Plant Cell Reports (2018) 37:1353–1356
https://doi.org/10.1007/s00299-018-2299-0

FOCUS ARTICLE

Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing

Shouwei Tian[1] · Linjian Jiang[2] · Xiaxia Cui[1] · Jie Zhang[1] · Shaogui Guo[1] · Maoying Li[1] · Haiying Zhang[1] · Yi Ren[1] · Guoyi Gong[1] · Mei Zong[1] · Fan Liu[1] · Qijun Chen[3] · Yong Xu[1]

Received: 13 April 2018 / Accepted: 11 May 2018 / Published online: 24 May 2018

Keywords Base-editing · Herbicide-resistant watermelon · Transgene-free

Planted to two million ha farmland and yielding more than 70 million tons per year, watermelon dominates the fruit market in China. However, with a low planting density and short canopy, field watermelon is severely threatened by weeds. Currently, only five herbicides, providing poor or no control on broadleaved weeds, are registered for watermelon weed control in China; therefore, technical solutions for broadleaved weed control are greatly needed by watermelon growers (Grey et al. 2000). Although this issue could be well addressed by developing herbicide-resistant watermelon, no such varieties were commercialized, largely due to the difficulties to generate herbicide-resistant watermelon germplasm via traditional breeding.

Precise genome editing technologies, especially CRISPR/Cas9, offer revolutionary solutions to create herbicide-resistant germplasm. By integrating a DNA template that contains herbicide-resistant mutations into the DNA double strand break (DSB) caused by CRISPR/Cas9, herbicide-resistant traits conferred by point mutations were successfully generated in soybean (Li et al. 2015) and maize (Svitashev et al. 2015). The difficulties to deliver sufficient template DNA at the time of repairing DSB significantly hinder its application on plant genome editing. Notably, a novel Cas9 variant fused with cytidine deaminase (named BE3) achieved single nucleotide conversion at precise positions without incorporating a donor DNA template (Komor et al. 2016), making base editing of plant genome much more feasible to conduct. Herbicide-resistant rice (Shimatani et al. 2017) and *Arabidopsis* (Chen et al. 2017) have been recently obtained via base-editing. However, whether this new base-editing system could generate herbicide-resistant germplasm in other economically important crops needs urgent testing. In this report, novel transgene-free herbicide-resistant watermelon varieties were created by base-editing with a potential of immediate field application to facilitate broadleaved weed control.

In this research, we selected watermelon *acetolactate synthase* (*ALS*) gene as the target for base-editing. ALS is the key enzyme for biosynthesis of branched-chain amino acids, valine, leucine and isoleucine, in plants. Single point mutations at several conserved positions of *ALS* genes are known to confer high level of herbicide resistance in different plant species (Yu and Powles 2014). Close analysis of watermelon *ALS* gene, *ClALS* (*Cla019277*, ICuGI database), revealed that converting C to T in the codon of Pro190 (CCG) could result in amino

Communicated by Neal Stewart.

Shouwei Tian and Linjian Jiang contributed equally to the work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00299-018-2299-0) contains supplementary material, which is available to authorized users.

✉ Linjian Jiang
jianglinjian@cau.edu.cn

✉ Yong Xu
xuyong@nercv.org

[1] Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (North China), Beijing Key Laboratory of Vegetable Germplasm Improvement, National Engineering Research Center for Vegetables, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China

[2] Key Lab of Pest Monitoring and Green Management, MOA; Department of Plant Pathology, College of Plant Protection, China Agricultural University, Beijing 100193, China

[3] State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China

acid change, a mutation likely to confer herbicide resistance in watermelon (Fig. 1a; Fig. S1). The selected target sequence (Fig. 1b) was cloned into pBSE901 (Chen et al. 2017), in which BE3 was driven by double 35S promoter (Fig. 1b) and would change C bases at 3–9 positions of the target on watermelon genome (Fig. 1a). This binary vector was then transformed into cotyledon of watermelon ZG94 mediated by *Agrobacterium* strain EHA105 following the method described by Tian et al. (2017). ZG94 is an elite inbred variety with white flesh and low sugar content for production of edible seeds, and it can also be used as a parent line to produce commercial hybrid seeds.

A total of 199 transgenic plants were obtained and PCR aimed to amplify *BE3* and *gRNA* sequences showed that all of the candidate plants contained genes of *BE3* and *gRNA*. Primers ALS-F and ALS-R were used to amplify the fragment spanning Pro190 region of *ALS* gene. All primers used in this report are listed in Table S1.

Sanger sequencing results revealed that 45 out of 199 transgenic plants had C to T mutations, indicating that the base-editing efficiency is 23% at T0 generation. As both C bases in the codon of Pro190 could be converted into T independently or simultaneously, 34 plants contained C to T conversion only at position 7 (C_7 to T_7, counting the distal

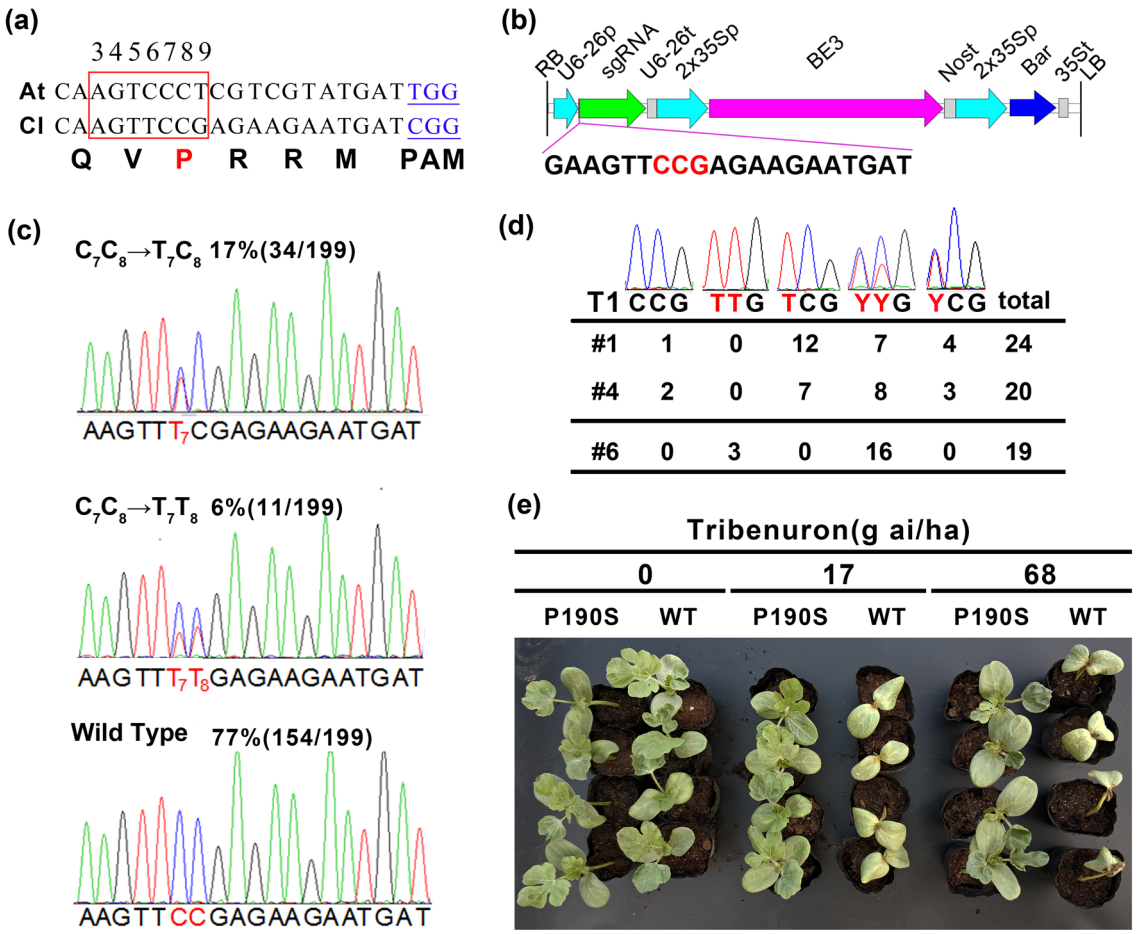

Fig. 1 Targeted base-editing on *ALS* gene efficiently creates herbicide-resistant watermelon. **a** The alignment of target regions of *ClALS* and *AtALS* gene (GenBank accession No. X51514). The coded amino acids corresponding to *ClALS* are listed and Pro190 of *ClALS* gene are shown in red. The base-editing window, where Cs can be converted into Ts, is boxed in red. **b** Physical map of base-editing vector pBSE901 (only the T-DNA region is shown), harboring the target sequence. As U6-26 promoter prefers "G" as the first transcribed nucleotide, the transcribed sgRNA was designed to have 19-base matches with the targets (Chen et al. 2017). **c** Sanger sequencing revealed that 45 out of 199 transgenic T0 plants contained base-edited alleles. The frequency and number of each genotype are indicated. Note the mixed signal on the chromatograms. **d** Base-edited alleles were effectively passed to next generation. The number of each genotype of T1 plants, progenies of base-edited T0 lines #1, #4 and #6, were listed. Note that some T1 plants contained new edited alleles (C_7C_8 to T_7T_8) compared with ancestral T0 lines #1 and #4. Y stands for mixed signals of C and T. **e** The homozygous P190S plants showed high level of resistance to tribenuron herbicide. The homozygous and wild type plants were grown in a greenhouse under the same conditions. Tribenuron herbicide was applied to watermelon plants at 0, 17 (the recommended field application rate), and 68 g ai/ha. Pictures were taken at 14 days after herbicide treatment. (Colour figure online)

end to the PAM as position 1, resulting in P190S), and the other 11 plants had C to T changes at position 7 and 8 (C_7C_8 to $T_7T_{8,}$ resulting in P190L) (Fig. 1c). PCR product cloning indicated that these 45 base-edited T0 plants were heterozygous or chimeric.

To test whether the base-edited mutations could be efficiently passed to next generation, we randomly chose lines #1 and #4, both T0 plants had heterozygous $C_7C_8G_9$ to $T_7C_8G_9$ change, and line #6 whose T0 plant had heterozygous $C_7C_8G_9$ to $T_7T_8G_9$ changes for analysis. The genotyping results showed that at T1 generation the respective frequency of homozygous ($T_7C_8G_9$; P190S), heterozygous and WT plants was 50% (12/24), 46% (11/24) and 4% (1/24) for lines #1, and 35% (7/20), 55% (11/20) and 10% (2/20) for line #4 (Fig. 1d). This observation did not fit the predictions of the Mendel's law of segregation, because the WT allele could be re-edited at reproduction stage of T0 plants. Indeed, we observed 7/24 and 8/20 plants harbored $C_7C_8G_9$ to $T_7T_8G_9$ mutations, respectively, at T1 generation of lines #1 and #4, whose parents contained only $C_7C_8G_9$ to $T_7C_8G_9$ mutation. Re-editing event was more evident at T1 generation of line #6, as a result, 16% (3/19) were homozygous mutant ($T_7T_8G_9$; P190L) plants, and the remaining 84% (16/19) were heterozygous (Fig. 1d). Lacking of WT plants was also a result of re-editing of WT allele. Although the occurrence of re-editing largely complicates segregation of edited alleles, the results clearly showed that base-edited point mutations were passed to next generation at high efficiency.

Notably, we identified 7/24, 6/20 and 4/19 plants from T1 generation of lines #1, #4 and #6, respectively, contained base-edited alleles but no *BE3* gene as revealed by PCR. Colloidal gold immunoassay strip (Artron BioResearch Inc. Canada) test showed that these plants did not contain PAT protein translated by bar gene on the T-DNA region of the construct. Moreover, individual leaves of these plants were severely damaged when treated with glufosinate. These results indicated that base-edited transgene-free plants were readily recovered at T1 generation. It should be stressed that two transgene-free plants were identified to contain homozygous P190S (TCG) alleles.

To test whether these point mutations could endow watermelon plants herbicide resistance, we used these two transgene-free homozygous P190S plant to produce homozygous P190S seeds. The homozygous P190S plants together with WT controls were treated with tribenuron, a herbicide highly effective on broadleaved weed control. While all WT plants were severely damaged by tribenuron at 17 g ai/ha (the recommended field application rate) at 14 days after treatment, homozygous P190S plants were not affected (Fig. 1e). Although slight injury occurred at 68 g ai/ha, homozygous P190S plants resumed normal growth within 30 days after treatment. Notably, Yu and Powles (2014) pointed out that plants with P190S mutation would resist all sulfonylurea herbicides including tribenuron, suggesting that nearly 35 different commercially-available sulfonylurea herbicides could be potentially coupled with this herbicide-resistant watermelon variety created in this report to effectively address weed problems. Moreover, we observed that fruit and seed size, and seed yield of these transgene-free homozygous P190S plants did not differ from WT controls (Fig. S2), suggesting that P190S mutation had no fitness cost. Similarly, no growth penalties were observed on other herbicide-resistant plant species conferred by P190S mutation (Yu and Powles 2014).

To evaluate the possible off-target potential, whole genome searching via Blastn identified five locations, with up to five mismatches compared with the gRNA sequence (Table S2). No off-target edits were found on these potential locations as indicated by Sanger sequencing of these five different PCR products. We did not find any unexpected nucleotide changes and indels in all base-edited plants examined in this study.

The base-editing efficiency of our system is up to 23% at T0 generation, and similar base-editing efficiency (17–38%) was also achieved on rice (Ren et al. 2017). Although the majority of transgenic T0 plants were not base-edited, re-editing actively occurred at the reproduction stage. Of 11 transgenic non-edited T0 plants, eight plants produced seeds that contained base-edited alleles revealed by Sanger sequencing of T1 seedlings (Fig. S3). This re-editing property could largely facilitate its application on difficult-to-transform plant species.

In this study, we successfully established high-efficient base-editing system and created non-GM herbicide-resistant watermelon varieties. Because these transgene-free base-edited herbicide-resistant watermelon plants are genetically identical to those bred via traditional mutagenesis, no extra regulations should be applied to these transgene-free base-edited mutants (Huang et al. 2016). Without fitness cost associated with the base-edited point mutations, this non-GM herbicide-resistant watermelon is now ready for immediate field application. Our report provides the first solid example showing that the CRISPR/Cas9-mediated base-editing system is a tremendously powerful tool for improvement of watermelon varieties.

Author contribution statement YX and LJ managed and organized the project; YX, ST and LJ designed the experiments and led the data analysis; ST, XC performed most of the experiments with the help from JZ, SG, ML, HZ, YR, GG, MZ, FL, QC; YX, ST, and LJ wrote the manuscript.

Acknowledgements This work was supported by grants from the National Key R&D Program of China (2018YFD010062), Beijing

science & technology program (D171100007617001), Beijing Academy of Agricultural and Forestry Sciences (KJCX20180427), National Natural Science Foundation of China (31701943, 31471785), the Ministry of Agriculture of China (CARS-26), Beijing Scholar Program (BSP026), BaGui Scholar Program.

Compliance with ethical standards

Conflict of interest A patent application related to the findings of this report was filed to the patent office in Beijing, and key authors of this report were listed as inventors.

References

Chen Y, Wang Z, Ni H, Xu Y, Chen Q, Jiang L (2017) CRISPR/Cas9-mediated base-editing system efficiently generates gain-of-function mutations in Arabidopsis. Sci China Life Sci 60:520–523

Grey TL, Bridges DC, Nesmith DS (2000) Tolerance of cucurbits to the herbicides clomazone, ethalfluralin and pendimethalin. II Watermelon Hortscience 35:637–641

Huang S, Weigel D, Beachy RN, Li J (2016) A proposed regulatory framework for genome-edited crops. Nat Genet 48:109–111

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533:420–424

Li Z, Liu ZB, Xing A, Moon BP, Koellhoffer JP, Huang L, Ward RT, Clifton E, Falco SC, Cigan AM (2015) Cas9-guide RNA directed genome editing in soybean. Plant Physiol 169:960–970

Ren B, Yan F, Kuang Y, Li N, Zhang D, Lin H, Zhou H (2017) A CRISPR/Cas9 toolkit for efficient targeted base editing to induce genetic variations in rice. Sci China Life Sci 60:516–519

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol 35:441–443

Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM (2015) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol 169:931–945

Tian S, Jiang L, Gao Q, Zhang J, Zong M, Zhang H, Ren Y, Guo S, Gong G, Liu F, Xu Y (2017) Efficient CRISPR/Cas9-based gene knockout in watermelon. Plant Cell Rep 36:399–406

Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag Sci 70:1340–1350