View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

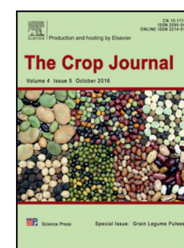
provided by Elsevier - Publisher Connector

HOSTED BY

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect



Short communication

Creation of targeted inversion mutations in plants using an RNA-guided endonuclease

Congsheng Zhang[a,b], *Changlin Liu*[a], *Jianfeng Weng*[a], *Beijiu Cheng*[b], *Fang Liu*[a], *Xinhai Li*[a], *Chuanxiao Xie*[a,*]

[a]*Institute of Crop Science, Chinese Academy of Agricultural Sciences, National Key Facility for Crop Gene Resources and Genetic Improvement, Beijing 100081, China*

[b]*Anhui Agricultural University, Hefei 230036, China*

ARTICLE INFO

Article history:
Received 30 May 2016
Received in revised form 8 July 2016
Accepted 2 November 2016
Available online 12 November 2016

Keywords:
RNA-guided endonuclease
CRISPR/Cas9
Targeted inversion
Genetic improvement

ABSTRACT

Inversions are DNA rearrangements that are essential for plant gene evolution and adaptation to environmental changes. We demonstrate the creation of targeted inversions and previously reported targeted deletion mutations via delivery of a pair of RNA-guided endonucleases (RGENs) of CRISPR/Cas9. The efficiencies of the targeted inversions were 2.6% and 2.2% in the *Arabidopsis FLOWERING TIME* (*AtFT*) and *TERMINAL FLOWER 1* (*AtTFL1*) loci, respectively. Thus, we successfully established an approach that can potentially be used to introduce targeted DNA inversions of interest for functional studies and crop improvement.

© 2016 Crop Science Society of China and Institute of Crop Science, CAAS. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Plant chromosomal rearrangements are widespread DNA polymorphisms that can be classified into inversions, deletions, duplications, and translocations [1]. Inversions are chromosome segments reversed end to end that occurs in a single chromosome undergoing break repair and rearrangement within itself. Widespread inversion polymorphisms contribute critically to local adaptation and speciation [2,3] and to gene evolution [4]. Extensive inversions have been found in plant genomes, such as in *Arabidopsis* [5] and in important crop species including rice [6], wheat [7], and maize [8,9]. To date, however, the biological significance of the widespread inversion rearrangements has been largely unknown because there has been no practical means of generating precise inversion mutants for functional and genetic improvement analyses.

Methodologies for precise genome editing could provide straightforward ways to target genes of interest and could have applications in both functional gene studies and genetic improvement designs. In recent years, zinc-finger nucleases (ZFNs) [10], transcription activator-like effector nucleases (TALENs) [11], and meganucleases [12] have been applied as target-specific nucleases and introduced into genomes to target double-strand breaks (DSBs) in DNA. Moreover, RNA-guided endonucleases (RGENs) based on clustered regularly interspaced short palindromic repeats (CRISPRs) and associated Cas9

* *Corresponding author.*
E-mail address: xiechuanxiao@caas.cn (C. Xie).
Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.

http://dx.doi.org/10.1016/j.cj.2016.08.001
2214-5141/© 2016 Crop Science Society of China and Institute of Crop Science, CAAS. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article as: C. Zhang, et al., Creation of targeted inversion mutations in plants using an RNA-guided endonuclease, The Crop Journal (2016), http://dx.doi.org/10.1016/j.cj.2016.08.001

nuclease (CRISPR/Cas9) have recently been developed [13] and have superseded ZFNs, TALENs, and meganucleases owing to their ease of use and flexibility of design. The CRISPR/Cas9 system has been demonstrated to be a versatile tool for genome editing and targeting in diverse species, including plants [6,14–19]. Targeted gene deletions can be created using pairs of TALENs or single guiding RNAs with the Cas9 nuclease [20]. In animal cell lines, inversion mutations mediated by a dual single-guide RNA (sgRNA) CRISPR/Cas9 targeting system have been frequently found [6,21,22], suggesting that this method is a valid way to create targeted inversion mutations and targeted gene deletions. In our previous study, a combinatory dual-sgRNA/Cas9 system was developed for the creation of targeted deletions and gene replacement in plants [23]. However, the characteristics and efficiency of inversion mutations induced by the dual CRISPR/Cas9 system have not yet been reported.

In the present study, the *Arabidopsis FLOWERING TIME* (*AtFT*) and *TERMINAL FLOWER 1* (*AtTFL1*) loci, a pair of homologous florigen genes with opposing functions (FT promotes flowering, whereas TFL1 represses flowering [24] were selected as a cross-validation system for creating targeted and precise inversion mutations using the dual-sgRNA CRISPR/Cas9 system. This study will demonstrate that the dual-sgRNA CRISPR/Cas9 system could be a useful approach for generating targeted inversions in plants.

2. Materials and methods

2.1. Plant materials and growth conditions

The *Arabidopsis thaliana* Col-0 ecotype was used as a wild-type control. Surface-sterilized seeds were germinated on Murashige–Skoog (MS) medium (pH 5.8). Three-week-old seedlings were transferred to soil and grown in a growth chamber at 23 °C under a 16-h light photoperiod and at 21 °C under an 8-h dark photoperiod. The humidity was approximately 50%.

2.2. Construction of Cas9 and sgRNA vectors

The codon-optimized coding sequence of hSpCas9 [25], driven by the CaMV 35S promoter, was cloned into a CPB vector with a Nos terminator using an In-Fusion PCR Cloning Kit (Clontech, Mountain View, California, USA). The AtU6-26 promoter used to drive sgRNA was cloned from the *A. thaliana* Col-0 ecotype using KOD DNA polymerase (TOYOBO, Japan). The target sequences and gRNA scaffold sequence are shown in Table 1 and Fig. S1. U6, gRNA, and sgRNA were connected using overlap extension PCR. The U6:gRNA:sgRNA construct was cloned into pCPB in a *Hin*dIII site using an In-Fusion PCR Cloning Kit. The Cas9 and different dual-sgRNA expression cassettes for *AtFT* and *AtTFL1* were manipulated to form construct #1 and construct #2. The M13R primer was used for sequencing vectors. The primer sequences used for vector construction are shown in Table S1.

2.3. Plant transformation

The vectors containing the Cas9 and dual-sgRNA expression cassettes were transformed into *Agrobacterium* strain GV3301 using electroporation. *Agrobacterium*-mediated *Arabidopsis* transformation was performed using the floral dip method [26]. The seeds of the T_0 generation were screened on MS medium (pH 5.8) containing 40 μmol L^{-1} glufosinate ammonium. Two weeks after screening, plants were transferred to soil, and PCR for detecting the bialaphos resistance protein encoded by the *bar* gene was used to identify transgenic plants.

2.4. Genomic DNA extraction and mutant identification

Genomic DNA extraction from leaves of *A. thaliana* transgenic plants was performed using TPS buffer (100 mmol L^{-1} Tris–HCl buffer containing 10 mmol L^{-1} EDTA and 1 mmol L^{-1} KCl) [27]. The genomic region surrounding the CRISPR target sites for *AtFT* and *AtTFL1* was PCR-amplified and sequenced for screening gene deletions and inversions. Primer Premier 5.0 was used for primer design. The primer pair for *AtFT* deletion screening included FT-F: 5′-ATCCCTGCTACAACTGGAACAAC-3′ and FT-R: 5′-CACAAAAA AGTGTATTA TGGAACCC-3′. The primer pair for *AtFT* inversion screening included FT-F1: 5′-AAAGTCTTCTTCCTC CGCAG-3′ and FT-R: 5′-CACAAAAAAGTGTATTATGGAACCC-3′. The primer pair for *AtTFL1* deletion screening included TFL1-F: 5′-ATGTCTC GGTCGTCTCTTTGTCT-3′ and TFL1-R: 5′-TCGACAGATCGTTA TCAATACTTCT-3′. The primer pair for *AtTFL1* inversion screening included TFL1-F1: 5′-GGCCATTG GAG ACTTGCTTC-3′ and TFL1-R: 5′-TCGACAGATCGTTATCAATACTTCT-3′. PCR products were analyzed by agarose gel electrophoresis. PCR products were purified using an AxyPrep DNA gel extraction kit (Axygen, CA, USA) and then cloned into a vector using the pEASY-Blunt Simple Cloning Kit (TransGene, Beijing, China). Single clones were picked and analyzed by DNA sequencing. The primer for sequencing was M13-R: 5′-CAGGAAACAGCTATGAC-3′. Inversion mutations were screened in T1-generation transgenic plants.

3. Results

3.1. Design of a dual sgRNA-directed gene deletion construct using the CRISPR/Cas9 system

To create inversion mutations in the target regions, we designed a single construct to express dual sgRNAs driven by a promoter and to express the *hSpCas9* nuclease driven by a CaMV35S promoter (Fig. 1A). The sites targeted by the dual-sgRNA/Cas9 construct were located in exon 4 of *AtFT* and exon 1 of *AtTFL1*

Table 1 – Targeted genes and the design of dual sgRNA-Cas9-mediated targeted deletions to create inversion mutations.

Gene	Locus	sgRNA1 target sequence with PAM and the expected excision site (↓)	sgRNA2 target sequence with PAM and the expected excision site (↓)	Designed target deletion or inversion region
AtFT	AT1G65480	5′-GACACGATGAATTCCTG↓CAGTGG-3′	5′-GTAATAAAAATGAGAGG↓GGGAGG-3′	Chr1:24331428..24333934 (−)
AtTFL1	AT5G03840	5′-GCCATTGATAATGGGGA↓GAGTGG-3′	5′-GGAAAACTTGTAAGAGG↓AAAAGG-3′	Chr5:1025496..1025750 (−)

Please cite this article as: C. Zhang, et al., Creation of targeted inversion mutations in plants using an RNA-guided endonuclease, The Crop Journal (2016), http://dx.doi.org/10.1016/j.cj.2016.08.001

ARTICLE IN PRESS

(Fig. 1B, C). Both sgRNA/Cas9 targeting activities within a single DNA segment would lead to two DSBs (Fig. 1D), which would be expected to result in a DNA inversion (Fig. 1D) instead of a deletion [23]. In this design, the deletion mutation could be screened by PCR using an "F" and "R" primer pair based on amplicon size. The inversion mutation events could also be screened by PCR because positive amplification could occur when the target region was inverted and rejoined end-to-end, based on the direction of the "F1" primer (Fig. 1D). PCR amplifications using an "F" and "R" primer pair outside the intended inversion region were implemented as internal controls of the PCR reactions (Fig. 1D). The mutations could then be confirmed by sequencing the amplicons. The sequence details of these designs are presented in Table 1 and Fig. S1.

3.2. Creation of targeted inversion mutations in the *AtFT* and *AtTFL1* loci

We selected *AtFT* and *AtTFL1* as target genes to test whether CRISPR/Cas9 could create inversion mutations. The two sites targeted by dual-CRISPR/Cas9 in *AtFT* are 341 bp apart (Table 1; Fig. 1B). In the *AtTFL1* locus, the two target sites were 255 bp apart (Table 1; Fig. 1C), which is also the same region we used in our previous study, wherein we designed a gene replacement system [23]. The inversion events were screened using primer pairs that are specific for inversion mutations (Table S1). Of 389 individual T1 plants, 10 were positively identified as harboring heritable inversion events, yielding an efficiency of 2.6%. At the *AtTFL1* locus, 6 of 272, representing an efficiency of 2.2%, showed the positive inversion pattern. Comparing the mutation efficiencies between inversion and deletion mutations (Fig. S2), we found that inversions were 11.6% and 9.1% of deletion mutations in *AtFT* and *AtTFL1* (Table 2), respectively. These observations indicated that approximately one-tenth of the targeted deletion mutations could reverse end to end and result in inversion mutations in populations.

To further verify the inversions identified in the *AtFT* and *AtTFL1* loci, we sequenced the targeted loci in the region flanking the inversion (Fig. 1D). The reconnection of the

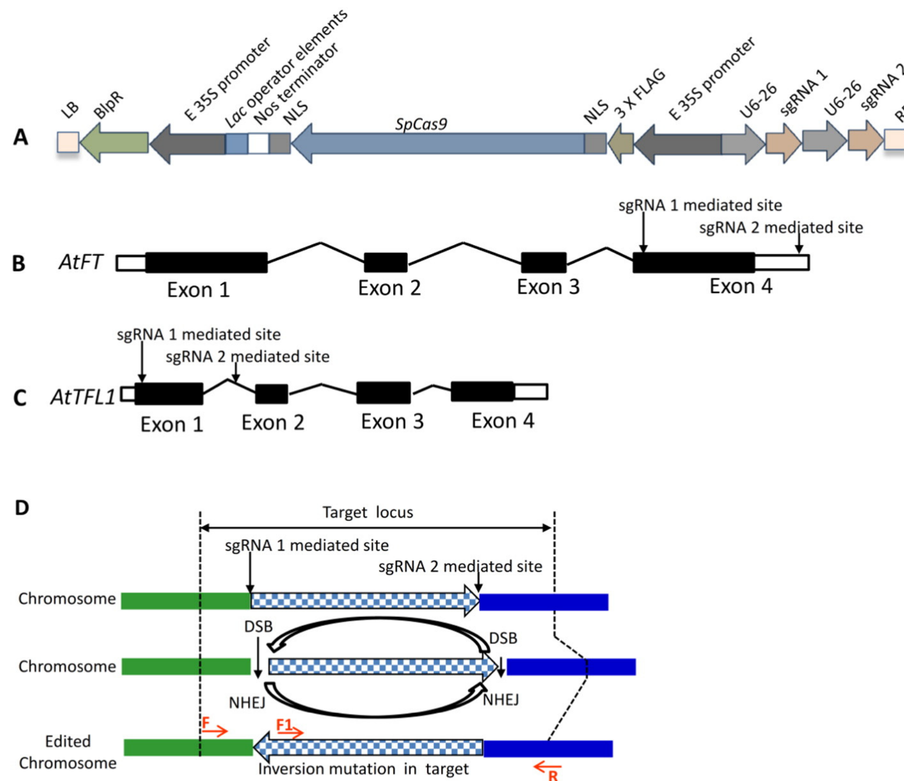


Fig. 1 – Schematic illustration of the design of the targeted gene deletion and inversion in the *AtFT* and *AtTFL1* loci using a dual-sgRNA/Cas9 system. (A) The components of the dual sgRNA CRISPR–Cas9 constructs: 3 × FLAG, 3 × FLAG-tag; BlpR, bialaphos resistance marker; E 35S promoter, enhanced 35S promoter; *Lac* operator elements, *E. coli Lac* operator elements; LB, T-DNA left border; NLS, nuclear location signal sequence; PAM, protospacer-adjacent motif sequence (sequence NGG in red and underlined); RB, T-DNA right border; sgRNA, single guiding RNA; *SpCas9*, *Streptococcus pyogenes Cas9*. (B) Schematic illustration of the designed targeted region within the *AtFT* gene structure. (C) Schematic illustration of the designed targeted region within the *AtTFL1* gene structure. (D) Schematic illustration of the rationale for the targeted inversion mutations mediated by the dual-sgRNA CRISPR/Cas9 system and by PCR screening of the targeted inversion mutation. The red arrows with an F (forward primer) and an R (reverse primer) represent primers designed for deletion mutation screening and sequencing. The red arrow with an F1 was the primer designed for screening inversion mutations that could form primer pairs only with the R primer as inversions were generated. DSB, double-strand break; NHEJ, non-homologous end-joining repair; F, forward primer; F1, a designed inverted primer; R, reverse primer. Note: the PCR primer pair of F1 and R could only perform exponential amplification on inversion mutation events.

Please cite this article as: C. Zhang, et al., Creation of targeted inversion mutations in plants using an RNA-guided endonuclease, The Crop Journal (2016), http://dx.doi.org/10.1016/j.cj.2016.08.001

Table 2 – Observed mutations in the inversion and deletion region in T1 transgenic plants.

	Screened plants (No.)	Inversion individuals and mutation rates	Deletion mutation individuals and mutation rates	Inversions/deletions
AtFT	389	10 (2.6%)	86 (22.1%)	11.6%
AtTFL1	272	6 (2.2%)	66 (24.3%)	9.1%

mutated sequences, in which the sequences were reversed end-to-end (Fig. 2B; Fig. 3B, C), and the generation of the inversion mutation mediated by the dual-sgRNA CRISPR system were verified by sequencing at least six individuals in the T_1 (1 plant) and T_2 (5 plants) generations (Fig. 2C, D; Fig. 3D). The sequencing results showed that these mutations rejoined precisely at the expected site after the inversion mutation occurred (Fig. 2B, C, D; Fig. 3B, C, D) and that those mutations were transmitted accurately across the T1 and T2 generations. The results indicated that the inversion mutations were stably inherited. Interestingly, a plant with both an inversion and a deletion, indicating a heterozygous mutation in the AtTFL1 locus, was also identified (Fig. 3C). The sequencing data showed that the targeted inversion could be expected to occur at a rate of approximately 2%–3% in plants.

4. Discussion

The dual-sgRNA CRISPR/Cas9 construct, along with other paired RGENs [28], can generate two DSBs, which should be capable of introducing multiple mutation types, including deletions, inversions, and translocations. With the supply of a repair donor, gene replacement could also be achieved [23]. The region between two cleavage sites could be deleted or inverted to create deletion or inversion mutations. Interestingly, target sites that could be the substrate of the RGENs and the dual-sgRNA CRISPR/Cas9 system were deleted or inverted to form the new sequences, which could no longer be targeted by the same RGENs because the original guiding RNA sequences were changed after the inversion or deletion mutations were generated. In contrast to inversion

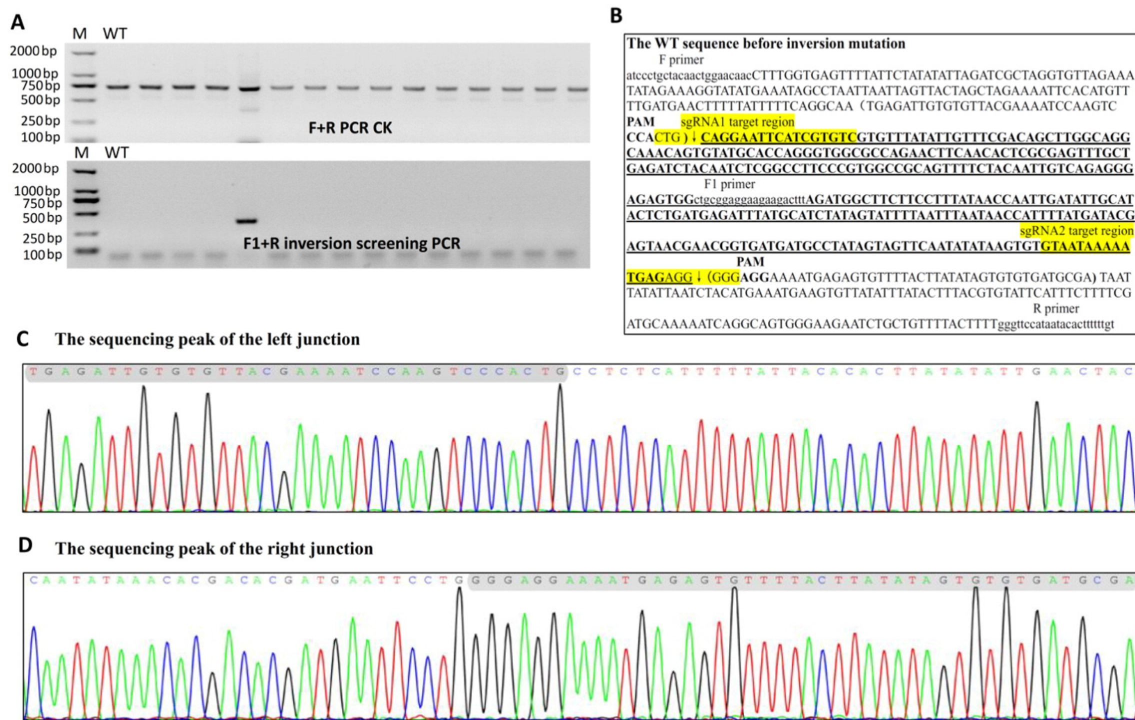


Fig. 2 – A targeted inversion mutation with a region deletion mutation induced by the dual sgRNA CRISPR/Cas9 system within the *AtFT* locus. (A) PCR screening profile of an inversion mutation event within the *AtFT* locus in the T_2 generation. The upper panel shows the control (F and R primer pair); the lower panel shows the inversion mutation PCR screening with the F1 and R primer pair. M: DNA size marker (Tiangen D2000, Tiangen Co., China). (B) The wild-type sequence and detail of the target sites along with the inversion region. The sequence is inverted where underlined. The primer sequence is shown in lower case. The vertical black arrow indicates the cut site. sgRNA target sites, along with their protospacer-adjacent motif (PAM) sequences and primer regions, are indicated between the sequence lines. (C) Sequencing peaks for the left junction region of the inversion mutant in the T_2 generation. The sequence highlighted in gray represents the sequencing in upper brackets in Fig. 2B. The sequence next to the highlighted gray region is expected to be the reverse complementary sequence of the underlined sequence. (D) Sequencing peaks for the right junction region of the inversion mutant in the T_2 generation. The sequence highlighted in gray represents the sequencing in lower brackets in Fig. 2B. The sequence before the highlighted gray region is expected to be the reverse complementary sequence of the underlined sequence.

Please cite this article as: C. Zhang, et al., Creation of targeted inversion mutations in plants using an RNA-guided endonuclease, The Crop Journal (2016), http://dx.doi.org/10.1016/j.cj.2016.08.001

ARTICLE IN PRESS

Fig. 3 – A targeted inversion mutation with a region deletion mutation induced by the dual sgRNA CRISPR/Cas9 system within the *AtTFL1* locus. (A) Screening profile of inversion mutations. The upper panel shows the control (F and R primer pair); the lower panel shows the inversion mutation PCR screening with the F1 and R primer pair. Accession 109 was the only one showing an inversion mutation that was verified by sequencing. M: DNA size marker (Tiangen D2000, Tiangen Co. China). (B) The wild-type sequence of the target region before the inversion event and the deletion mutation. (C) Edited sequence around the target region in *AtTFL1*. (D). Sequencing peaks surrounding the inverted sequence with evidence of both end junction sites in the T_2 generation. A 126-bp region previously highlighted in (B) and one "A" (also highlighted in gray in B) within the protospacer-adjacent motif (PAM) (CCA) of sgRNA 1 were deleted. The inverted mutation sequence is shown in red and highlighted in green in (C); the inverted sequence with both end junctions is shown in (D).

mutations, deletion mutations play a dominant role in the mutations introduced by dual RGENs [23,29]. In this study, inversions occurred at a frequency approximately one-tenth that of deletion mutations. This result was consistent with the previous report on ZFNs [28]. Although the inversion rate was lower, it should still be adequate for screening enough intended mutations with an acceptable work load, as a stable RGEN-transformed individual plant could produce many offspring harboring diverse mutation types.

Inversions contribute to species divergence in a variety of organisms, including plants and even humans [3]. In animals, inversion rearrangement mutations are likely associated with genetic diseases, and the lengths of the rearrangements range from hundreds of bp to tens of Mbp [21]. The widespread inversion rearrangement mutations in plants are likely associated with environmental adaptation [9]. However, the functions of inversion mutations in plant genomes are largely unknown, owing to a lack of tools for creating targeted inversion mutations. In the present study, we demonstrated the creation of targeted inversion mutations using RGENs as genome editing tools. In addition to inversion and deletion mutations, translocations could also be generated when the two DSBs occur on different chromosomes [30]. Thus, the CRISPR/Cas9 system should be a versatile tool for producing multiple targeted DNA rearrangements across diverse species, including crop plants.

Based on the data from both this study and our previously reported deletion work [23], we found that the mutation sites were located only between the third and fourth bp before the protospacer-adjacent motif sequence. Compared with that of reported knockout mutations based on single RGENs [13,25], the precision of the deletion or inversion mutation sequences induced by the dual-sgRNA system was highly improved and enabled the generation of mutations at the designed sites.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 31361140364), the National Major Project for Developing New GM Crops (No. 2016ZX080009-001), and the Agricultural Science and Technology Innovation Program (ASTIP) of CAAS to Chuanxiao Xie.

Appendix A. Supplementary data

Supplementary data for this article can be found online at http://dx.doi.org/10.1016/j.cj.2016.08.001.

REFERENCES

[1] T.S. Painter, A new method for the study of chromosome rearrangements and the plotting of chromosome maps, Science 78 (1933) 585–586.

ARTICLE IN PRESS

[2] D.B. Lowry, J.H. Willis, A widespread chromosomal inversion polymorphism contributes to a major life-history transition, local adaptation, and reproductive isolation, PLoS Biol. 8 (2010), e1000500.

[3] A. Navarro, N.H. Barton, Chromosomal speciation and molecular divergence-accelerated evolution in rearranged chromosomes, Science 300 (2003) 321–324.

[4] E. Allen, Z.X. Xie, A.M. Gustafson, G.H. Sung, J.W. Spatafora, J.C. Carrington, Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*, Nat. Genet. 36 (2004) 1282–1290.

[5] G. Blanc, A. Barakat, R. Guyot, R. Cooke, M. Delseny, Extensive duplication and reshuffling in the *Arabidopsis* genome, Plant Cell 12 (2000) 1093–1101.

[6] J.F. Chen, Q.F. Huang, D.Y. Gao, J.Y. Wang, Y.S. Lang, T.Y. Liu, B. Li, Z.T. Bai, J.L. Goicoechea, C.Z. Liang, C.B. Chen, W.L. Zhang, S.H. Sun, Y. Liao, X.M. Zhang, L. Yang, C.L. Song, M.J. Wang, J.F. Shi, G. Liu, J.J. Liu, H.L. Zhou, W.L. Zhou, Q.L. Yu, N. An, Y. Chen, Q.L. Cai, B. Wang, B.H. Liu, J.M. Min, Y. Huang, H.L. Wu, Z.Y. Li, Y. Zhang, Y. Yin, W.Q. Song, J.M. Jiang, S.A. Jackson, R.A. Wing, J. Wang, M.S. Chen, Whole-genome sequencing of *Oryza brachyantha* reveals mechanisms underlying *Oryza* genome evolution, Nat. Commun. 4 (2013) 1595.

[7] J. Ma, J. Stiller, Y.M. Wei, Y.L. Zheng, K.M. Devos, J. Doležel, C.L. Liu, Extensive pericentric rearrangements in the bread wheat (*Triticum aestivum* L.) genotype "Chinese Spring" revealed from chromosome shotgun sequence data, Genome Biol. Evol. 6 (2014) 3039–3048.

[8] C. Yu, J. Zhang, T. Peterson, Genome rearrangements in maize induced by alternative transposition of reversed Ac/Ds termini, Genetics 188 (2011) 59–67.

[9] P.S. Schnable, D. Ware, R.S. Fulton, J.C. Stein, F.S. Wei, S. Pasternak, C.Z. Liang, J.W. Zhang, L. Fulton, T.A. Graves, P. Minx, A.D. Reily, L. Courtney, S.S. Kruchowski, C. Tomlinson, C. Strong, K. Delehaunty, C. Fronick, B. Courtney, S.M. Rock, E. Belter, F.Y. Du, K. Kim, R.M. Abbott, M. Cotton, A. Levy, P. Marchetto, K. Ochoa, S.M. Jackson, B. Gillam, W.Z. Chen, L. Yan, J. Higginbotham, M. Cardenas, J. Waligorski, E. Applebaum, L. Phelps, J. Falcone, K. Kanchi, T. Thane, A. Scimone, N. Thane, J. Henke, T. Wang, J. Ruppert, N. Shah, K. Rotter, J. Hodges, E. Ingenthron, M. Cordes, S. Kohlberg, J. Sgro, B. Delgado, K. Mead, A. Chinwalla, S. Leonard, K. Crouse, K. Collura, D. Kudrna, J. Currie, R.F. He, A. Angelova, S. Rajasekar, T. Mueller, R. Lomeli, G. Scara, A. Ko, K. Delaney, M. Wissotski, G. Lopez, D. Campos, M. Braidotti, E. Ashley, W. Golser, H. Kim, S. Lee, J.K. Lin, Z. Dujmic, W. Kim, J. Talag, A. Zuccolo, C.Z. Fan, A. Sebastian, M. Kramer, L. Spiegel, L. Nascimento, T. Zutavern, B. Miller, C. Ambroise, S. Muller, W. Spooner, A. Narechania, L.Y. Ren, S. Wei, S. Kumari, B. Faga, M.J. Levy, L. McMahan, P. Van Buren, M.W. Vaughn, K. Ying, C.T. Yeh, S.J. Emrich, Y. Jia, A. Kalyanaraman, A.P. Hsia, W.B. Barbazuk, R.S. Baucom, T.P. Brutnell, N.C. Carpita, C. Chaparro, J.M. Chia, J.M. Deragon, J.C. Estill, Y. Fu, J.A. Jeddeloh, Y.J. Han, H. Lee, P.H. Li, D.R. Lisch, S.Z. Liu, Z.J. Liu, D.H. Nagel, M.C. McCann, P. SanMiguel, A.M. Myers, D. Nettleton, J. Nguyen, B.W. Penning, L. Ponnala, K.L. Schneider, D.C. Schwartz, A. Sharma, C. Soderlund, N.M. Springer, Q. Sun, H. Wang, M. Waterman, R. Westerman, T.K. Wolfgruber, L.X. Yang, Y. Yu, L.F. Zhang, S.G. Zhou, Q.H. Zhu, J.L. Bennetzen, R.K. Dawe, J.M. Jiang, N. Jiang, G.G. Presting, S.R. Wessler, S. Aluru, R.A. Martienssen, S.W. Clifton, W.R. McCombie, R.A. Wing, R.K. Wilson, The B73 maize genome: complexity, diversity, and dynamics, Science 326 (2009) 1112–1115.

[10] M.H. Porteus, D. Carroll, Gene targeting using zinc finger nucleases, Nat. Biotechnol. 23 (2005) 967–973.

[11] D. Reyon, S.Q. Tsai, C. Khayter, J.A. Foden, J.D. Sander, J.K. Joung, FLASH assembly of TALENs for high-throughput genome editing, Nat. Biotechnol. 30 (2012) 460–465.

[12] H.R. Gao, J. Smith, M.Z. Yang, S. Jones, V. Djukanovic, M.G. Nicholson, A. West, D. Bidney, S.C. Falco, D. Jantz, L.A. Lyznik, Heritable targeted mutagenesis in maize using a designed endonuclease, Plant J. 61 (2009) 176–187.

[13] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821.

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

[15] J. Miao, D.S. Guo, J.Z. Zhang, Q.P. Huang, G.J. Qin, X. Zhang, J.M. Wan, H.Y. Gu, L.J. Qu, Targeted mutagenesis in rice using CRISPR–Cas system, Cell Res. 23 (2013) 1233–1236.

[16] Q.W. Shan, Y.P. Wang, J. Li, Y. Zhang, K.L. Chen, Z. Liang, K. Zhang, J.X. Liu, J.J. Xi, J.L. Qiu, C.X. Gao, Targeted genome modification of crop plants using a CRISPR–Cas system, Nat. Biotechnol. 31 (2013) 686–688.

[17] H.Y. Wang, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, R. Jaenisch, One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, Cell 153 (2013) 910–918.

[18] K.B. Xie, Y.N. Yang, RNA-guided genome editing in plants using a CRISPR–Cas system, Mol. Plant 6 (2013) 1975–1983.

[19] H. Zhang, J.S. Zhang, P.L. Wei, B.T. Zhang, F. Gou, Z.Y. Feng, Y.F. Mao, L. Yang, H. Zhang, N.F. Xu, J.K. Zhu, The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation, Plant Biotechnol. J. 12 (2014) 797–807.

[20] C. Brandl, O. Ortiz, B. Röttig, B. Wefers, W. Wurst, R. Kühn, Creation of targeted genomic deletions using TALEN or CRISPR/Cas nuclease pairs in one-cell mouse embryos, FEBS Open Bio 5 (2015) 26–35.

[21] P.S. Choi, M. Meyerson, Targeted genomic rearrangements using CRISPR/Cas technology, Nat. Commun. 5 (2014) 3728.

[22] P. Essletzbichler, T. Konopka, F. Santoro, D. Chen, B.V. Gapp, R. Kralovics, T.R. Brummel, S.M.B. Nijman, T. Bürckstümmer, Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line, Genome Res. 24 (2014) 2059–2065.

[23] Y.P. Zhao, C.S. Zhang, W.W. Liu, W. Gao, C.L. Liu, G.Y. Song, W.X. Li, L. Mao, B. Chen, Y.B. Xu, X.H. Li, C.X. Xie, An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design, Sci. Rep. 6 (2016) 23890.

[24] D.P. Wickland, Y. Hanzawa, The *FLOWERING LOCUS T/TERMINAL FLOWER 1* gene family: functional evolution and molecular mechanisms, Mol. Plant 8 (2015) 983–997.

[25] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (2013) 819–823.

[26] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.

[27] L. Monna, N. Kitazawa, R. Yoshino, J. Suzuki, H. Masuda, Y. Maehara, M. Tanji, M. Sato, S. Nasu, Y. Minobe, Positional cloning of rice semidwarfing gene, *sd-1*: rice "green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis, DNA Res. 9 (2002) 11–17.

[28] H.J. Lee, J. Kweon, E. Kim, S. Kim, J.S. Kim, Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases, Genome Res. 22 (2012) 539–548.

[29] Y.P. Qi, X.H. Li, Y. Zhang, C.G. Starker, N.J. Baltes, F. Zhang, J.D. Sander, D. Reyon, J.K. Joung, D.F. Voytas, Targeted deletion and inversion of tandemly arrayed genes in *Arabidopsis thaliana* using zinc finger nucleases, G3-Genes Genom. Genet. 3 (2013) 1707–1715.

[30] R. Torres, M. Martin, A. Garcia, J.C. Cigudosa, J. Ramirez, S. Rodriguez-Perales, Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR–Cas9 system, Nat. Commun. 5 (2014) 3964.

Please cite this article as: C. Zhang, et al., Creation of targeted inversion mutations in plants using an RNA-guided endonuclease, The Crop Journal (2016), http://dx.doi.org/10.1016/j.cj.2016.08.001