Brief Communication

Developing a novel artificial rice germplasm for dinitroaniline herbicide resistance by base editing of OsTubA2

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Received 13 February 2020; revised 15 March 2020; accepted 20 April 2020.
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Keywords: base editing, OsTubA2, herbicide resistance, dinitroaniline, rice.

Chemical herbicides, which are commonly used to kill weeds in the field, have been extensively applied and transformed the way of weed management in modern agriculture due to its efficiency, ease of use and relatively low cost. In the past decades, a large number of transgenic herbicide-tolerant crops (i.e. corn, soybean, cotton, rice) have been developed and commercialized, reshaping the global seed market (Schutte et al., 2017). However, the use of chemical herbicides (i.e. ALS inhibitors, EPSPS inhibitors, ACCCase inhibitors) has also drastically increased, resulting in the occurrence of weed species resistant to these herbicides. Thus, developing novel herbicide-resistant crops to diversify weed management is of great value in slowing the evolution of weed resistance to herbicides and to maintain sustainable crop production in the future. Thanks to cutting-edge CRISPR-mediated base editing technologies which have emerged lately (Ren et al., 2019; Wang et al., 2020; Yan et al., 2018); important genetic variations related to any herbicide resistance can be manipulated directly and rapidly by the relevant base editors. Tubulin genes have been reported to endow resistance to trifluralin and other dinitroaniline herbicides in a number of crops (Chu et al., 2018; Lyons-Abbott et al., 2010). Compared to other major herbicides, the frequency of dinitroaniline resistance in weeds is quite low (Heap, 2014). This is likely because mutations in tubulin genes could affect microtubule polymerization, an important process in cell division and elongation, and consequently lead to plant death. Therefore, tubulin genes are promising target genes to develop herbicide-resistant germplasms for future crop breeding. In this study, we successfully generated a novel artificial rice germplasm with trifluralin and pendimethalin resistance without fitness penalty by precisely editing the endogenous OsTubA2 gene within the rice genome. Theoretically, this important trait can also be rapidly introduced into other major crops using CRISPR-mediated adenine base editors.

It has been previously shown that a Met-268 Thr mutation in the \( \alpha \)-tubulin gene EITUA1 correlates with dinitroaniline resistance in a number of goosegrass (Eleusine indica) biotypes (Yamamoto et al., 1998). Proteins belonging to the \( \alpha \)-tubulin and \( \beta \)-tubulin family are highly conserved, showing up to 88% amino acid similarity (Rao et al., 2016). Therefore, we hypothesized that introduction of this point mutation into the rice genome might transform common rice into a herbicide-tolerant variety. To this end, precise base editing of the \( \alpha \)-tubulin homologue gene OsTubA2 (LOC_Os11g42220) was carried out using the previously described rice adenine base editor rBE14 (Yan et al., 2018). A sgRNA corresponding to a NGG PAM was designed to target the complementary genomic DNA strand of OsTubA2 at T1981 site (Figure 1a). The oligos were synthesized, constructed and shuttled into rBE14 binary vector through Gateway recombination as described previously (Yan et al., 2018). The rBE14/sgRNA system was then introduced into rice cultivar Kitaake (Oryza sativa spp. japonica) through Agrobacterium-mediated transformation.

Rice plants were regenerated from the independent calli and genotyped directly by Sanger sequencing of the target region. Out of 63 independent rice lines obtained, 8 lines (12.7% efficiency) were identified with A > G conversion occurring at T1981, replacing the Met268 residue with threonine residue (Figure 1b). All eight mutant lines were heterozygous and had no random indels detected. The potential off-targets of OsTubA2 targeting rBE14/sgRNA in the rice genome were predicted. No nucleotide changes at the potential off-target sites were detected in the eight positive transgenic lines (Figure 1c).

Next, T1 progenies of each OsTubA2-edited T0 lines were genotyped to determine the lines in which the T-DNA was segregated out. PCR amplification with specific primers corresponding to the Cas9, sgRNA and Hyg transgenes was conducted. As shown in Figure 1d and e, some T1 individuals lacked the transgenes due to genetic segregation. Furthermore, Sanger sequencing revealed that some plants (i.e. #2-5, #2-10 etc.) were homozygous. Given that germination of wild-type Kitaake seeds is sensitive to dinitroaniline herbicide treatment (Figure 1f), a phenotypic and genotypic analysis was further conducted. Rice seeds of T1 population of heterozygous T0 lines #2 and #5 were germinated in cylinders containing 6.6 mg/L pendimethalin which is sufficient to inhibit the hypocotyl and root growth of wild-type rice. All homozygous seeds carrying the M268T mutation showed resistance to pendimethalin treatment, whereas germination of wild-type seed was arrested. On the other hand, heterozygous
Figure 1 Generation of a novel artificial rice germplasm for dinitroaniline herbicide resistance by precise editing of the endogenous OsTubA2 gene. (a) The target site in the OsTubA2 gene in rice. Exons are indicated by black boxes. (b) Representative Sanger sequencing chromatogram of the rBE14-edited OsTubA2 allele in a T0 transgenic line. The nucleotide change is underlined, and the PAM sequence is marked in box. (c) The potential off-target sites of OsTubA2-targeting sgRNA in the rice genome. (d) Isolation of T1 plants without T-DNA insert. The presence and absence of individual genes were detected by PCR amplification with gene-specific primers (Cas9, Hyg, sgRNA). (e) The genotype of heterozygous T0 line #2 and representatives of its homozygous T1 offspring #2-5 and #2-10. (f) Herbicide tolerance assay of wild-type Kitaake seeds. Wild-type Kitaake seeds were germinated in cylinders complemented with 0, 0.7, 1.3, 3.3, 6.6, 13.2 mg/L of pendimethalin and 0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/L of trifluralin, respectively. Samples were photographed 14 days after treatment. (g) Genotype–phenotype association analysis of dinitroaniline herbicide-tolerated rice plants in T1 generation. T1 seeds of independent T0 line #2 and #5 were germinated in 1/2 MS with 6.6 mg/L pendimethalin or 4.0 mg/L trifluralin, respectively. R = resistance; S = susceptible. (h) Pendimethalin and trifluralin resistance of homozygous OsTubA2(M268T) seedlings. Samples were photographed 14 days after treatment. (i) Plant morphology of wild-type and OsTubA2(M268T) plants at the heading stage. (j) The 1000-grain weight and germination rate of the homozygous OsTubA2(M268T) T2 seeds. The 1000-grain weight of the homozygous mutants was 23.43, and the wild type was 23.86. P > 0.05. The germination rate of the homozygous mutant was 95.38%, and the wild type was 98.02%. P > 0.05. (k) Genetic variations of OsTubA2 in the 4,726 rice accessions (http://ricevarmap.ncpgr.cn/v2). The naturally occurring SNVs and novel M268T substitution generated in this study are indicated by bars in black and red, respectively. The location of OsTubA2 on rice chromosome 11 is indicated on the top. (l) The multiple nucleotide sequence alignment of the Met268 region in α-tubulin genes of several major crops. Met268 is highly conserved in all 8 α-Tubulin proteins. The conserved PAM and sgRNA sequences are marked on the top. The conserved thymine is marked in red box. In (a), (c) and (e), the target sequences, PAM sequences, target cytosines and detected nucleotide changes/corresponding amino acids are highlighted in bold, green, red and blue, respectively.

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plants exhibited different resistance phenotypes according to the growth of roots and hypocotyls under the conditions tested, it likely results from the dosage effect of M268T mutation (Figure 1g and h). We also analysed the resistance to 4.0 mg/L trifluralin (another type of dinitroaniline herbicides) which can inhibit the root and hypocotyl growth of wild-type rice. When trifluralin was applied to T1 progenies, similar phenotypes were observed (Figure 1f). Thus, the M268T mutation in OsTubA2 truly confers resistance to both trifluralin and pendimethalin herbicides in rice and is stably inherited in the subsequent generations.

All the OsTubA2-edited plants lacking the T-DNA transgenes were grown under natural light conditions in the greenhouse. The plants were morphologically indistinguishable from the wild-type plants (Figure 1). Furthermore, the thousand-grain weight and germination rate of T2 seeds were investigated, no significant difference was observed between M268T and wild-type plants (Figure 1j). Together, our results indicate that introduction of Met-268-Thr mutation in OsTubA2 by base editing does not cause growth penalty in rice.

Genetic diversity of OsTubA2 gene in 4,726 re-sequenced rice accessions was investigated using RiceVarMap v2.0 (Zhao et al., 2015). The data indicate that M268 in OsTubA2 has not been targeted by natural or human selection during rice domestication (Figure 1k). Thus, M268T plants are a novel artificial germplasm with a great potential for future rice improvement. A number of α-tubulin genes from other major economic crops, including wheat, maize, barley, sorghum, oilseed rape, cotton and soybean, were further analysed. Sequence alignments revealed that the sequence of M268 locus is highly conserved among different species (Figure 1), opening the possibility that M268T-mediated dinitroaniline resistance can be rapidly introduced into other crops through precise base editing.

In summary, our study shows that the M268T mutation in the endogenous OsTubA2 gene, generated by adenine base editor, endows dinitroaniline herbicide resistance in rice without inducing fitness cost. Any other rice cultivars or cash crops can be enhanced with the herbicide resistance trait using this strategy in the future.

Acknowledgements

This study was supported by a grant from the Joint Project between NSFC and RCN (31861133020) to X.W., and grants from the National Natural Science Foundation of China (31871948) and the National Key Research and Development Program of China (2017YFD0200900) to H.Z.

Author Contributions

H.Z., X.W., X.Z. and X.L. designed the experiments; L.L., Y.K., B.R., F.Y. and G.G. performed experiments; S.L. carried out bioinformatics analysis; H.Z., G.G. and C.S wrote the paper with input from all other authors; all authors participated in discussion and revision of the manuscript.

Conflict of interests

The authors declare no conflict of interests.

References

Chu, Z., Chen, J., Nyporko, A., Han, H., Yu, Q. and Powles, S. (2018) Novel α-tubulin mutations conferring resistance to dinitroaniline herbicides in Lolium rigidum. Front. Plant Sci. 9, 97.

Heap, I. (2014) Global perspective of herbicide-resistant weeds. Pest Manag. Sci. 70, 1306–1315.

Lyons-Abbott, S., Sackett, D.L., Woga, D., Gaertig, J., Morgan, R.E., Werbovetz, K.A. and Morrisette, N.S. (2010) α-tubulin mutations alter oryzalin affinity and microtubule assembly properties to confer dinitroaniline resistance. Eukaryot. Cell, 9, 1825–1834.

Rao, G., Zeng, Y., He, C. and Zhang, J. (2016) Characterization and putative post-translational regulation of α- and β-tubulin gene families in Salix arbutifolia. Sci. Rep. 6, 19258.

Ren, B., Liu, L., Li, S., Kuang, Y., Wang, J., Zhang, D., Zhou, X. et al. (2019) Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. Mol. Plant, 12, 1015–1026.

Schutte, G., Eckerstorfer, M., Rastelli, V., Reichenbecher, W., Restrepo-Vassalli, S., Ruohon-Lehto, M., Saucy, A.W. et al. (2017) Herbicide resistance and biodiversity: agronomic and environmental aspects of genetically modified herbicide-resistant plants. Environ. Sci. Eur. 29, 5.

Wang, M., Xu, Z., Gosavi, G., Ren, B., Cao, Y., Kuang, Y., Zhou, C. et al. (2020) Targeted base editing in rice with CRISPR/Cas9 system. Plant Biotech. J. https://doi.org/10.1111/pbi.13330. [Epub ahead of print].

Yamamoto, E., Zeng, L. and Bard, W.V. (1998) α-tubulin missense mutations correlate with antimicrotubule drug resistance in Eleusine indica. Plant Cell, 10, 297–308.

Yan, F., Kuang, Y., Ren, B., Wang, J., Zhang, D., Lin, H., Yang, B. et al. (2018) Highly efficient A.T to G.C base editing by Cas9n-guided RNA adenosine deaminase in rice. Mol. Plant, 11, 631–634.

Zhao, H., Yao, W., Ouyang, Y., Yang, W., Wang, G., Lian, X., Xing, Y. et al. (2015) RiceVarMap: a comprehensive database of rice genomic variations. Nucleic Acids Res. 43, D1018–1022.