Expanding the target range of base editing in plants without loss of efficiency by blocking RNA-silencing

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Bacterial-derived CRISPR/Cas systems are versatile platforms to engineer site-specific gene editing tools. Compared to the canonical Cas9-mediated DNA cleavage systems, which induce a high-proportion of frame-shift mutations, the recently developed base editing (BE) tools allow more precise and predictable base substitutions within a CRISPR/Cas9-defined editing window. Initially, such tools made use of engineered cytosine deaminases or evolved adenine deaminases to catalyse base deamination when fused to a Cas9 nickase (nCas9) (Rees and Liu, 2018). The deamination of cytosines and adenines results in the conversion of C-G and A-T pairing to T-A and G-C respectively. Since this method facilitates precise gene editing in a repair template-independent manner, it was soon applied to a wide range of species, including plants (Mao et al., 2019).

However, the narrow target range and low editing activity still limit the application of BEs to gene function studies and crop breeding. As a solution, engineered SpCas9 nickase variants with relaxed PAM preferences, such as Cas9-NG and SpRY, were used to produce newer versions of BE tools (Nishimasu et al., 2018; Walton et al., 2020). Although this strategy helped to expand the target range of BE systems, efficiency losses were usually observed (Zhong et al., 2019). To overcome this limitation, two previously developed rice-optimized BE systems (based on nCas9-NG fused to Anc689B6max and ABEmax respectively) were further adapted for use in Arabidopsis (Koblan et al., 2018; Wang et al., 2019). The four recombinant base editors, CBE/ABEmax-nCas9 and CBE/ABEmax-nCas9NG, were expressed using an Arabidopsis-optimized vector under the control of the RPP5a promoter and NOS terminator.

Previous studies in Arabidopsis have shown that CRISPR/Cas9 gene editing efficiencies can be significantly improved by suppressing the plant RNA silencing pathways (Mao et al., 2018). To minimize the silencing effects, we fused a commonly used silencing suppressor (p19) to the above BE systems, generating the CBE/ABEmax-nCas9-p19 and CBE/ABEmax-nCas9NG-p19 systems (Figure 1a).

We first investigated the effect of co-expressing p19 in the two CBE systems by targeting a site in the Arabidopsis Acetolactate Synthase (ALS) gene with a canonical NGG PAM. The chosen target, ATALS-599, contains three successive cytosines (C5, C6 and C7) within the editing window (Figure 1b). Transgenic lines were produced by the Agrobacterium-mediated floral dipping method. At least 20 10-day-old seedlings were bulked as a single biological replicate for DNA extraction and SNP detection using next-generation sequencing (NGS). For each construct, three biological replicates were analysed. Compared to the nCas9 control, the editing efficiencies of nCas9NG at the three cytosines were decreased by about eight-folds. However, co-expression of p19 substantially increased the editing efficiencies of the nCas9NG system to the nCas9 levels. Interestingly, co-expression of p19 only had a marginal enhancing effect on the activity of the canonical nCas9-based BE system (Figure 1b).

To study whether the beneficial effect of p19 extends to other targets as well as different BE systems, a second site in the ALS gene was targeted for editing. The ATALS-1700 site contains two cytosines (C6, C7) and two adenines (A4, A5) located within the editing window (Figure 1c,d). Analysis of editing efficiency in transgenic Arabidopsis lines again showed that the non-canonical nCas9NG systems were 5- to 10-fold less efficient than the wild-type nCas9 systems for both cytosine and adenine base editing. Nevertheless, as observed above, the lower nCas9NG efficiencies can be restored to nCas9 levels by p19 co-expression (Figure 1c,d).

To evaluate the efficiency of the relaxed PAM BE systems at targets with NG PAMs, four overlapping targets within the Arabidopsis At7tc236 gene (AT2G25660) were selected for editing with the CBE/ABEmax-nCas9NG system using four different sgRNAs (sgR1 to sgR4) (Figure 1e). Based on the NGS data of bulked T1 plants, none of the cytosines within the editing window exhibited an editing frequency over 10% on average from the three replicates (Figure 1f, i). Co-expression of p19 improved efficiencies overall, although the effect was heavily dependent on the guide RNA and the nucleotide position within the editing window. In some cases, spectacular efficiency improvements were observed, with the editing frequency of C14 increasing from 2.1% to 11.9% and from 8.7% to 41.4% by using the guides sgR1 and sgR2 respectively. In other cases, such as for C6 and C8, editing frequencies were increased by 15–20 folds, but the absolute values were still very low (<10%) (Figure 1h,i).

Although p19 had a positive effect on BE efficiencies, it also induced strong developmental phenotypes when highly expressed in plants. Counter selection for T-DNA free BE plants
in subsequent generations is necessary for removing this side effect (Mao et al., 2018). We queried whether the efficiency of non-canonical BE systems could be improved using an alternative strategy, without causing severe developmental phenotypes. We targeted the same AtTIC236 gene sites in the Arabidopsis dicer-like (DCL) 2/3/4 triple mutant background using the CBEmax-nCas9NG system (Henderson et al., 2006). We observed a good correlation between the results in the dcl234 mutants and those observed using the p19 co-expression strategy. Nucleotides showing efficiency improvements by p19 co-expression in WT plants also showed improvements in the dcl234 mutant without p19 co-expression. Overall, the use of dcl234 mutant was more efficient than p19 co-expression (Figure 1h,i). Our results suggest that the efficiencies of non-canonical BE systems are constrained at the post-transcriptional level.

To determine whether the relaxed PAM systems can generate heritable BE events, we selected two dcl234 T1 populations transformed with CBEmax-nCas9NG for further analysis. The C/T editing frequency of the TIC236 C14 site using sgR1 and sgR2 was evaluated by Hi-Tom sequencing. Forty-eight individual T1 seedlings were sequenced for each population. Four of the T1 lines for sgR1 and 23 lines for sgR2 edited each nucleotide above 50% in the sampled leaves (Figure 1o). In the T2 generation of two of them (sgR1–#6 and sgR2–#48), heritable C/T conversion events were validated by Sanger sequencing with three different editing outcomes observed, suggesting stable inheritance of BE events (Figure 1n).

Our results suggest that the efficiency loss observed in non-canonical BE systems could be due to suboptimal expression of the engineered base editors. Increasing expression levels using strong promoters can overcome this limitation as shown in a previous study, demonstrating that the use of the AtRPP5a-promoter enables ABE7.10–nCas9-mediated base editing in Arabidopsis (Kim, 2018). An alternative strategy is to reduce RNA degradation using silencing suppressors as shown in this work where we demonstrated that relaxed PAM base editing can be achieved in Arabidopsis seedlings with acceptable efficiencies.

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Conflict of interests

The authors declare no competing financial interests.

Author contributions

Y.M. and M.W. designed the experiments; Y.M., Y.Z., Q.W. and B.H. performed the experiments; Q.Z. analysed the data; Y.M. wrote the manuscript and J.B. and J.-K.Z. edited the manuscript; J.-K.Z. supervised the project.

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