Disruption of *Phytoene Desaturase* Gene using Transient Expression of Cas9: gRNA Complex

Muhammad Salman Mubarik1, Sultan Habibullah Khan12*, Aftab Ahmad2, Zulqurnain Khan1, Muhammad Sajjad3 and Iqra Ahmad Khan4

1Centre of Agricultural Biochemistry & Biotechnology (CABB), University of Agriculture Faisalabad 38040, Pakistan
2U.S.-Pakistan Center for Advanced Studies in Agriculture and Food Security (US-PCAS-AFS), University of Agriculture Faisalabad 38040, Pakistan
3Department of Environmental Sciences, COMSATS Institute of Information Technology, Vehari 61100, Pakistan
4Institute for Horticultural Sciences, University of Agriculture Faisalabad 38040, Pakistan

*For correspondence: sultan@uaf.edu.pk*

Abstract

Engineered nucleases have emerged as a powerful tool for site specific gene manipulation in plants. Based on Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated (CRISPR/Cas) system, engineered Cas9:gRNA complex can be used to cleave specific DNA sequences in the genome. In the present study, *Nicotiana benthamiana Phytoene Desaturase* (*NbPDS*) gene was targeted by CRISPR/Cas9 system. The plant codon optimized (pcoCas9) along with guided RNA (gRNA) was cloned in plant expression vector pGreen0029, to target *NbPDS* gene. The *NbPDS* gene was disrupted transiently by agroinfiltration of pcoCas9-gRNA complex. Visible albino spots were observed on *agro*-infiltrated leaves of *N. benthamiana* plants after 7 days of infiltration. The observed albino spots were analyzed through PCR amplification of gRNA-target, fluorescent microscopy and chlorophyll contents measurements. Our results support the notion that CRISPR/Cas9 system is a swift, robust and useful tool for targeted gene disruption, deletion and editing. © 2016 Friends Science Publishers

Keywords: Site specific genome editing; CRISPR/Cas9 system; *NbPDS* gene; Transient expression

Introduction

After years of research, biologists eventually developed tools to precisely identify and target specific DNA sequence. This nature’s DNA recognition system resulted into a general system for manipulating genes. Three precise genome manipulation approaches have been established which include zinc finger nucleases (ZFNs), transcription activators like effector nucleases (TALENs) and CRISPR/Cas system (Zhang et al., 2014). These targeted genome manipulation technologies provide a new generation of tools to answer core biological questions that could include DNA repair mechanism, recombination, metabolism and stress response. These systems could also have medical applications through their capability of introducing precise mutations to cure genetic diseases and supplying the correct template for DNA repair pathways to adopt and re-write the mutated sequence (Sander and Joung, 2014).

Targeted genome manipulation techniques have the potential to rectify concerns over insertion of foreign DNA into natural systems and the random DNA integration process. This would help in casting away the doubts of genetically modified crops in the public. These new biotechnological advances would also improve the overall quality and quantity of current crop production, and accelerate breeding of climate resilient cultivars by combining beneficial traits. ZFNs, TALENs and CRISPR/Cas systems are utilized to produce double strand breaks (DSBs) at specific genomic sites (Weinthal et al., 2010). The DSBs can then be directed to repair by Non Homologous End Joining (NHEJ) to cause mutation or through Homologous Directed Recombination (HDR) to introduce new DNA sequences (Miao et al., 2013). Thus, these technologies can be used to replace the native DNA sequences with foreign DNA, to integrate the targeted transgene into native DNA, to stimulate the repair of defective genes, and site specific mutants in crop plants. These approaches can help researchers in a broad range of applications in genome wide experiments in crop plants and the production of multi-resistant disease models. Methods for introducing site-specific DSBs in genomic DNA have transformed our ability to engineer eukaryotic organisms by initiating DNA repair pathways that lead to targeted genetic re-programming. Although, ZFNs and TALENs have proved effective for such genomic manipulation but their
use has been limited by the need to engineer a specific protein for each dsDNA target site and by off-target activity (Urnov et al., 2005; Bogdanove and Voytas, 2011). Thus, alternative strategies for triggering site-specific DNA cleavage in eukaryotic cells are of great interest. CRISPR/Cas9 system has been becoming one of the powerful tools in plant biotechnology. Due to cheap and quick method of inducing site-specific genome modification, the CRISPR/Cas system could potentially transform next generation genome scale studies. It makes possible to introduce plant genome modifications, which are indistinguishable from those introduced by conventional breeding and chemical or physical mutagenesis. Unlike its predecessors, the CRISPR/Cas9 system does not require any protein engineering steps, making it much more straightforward to test multiple gRNAs for each target gene. Furthermore, only 20 nucleotides in the gRNA sequence need to be changed to confer a different target specificity, which means that cloning is also unnecessary (Cho et al., 2013). A very peculiar feature of this system is that the crops produce by this system may be classified as non-GM crops. It would have an enormous positive impact on the development of the plant biotechnology and breeding sector (Belhaj et al., 2013; Lusser and Davies, 2013).

The CRISPR/Cas system is a natural system used by about 40% of bacteria and 90% of archaea as a form of adaptive immunity against invading viral or plasmid DNA sequences (Redder et al., 2009). The bacterial CRISPR/Cas system consists of Cas protein operons, CRISPR locus, and two non-coding RNAs. The main functional element in this system is the Cas protein, providing the nuclease activity. The invading mobile genetic element integrate into CRISPR locus. It is transcribed and processed into CRISPR RNAs (crRNAs). These crRNAs specifically guide the Cas protein machinery to their complementary targets (invading viruses or plasmids DNA). Thus, the CRISPR/Cas system can provide the host with acquired and heritable resistance (Marraffini and Sontheimer, 2008).

The easiness and versatility of the CRISPR/Cas9 system enable biologists to develop selectable marker free gene engineering with high accuracy in different crop plants (Li et al., 2013). Unique features (tolerates DNA methylation and different from ZFN and TALEN) and high efficiency of CRISPR/Cas system gives leverage for genome manipulation in plants such as rice (Ma et al., 2015). In rice and wheat, sequence specific genome modification has been induced by Cas9: sgRNA (Shan et al., 2013; Li et al., 2016). The Arabidopsis, tobacco and sorghum genome modifications also achieved by CRISPR/Cas system (Jiang et al., 2013). In rice OsPDS-SP1 gene mutations were identified in 9 of 96 independent transgenic plants with 9.4% mutation efficiency, and in rice OsBADH2 gene mutations were identified in 7 of 98 transgenic plants with 7.1% mutation efficiency (Shan et al., 2013).

Over the years, *Nicotiana benthamiana* has been used as a model system to study gene functions especially using transient expression of genes via agro-infiltration. *Agrobacterium* mediated transient gene expression has been established for processes such as assigning gene functions, promoter element analysis (Hellens et al., 2005). *N. benthamiana* is especially useful for transiently expressing genes via agroinfiltration (Goodin et al., 2008). Furthermore, *N. benthamiana* can be transformed with high efficiency and easily maintained due to its short stature, short regeneration time and high seed production (Goodin et al., 2008). The *N. benthamiana* plants can be transformed easily with remarkably high regeneration capacity. It is also very useful tool to study virus induced gene silencing and expressing genes transiently using agro-inoculation. It is very popular system to study protein localization, protein-protein interaction and expression of proteins which can be easily purified. Agro infiltration has been used in different types of experiments, gene function studies (Wroblewski et al., 2005), host pathogen interaction (Tang et al., 2002), protein production (Vaquero et al., 1999), protein-protein interaction (Obori et al., 2007) and protein localization (Bhat et al., 2007). In a variety of plant species, agroinfiltration has been applied successfully including *N. benthamiana*, *Arabidopsis*, tomato, pea, pepper and rose (Abramovitch et al., 2006). The major advantage of agroinfiltration on stable transformation procedures is its ease and speed. In addition, comparing with other transient expression systems like protoplast transformation (Sheen 2001), gene gun mediated transformation (Schweizer et al., 1999) and microinjection (Bilang et al., 1993), agroinfiltration has the advantages that it is simple, cheap and can be exploited for intact plant leaves, hence a relative large leaf area can be transformed (Kapila et al., 1997).

The objective of our study was to establish CRISPR/Cas9 system to evaluate the gene function through transient expression of pcoCas9-gRNA cassettes. The results demonstrated the feasibility of CRISPR/Cas9 system in gene functional studies and its potential utilization in transgenic approaches.

### Materials and Methods

#### Plant Growth

Seeds of *N. benthamiana* were grown in plastic pots containing standard germination soil at 25°C with 75 μmol light intensity under photoperiods of 16-h light and 8-h dark. Seedlings were transferred to new pots after 8 days of sowing containing potting soil (one seedling per pot). The plants were raised at 25°C with mentioned light intensity. Three weeks after transplanting, plants attained optimal developmental stage to be used for agro-infiltration. At this stage the plants had 4–5 fully developed true leaves and no visible flower buds (Fig. 1A).
Synthesis of Guided RNA

We targeted the NbPDS gene to demonstrate RNA-guided genome editing in plants. Twenty nucleotides of NbPDS gene (5′-CAGGCTTATCCACTGAGT-3′) were manually selected (Hwang et al., 2013) as guided RNA target region. The essential criterion of target selection was the presence of NGG tri-nucleotide protospacer adjacent motif (PAM) at the 3′ end of target region. The chimeric gRNA containing 20 nucleotide target sequence and 80 nucleotide scaffold sequence was commercially synthesized (e-oligos, Gene Link™ NY, USA) to target NbPDS gene.

Cloning of pcoCas9 and gRNA in pGreen0029

Plant expression vectors were constructed by cloning of pcoCas9 and gRNA into pGreen0029 vector. Firstly, both pcoCas9 and gRNA were cloned in a single pGreen0029 vector then both pcoCas9 and gRNA were also cloned separately in pGreen0029. The HBT-pcoCas9 vector containing pcoCas9 gene fused with 35SPPDK promoter, FLAG tag, nuclear localization signal and NOS terminator was obtained from Department of Molecular Biology, Harvard Medical School, USA (Li et al., 2013). The gRNA cassette was taken from commercially synthesized pJET-gRNA construct (Gene Link™ NY, USA).

Agro-inoculation of Nicotiana benthamiana Leaves

Agrobacterium tumefaciens strain GV3101 harboring pGreen0029-pcoCas9, pGreen0029-gRNA and pGreen0029-pcoCas9:gRNA were introduced into N. benthamiana leaves through agro-inoculation. pcoCas9 and gRNA were used in different combinations: i) pGreen0029-pcoCas9:gRNA, ii) pGreen0029-pcoCas9 and pGreen0029-gRNA, iii) pGreen0029 empty vector. Three to four weeks old N. benthamiana plant leaves were infiltrated with using the standard protocols (Van der Hoorn et al., 2000) (Fig. 1B) The pcoCas9-gRNA mediated disruption of NbPDS gene was examined 7 days’ post infiltration (Li et al., 2013).

Phenotypic Screening of NbPDS Disruption

The NbPDS gene is involved in oxidoreductase activity, acting on paired donors; it incorporates or reduces molecular oxygen during the carotenoid biosynthetic pathway. PDS is the first enzyme in the carotenoid biosynthetic pathway to convert the colorless phytoene to colored carotenoids in plants (Li et al., 2013). Disruption of PDS results into clearly visible albino spots on leaves was easily observed.

Chlorophyll Contents Measurement

Chlorophyll contents were measured to confirm NbPDS gene disruption. The chlorophyll contents were measured with a SPAD-502 chlorophyll meter (Konica Minolta, Singapore). Triplicate measurements were made for each infiltrated and control plants, on each individual leaves from the rosette of 3 to 4 weeks old N. benthamiana plants. Analysis of variance (ANOVA) was applied on SPAD value indicating chlorophyll contents.

Fluorescent Microscopy

Careful screening of infiltrated leaves after the degradation of existing chlorophyll is necessary for further characterization using fluorescent microscopy. We performed Photo bleaching experiments using the Olympus IX51 (Olympus America Inc.). Briefly, 25 mm² sections of leaf tissues were excised from control and agro-infiltrated leaves and mounted on glass slides in water, slides were covered with a glass coverslip. Images were acquired prior to agro-infiltration, followed by an additional image to monitor photo bleached cells after agro-infiltration.

PCR Detection of Plant Expression Vectors in Infiltrated Leaves

Primers used (gRNA forward 5′-TCCAGGTTAATTCTATTAC-3′ and gRNA reverse 5′-CGAAGATTGAACAGATTT-3′) to detect mutation in NbPDS gene were designed from gRNA-target region (gRNA reverse primer was designed within target site of gRNA) (Supplementary Fig. 1). PCR amplification of control and infiltrated leaves was carried out using Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Massachusetts, USA). A 20 μL reactions containing 10 μL 2X Phire Plant PCR Buffer, 0.4 μL Phire Hot Start II DNA Polymerase, 0.5 mm diameter leaf sample, 0.5 μM of each primer and add H₂O to 20 μL reaction. The thermal cycle profile was as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 45 Sec, extension at 72°C for 30 Sec, final extension at 72°C for 5 min, and cooling at 4°C. The PCR products were separated on a 1% agarose gel. Amplification of DNA from untransformed N. benthamiana leaf was used as control.

Results

Target Selection and gRNA Designing

We targeted NbPDS gene to demonstrate the RNA-guided pcoCas9-based gene editing in plants. Target site of 20 nucleotides was selected manually and chimeric gRNA was commercially synthesized as described by Hwang et al. (2013). The PAM sequence is absolutely necessary for target binding and the exact sequence is dependent upon the species of Cas9 (5′-NGG-3′ for S. pyogenes Cas9). We used Cas9 from S. pyogenes as it is currently the most widely used in plant genome engineering. We used 20 nucleotide target sequence of NbPDS gene fused with 80 nucleotide RNA scaffold sequence as described previously (Li et al., 2013).

Construction of Plant Expression Vectors

For the construction of plant expression vectors (Fig. 2), Firstly, HBT-pcoCas9 and pGreen0029 vectors were digested with BanHI and EcoRI. Required digested fragments were purified from agarose gel and ligated to construct pGreen0029-pcoCas9 expression vector (Fig. 3a). Similarly, pGreen0029 and pJET-gRNA vector were digested with EcoRI and SacI and excised fragments were purified from gel and finally ligated to complete pGree0029-gRNA expression vector (Fig. 3b). To combine pcoCas9 and gRNA cassettes in one vector, pGreen0029-pcoCas9 and pJET-gRNA vectors were digested with KpnI and XhoI and the required fragments were gel purified and ligated to complete pGreen0029-pcoCas9:gRNA vector containing pcoCas9 and gRNA cassettes (Fig. 3c).

We studied the efficiency of the RNA-guided pcoCas9 system to introduce mutations at a specified genomic region using agroinfiltration mediated plant transformation method. Different three plant expression vectors containing 35SPPDKpcoCas9, U6PoIII:gRNA and 35SPPDK:pcoCas9,::U6PoIII:gRNA cassettes were transformed into N. benthamiana in different combinations. No phenotypic albino spots were observed when N. benthamiana leaves were agro-infiltrated only with pcoCas9 or gRNA cassettes (Fig. 4a, b). When both pcoCas9 and gRNA expression cassettes were co-inoculated in 1:1 ratio, we observed few albino spots on infiltrated area (Fig. 4c, d). However, frequent and clearly visible albino spots were observed on leaves infiltrated with pcoCas9-gRNA after 7 days. Results clearly revealed that pcoCas9-gRNA is more effective due to close vicinity and high mutation rate in NbPDS gene as compared to when inoculated in separate plasmids (Fig. 4e, f).

PCR Amplification of Target Region

Genomic DNA samples isolated from infiltrated and control N. benthamiana leaves were used as template in PCR amplification. Primers designed on gRNA-target site of NbPDS gene produced 168 bp product of varying intensity (less intense bands in the DNA isolated from infiltrated leaf sections) which shows the disruption of targeted nucleotide sequence (Fig. 5).

Chlorophyll Contents in Photobleached Phenotype

Agroinfiltration experiment was performed to evaluate the suppression of NbPDS gene expression in leaves. Infiltrated leaves showed different levels of albinism, from just discernable to clearly distinguishable. The chlorophyll contents were decreased significantly in agro-infiltrated leaves as compared to control leaves. Chlorophyll contents were measured using SPAD-502 meter by taking three readings for each leaf.

![Plant expression vectors used in this study. (a) A pGreen0029 based plant expression vector having pcoCas9; (b) A plant expression vector containing gRNA expression cassette; (c) A plant expression vector containing both pcoCas9 and gRNA expression cassettes](image-url)

The analysis of variance for chlorophyll contents showed that treatments have significant effects on chlorophyll content reduction. The range of SPAD value for chlorophyll contents in control plants vary from 30.7 to 33.2, whereas, SPAD value for chlorophyll contents in plants infiltrated with pcoCas9 and gRNA in two different vectors and in same vector varies from 1.4 to 2.5 and 0.0 to 0.0 respectively (Table. 1).

Fluorescent Microscopy to Observe the Photobleached Cells

We carefully examined N. benthamiana leaves before and after the agro infiltration to check the degradation of
existing chlorophyll due to mutation in NbPDS gene using fluorescent microscope. Leaves having phenotypically visible albino spots showed photo bleached cells (Fig. 6a, b) and control leaves cells were normal under fluorescent microscope (Fig. 6c, d). Infiltrated leaves with less albino symptoms also showed photo bleached cells along with normal cells (Fig. 6e, f).

Discussion

The CRISPR/Cas9 system is much simpler than the other targeted genome modification systems such as ZFN and TALEN (Zhang et al., 2010). High-efficiency editing can be achieved even in large and complex plant genomes at each of the multiple targeted locations using the CRISPR/Cas9 system. Because of the simplicity in designing gRNA and the applicability to a wide variety of plants and animals, the CRISPR/Cas9 system has been proved as a useful tool for reverse genetics as well as functional genomics studies (Cong et al., 2013).

The application of the CRISPR/Cas9 system has been demonstrated in a variety of organisms like bacteria, yeast and animal cells with a high percentage of mutations (Cong et al., 2013; DiCarlo et al., 2013; Jinek et al., 2013; Mali et al., 2013). The efficacy of CRISPR/Cas9 system has been demonstrated in model plants, Arabidopsis (Li et al., 2013) and N. benthamiana (Nekrasov et al., 2013), as well as in important crops; rice (Zhang et al., 2014), sorghum (Jiang et al., 2013) and wheat (Wang et al., 2014). Our demonstration further strengthens the potential of using CRISPR/Cas9 system as molecular scissors for targeted gene disruption.

PDS gene is involved in carotenoids biosynthetic pathway which plays an important role in a large number of physiological processes in plants. Carotenoids act as accessory pigments in photosynthesis and form the basic structural units of photosynthetic antennae (Qin et al., 2007). The disruption of NbPDS gene affects the pigmentation and results in albino phenotype clearly visible with naked eye and can be used as morphological parameter. In plants, the efficacy of CRISPR/Cas9 system gene disruption has also been reported by several researchers using transient expression by agroinfiltration and protoplast transformation (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), but in previous studies albino spots or photo-bleaching were not observed clearly using agroinfiltration based transient expression (Li et al., 2013).
Biallelic disruption of NbPDS gene abolishes carotenoid biosynthesis and promotes chlorophyll oxidation causing photobleached phenotype. As described earlier, Li et al. (2013) did not observe visible albino spots on agro-infiltrated leaves of N. benthamiana plants after 7 days of infiltration. It suggested that there were either no cells with biallelic disruption of NbPDS gene or the population of photobleached cells was too small due to cessation of cell division in the infiltrated leaves. Our results of microscopy and SPAD-502 values clearly showed the visible albino spots and significant difference between chlorophyll values of control and infiltrated leaves respectively. These results are in accordance with Li et al. (2013), fluorescent microscopy was necessary to study photobleached cells. Moreover, results of PCR and chlorophyll contents of albino phenotype also confirmed the mutation in leaves.

We performed screening of photobleached cells of infiltrated leaves under fluorescent microscope. Moreover, PCR amplification of gRNA target site and chlorophyll contents measurement using SPAD-502 meter also indicated disruption of NbPDS gene. These results suggest that the method is useful for targeted genome editing and for the development of knockout mutants.

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Fig. 6: Screening of Photo bleached cells under Fluorescent microscope. (a, b) Photo bleached cells in leaves infiltrated with pGreen0029-pcoCas9:gRNA vector; (c) Control leaf under fluorescent microscope; (d) Leaf infiltrated either with pGreen0029-pcoCas9 or pGreen0029-gRNA; (e, f) N. benthamiana leaves agro-infiltrated with both pGreen0029-pcoCas9 and pGreen0029-gRNA binary vectors.

Therefore, careful screens of infiltrated area after the degradation of existing chlorophyll using fluorescent microscopy is necessary for further characterization. We have demonstrated that pcoCas9-gRNA mediated gene disruption can be achieved in N. benthamiana through agro-infiltration method. We showed that the possible mutation in NbPDS gene resulted into distinguishable albino phenotype that could be achieved by transiently expressing pcoCas9-gRNA cassettes in a single vector. The visible albino phenotype was possibly due to presence of both pcoCas9 and gRNA in single vector suggested that both genes were expressed simultaneously in close vicinity. However, some visible albino spots were also appeared on leaves co-infiltrated with pcoCas9 and gRNA cassettes in two separate vectors. Moreover, control plants infiltrated with empty pGreen0029 and either with pGreen0029-pcoCas9 or pGreen0029-gRNA remained normal and did not show albino spots.


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