

Lithtauer S, Battle MW, Lawson T, Jones MA. 2015. Phototropins maintain robust circadian oscillation of PSL1 operating efficiency under blue light. The Plant Journal 83, 1034–1045.

Liu D, Chen M, Mendoza B, Cheng H, Hu R, Li L, Trinh CT, Tuskan GA, Yang X. 2019. Data from: CRISPR/Cas9-mediated targeted mutagenesis for functional genomics research of crassulacean acid metabolism plants. Dryad Digital Repository. https://datadryad.org/stash/dataset/doi:10.5061/dryad.3qk1303.

Liu D, He S, Zhai H, Wang L, Zhao Y, Wang B, Li R, Liu Q, Culture O, 2014. Overexpression of bPSKR enhances salt tolerance in transgenic sweetpotato. Plant, Tissue and Organ Culture 117, 1–16.

Liu D, Hu R, Palla KJ, Tuskan GA, Yang X. 2016. Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research. Current Opinion in Plant Biology 40, 70–77.

Liu D, Palla KJ, Hu R, et al. 2018. Perspectives on the basic and applied aspects of crassulacean acid metabolism (CAM) research. Plant Science 274, 394–401.

Ma X, Zhu Q, Chen Y, Liu YG. 2016. CRISPR/Cas9 platforms for genome editing in plants: developments and applications. Molecular Plant 9, 961–974.

Males J, Griffiths H. 2017. Stomatal biology of CAM plants. Plant Physiology 174, 550–560.

Mendoza BJ, Trinh CT. 2018. Enhanced guide-RNA design and targeting efficiency of CRISPR/Cas9 genome editing of single and consortia of industrially relevant and non-model organisms. Bioinformatics 34, 16–23.

Puchta H. 2017. Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. Current Opinion in Plant Biology 36, 1–8.

Salesse-Smith CE, Sharwood RE, Busch FA, Kromdijk J, Bardal V, Stern DB. 2018. Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize. Nature Plants 4, 802–810.

Shan Q, Wang Y, Li J, et al. 2013. Targeted genome modification of crop plants using a CRISPR–Cas system. Nature Biotechnology 31, 686–688.

Shimatan Z, Kashojoya S, Takayama M, et al. 2017. Targeted base editing in rice and tomato using a CRISPR–Cas9 cytidine deaminase fusion. Nature Biotechnology 35, 441–443.

Tallman G, Zhu J, Mawson BT, Amodeo G, Nouchi Z, Levy K, Zeiger E. 1997. Induction of CAM in Mesembryanthemum crystallinum abolishes the stomatal response to blue light and light-dependent xanthophyll formation in guard cell chloroplasts. Plant and Cell Physiology 38, 236–242.

Winter K. 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. Journal of Experimental Botany 70, 6495–6508.

Xie K, Minkenberg B, Yang Y. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proceedings of the National Academy of Sciences, USA 112, 3570–3575.

Xie S, Shen B, Zhang C, Huang X, Zhang Y. 2014. sgRNAas9: a software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. PLoS One 9, e100448.

Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biology 14, 327.

Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS. 2006. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiology 142, 429–440.

Yang X, Cushman JC, Borland AM, et al. 2015. A roadmap for research on crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. New Phytologist 207, 491–504.

Yang X, Hu R, Yin H, et al. 2017. The Kalancheø genome provides insights into convergent evolution and building blocks of crassulacean acid metabolism. Nature Communications 8, 1899.