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Full Length Article

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

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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

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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 (Ohori *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´-CACGACCCGAAGATTGACAA-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 be 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 mm^2 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′-TCCAAGGTAATTCAGCTTATC-3′ and gRNA reverse 5′-CGAAGATTGACAAAGGACTT-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 Fischer 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 H_2O 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 *Bam*HI and *Eco*RI. Required digested fragments were purified from agarose gel and ligated to construct pGree0029-pcoCas9 expression vector (Fig. 3a). Similarly, pGreen0029 and pJET-gRNA vector were digested with *Eco*RI and *Sac*I 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 *Kpn*I and *Xho*I 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. Three different plant expression vectors containing 35SPPDK:pcoCas9, 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 Nb*PDS* 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.

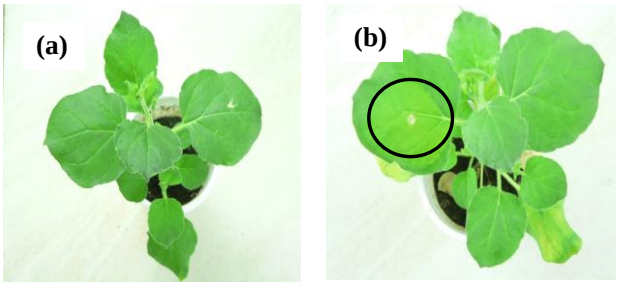

Fig. 1: Four weeks old *N. benthamiana* plants. (a) Control *N. benthamiana* plant; (b) *Agro*-infiltrated plant

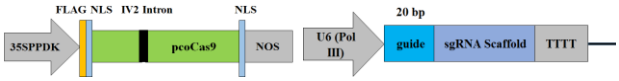

Fig. 2: Basic architecture of constructs used for disruption of *NbPDS* gene

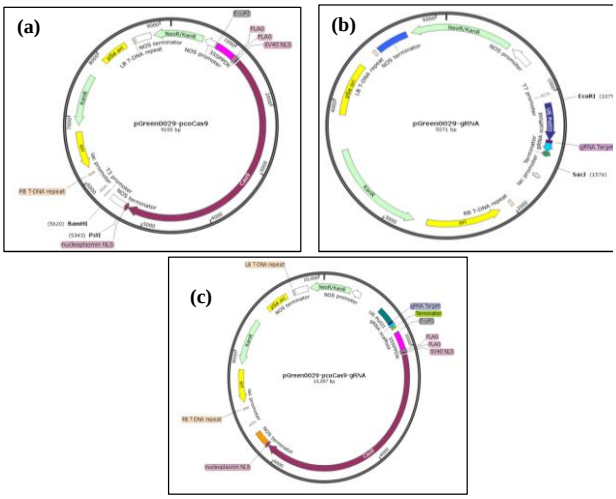

Fig. 3: Plant expression vectors used in this study. (a) A pGreen0029 based plant expression vector having pcoCas9; (b) A plant expression vector containing gRNA expression cassette; (c) A plant expression vector containing both pcoCas9 and gRNA expression cassettes

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

Table 1: ANOVA table for chlorophyll contents measurement

Source	DF	SS	MS	F	P
Genotypes	24	3.5	0.15	0.54	0.9468
Treatments	2	15997.9	7998.96	29350.4	0.0000
Error	48	13.1	0.27		
Total	74	16014.5			

CV 4.66%

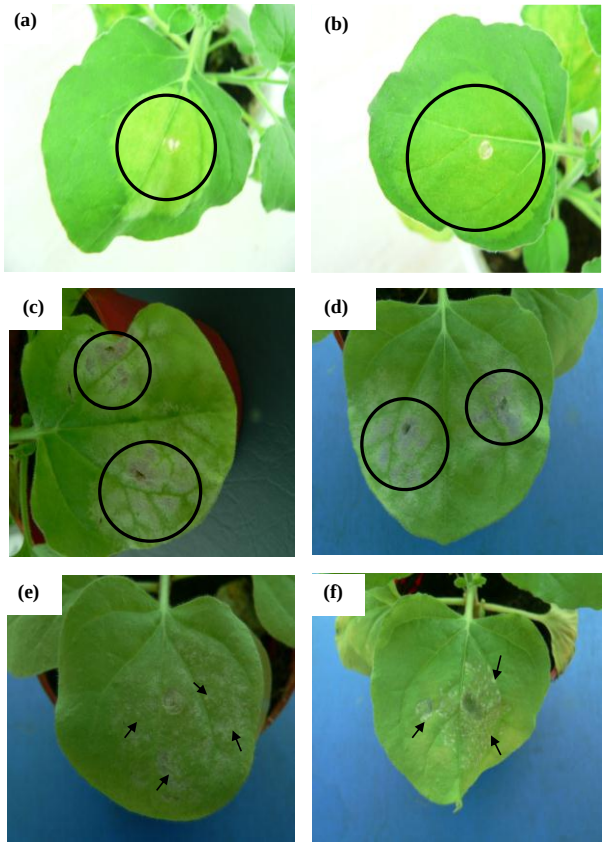

Fig. 4: *N. benthamiana* leaves 7 days' post infiltration. (a, b) *Agro*-infiltrated leaves with either pGreen0029-pcoCas9 or pGreen0029-gRNA vector; (c, d) Leaves are subjected to agroinfiltration with pGree0029-pcoCas9 and pGreen0029-gRNA vectors; (e, f) Leaves with visible albino spots, *agro*-infiltrated with pGreen0029-pcoCas9:gRNA vector

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

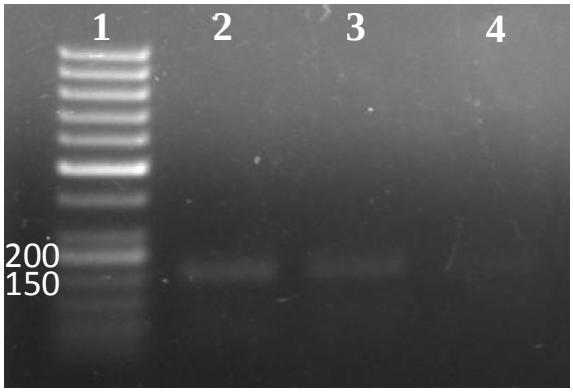

Fig. 5: PCR amplification of gRNA target of *NbPDS* gene. (1) 50 bp DNA ladder. (2) DNA amplification of control or untransformed plant leaf. (3) The DNA amplification from leaf infiltrated with pGreen0029-pcoCas9 and pGreen0029-gRNA vectors. (4) The DNA amplification of infiltrated leaf with pGreen0029-pcoCas9:gRNA vector

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).

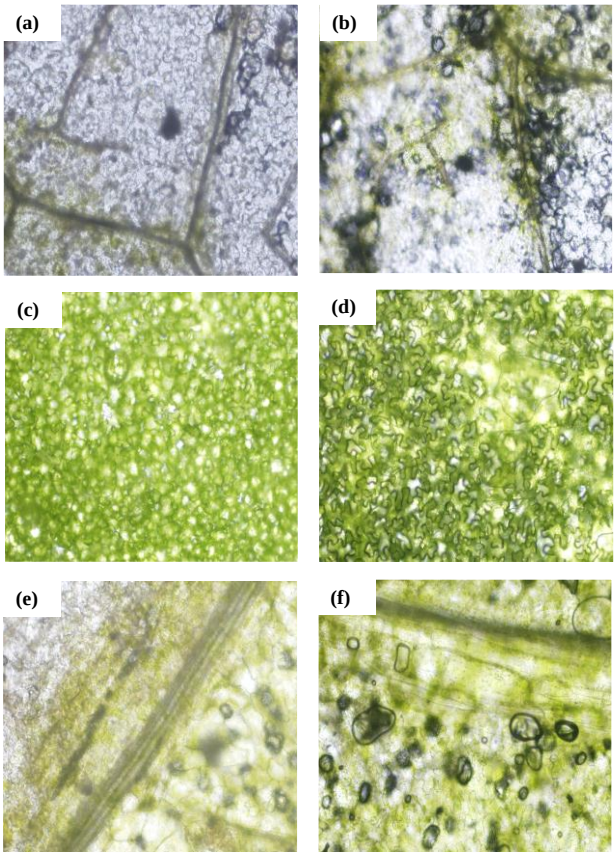

Fig. 6: Screening of Photo bleached cells under Fluorescent microscope. (a, b) Photo bleached cells in leaves infiltrated with pGreen0029-pcoCas9:gRNA vector; (c) Control leaf under fluorescent microscope; (d) Leaf infiltrated either with pGreen0029-pcoCas9 or pGreen0029-gRNA; (e, f) *N. benthamiana*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

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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References

Abramovitch, R.B., J.R. Stebbins and C.E. Martin, 2006. Type III effector *AvrPtoB* requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. *Proc. Natl. Acad. Sci.*, 103: 2851–2856

Belhaj, K., A.C. Garcia, S. Kamoun and V. Nekrasov, 2013. Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods*, 9: 1–10

Bhat, R., T. Lahaye and R. Panstruga, 2007. The visible touch: in planta visualization of protein-protein interactions by fluorophore-based methods. *Plant Methods*, 2: 12–16

Bilang, R., S. Zhang, N. Leduc, V.A. Iglesias, A. Gisel, J. Simmonds, I. Potrykus and C. Sautter, 1993. Transient gene expression in vegetative shoot apical meristems of wheat after ballistic micro targeting. *Plant J.*, 4: 735–744

Bogdanove, A.J. and D.J. Voytas, 2011. TAL effectors: customizable proteins for DNA targeting. *Science*, 33: 1843–1846

Cho, S.W., S. Kim, J.M. Kim and J.S. Kim, 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.*, 31: 230–232

Cong, L., F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini and F. Zhang, 2013. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*, 339: 819–823

DiCarlo, J.E., J.E. Norville, P. Mali, X. Rios, J. Aach and G.M. Church, 2013. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucl. Acids Res.*, 41: 4336–43

Goodin, M.M., D. Zaitlin, R.A. Naidu and S.A. Lommel, 2008. *Nicotiana benthamiana*: Its History and Future as a Model for Plant–Pathogen Interactions. *Mol. Plant Microbe Interact*, 21: 1015–1026

Hellens, R.P., A.C. Allan, E.N. Friel, K. Bolitho, K. Grafton, M. Templeton, S. Karunairetnam, A.P. Gleave and W.A. Laing, 2005. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*, 13: 1–13

Hwang, W.Y., Y. Fu, D. Reyon, M.L. Maeder, S.Q. Tsai, J.D. Sander, R.T. Peterson, J.R.J. Yeh and L.K. Joung, 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.*, 31: 227–229

Jiang, W., D. Bikard, D. Cox, F. Zhang and L.A. Marraffini, 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.*, 31: 233–239

Jinek, M., A. East, A. Cheng, S. Lin, E. Ma and J. Doudna, 2013. RNA-programmed genome editing in human cells. *eLife*, 2: 471–475

Kapila, J., R. Rycke, M.M. Van and G. Angenon, 1997. An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci.*, 122: 101–108

Li, J.F., J.E. Norville, J. Aach, M. McCormack, D. Zhang, J. Bush, G.M. Church and J. Sheen, 2013. Multiplex and homologous recombination mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol*., 31: 688–691

Li, M., X. Li, Z. Zhou, P. Wu, M. Fang, X. Pan, Q. Lin, W. Luo, G. Wu and H. Li, 2016. Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. *Front. Plant Sci*., 7: 377

Lusser, M. and H.V. Davies, 2013. Comparative regulatory approaches for groups of new plant breeding techniques. *Nat. Biotechnol*., 30: 437–446

Ma, X., Q. Zhang, Q. Zhu, W. Liu, Y. Chen and R. Qiu, 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant*, 8: 1274–1284

Mali, P., L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. Di-Carlo, J.E. Norville and G.M. Church, 2013. RNA-guided human genome engineering via Cas9. *Science*, 339: 823–826

Marraffini, L.A. and E.J. Sontheimer, 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science*, 322:1843–1845

Miao, J., D. Guo, J. Zhang, Q. Huang, G. Qin, X. Zhang, J. Wan, H. Gu and L.J. Qu, 2013. Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res.*, 23: 1233–1236

Nekrasov, V., B. Staskawicz, D. Weigel, J.D.G. Jones and S. Kamoun, 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol*., 31: 691–693

Ohori, Y.I., M. Nagano, S. Muto, H. Uchimiya and M.K. Yamada, 2007. Cell death suppressor Arabidopsis bax inhibitor-1 is associated with calmodulin binding and ion homeostasis. *Plant Physiol.*, 143: 650–660

Qin, G., H. Gu, L. Ma, Y. Peng, X.W. Deng, Z. Chen and L.J. Qu, 2007. Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Res.*, 17: 471–482

Redder, P., X. Peng, K. Brugger, S.A. Shah, F. Roesch, B. Greve, Q. She, C. Schleper, P. Forterre, R.A. Garrett and D. Prangishvili, 2009. Four newly isolated fuselloviruses from extreme geothermal environments reveal unusual morphologies and a possible inter-viral recombination mechanism. *Environ. Microbiol*., 3: 1–7

Sander, J.D. and J.K. Joung, 2014. CRISPR-Cas systems for editing regulating and targeting genomes *Nat. Biotechnol*., 32: 347–355

Schweizer, P., A. Christoffel and R. Dudler, 1999. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *Plant J*., 20: 541–552

Shan, Q., Y. Wang, J. Lil, J.L. Qiu and C. Gao, 2013. Targeted genome modification of crop plants using a CRISPR-Cas system wrote the paper. *Nat. Biotechnol*., 31: 686–688

Sheen, J., 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiol*., 127: 1466–1475

Tang, T.H., J.P. Bachellerie, T. Rozhdestvensky, M.L. Bortolin, H. Huber, M. Drungowski, T. Elge, J. Brosius and A. Huttenhofer, 2002. Identification of 86 candidates for small nonmessenger rnas from the archaeon archaeoglobus fulgidus. *Proc. Natl. Acad. Sci*., 99: 7536–7541

Urnov, F.D., J.C. Miller, Y.L. Lee, C.M. Beausejour, J.M. Rock, S. Augustus, A.C. Jamieson, M.H. Porteus, P.D. Gregory and M.C. Holmes, 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, 435: 646–651

Van der Hoorn, R.A., F. Laurent, R. Roth and P.J. De Wit, 2000. Agro infiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. *Mol. Plant Microbe Interact.*, 13: 439–446

Vaquero, C., M. Sack, J. Chandler, D. Jurgen, F. Schuster, M. Monecke, S. Schillberg and R. Fischer, 1999. Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc. Natl. Acad. Sci*., 96: 28–33

Wang, T., J.J. Wei, D.M. Sabatini and E.S. Lander, 2014. Genetic screens in human cells using the CRISPR/Cas9 system. *Science*, 343: 80–84

Weinthal, D., A. Tovkach, V. Zeevi and T. Tzfira, 2010. Genome editing in plant cells by zinc finger nucleases. *Trends Plant Sci*., 15: 308–321

Wroblewski, T., A. Tomczak and R. Michelmore, 2005. Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnol. J.*, 3: 259–273

Zhang, F., V. Gradinaru, A.R. Adamantidis, R. Durand, R.D. Airan, L. de Lecea and K. Deisseroth, 2010. Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. *Nat. Protocols*, 5: 439–456

Zhang, H., J. Zhang, P. Wei, B. Zhang, F. Gou, Z. Feng, Y. Mao, L. Yang, H. Zhang, N. Xu and J.K. Zhu, 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotehnol. J*., 12: 797–807

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