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Manipulating mRNA splicing by base editing in plants

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Precursor-mRNAs (pre-mRNA) can be processed into one or more mature mRNA isoforms through constitutive or alternative splicing pathways. Constitutive splicing of pre-mRNA plays critical roles in gene expressional regulation, such as intron-mediated enhancement (IME), whereas alternative splicing (AS) dramatically increases the protein diversity and gene functional regulation. However, the unavailability of mutants for individual spliced isoforms in plants has been a major limitation in studying the function of mRNA splicing. Here, we describe an efficient tool for manipulating the splicing of plant genes. Using a Cas9-directed base editor, we converted the 5′ splice sites in four *Arabidopsis* genes from the activated GT form to the inactive AT form. Silencing the AS of *HAB1.1* (encoding a type 2C phosphatase) validated its function in abscisic acid signaling, while perturbing the AS of *RS31A* revealed its functional involvement in plant response to genotoxic treatment for the first time. Lastly, altering the constitutive splicing of *Act2* via base editing facilitated the analysis of IME. This strategy provides an efficient tool for investigating the function and regulation of gene splicing in plants and other eukaryotes.

base editing, splicing, intron retention, alternative 5′ splicing site

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INTRODUCTION

Most protein coding genes in eukaryotic genomes are interrupted by introns (Reddy et al., 2013). To function properly, precursor-mRNAs (pre-mRNA) are processed by spliceosomes to generate mature mRNAs by removing introns and joining exons (Reddy et al., 2013). Transcriptome-wide RNA sequencing has revealed that alternative splicing (AS) events occur in more than 60% of plant intron-containing genes (Reddy et al., 2013). There are four common types of AS events, namely intron retention (IR), alternative 5′ splicing, alternative 3′ splicing, and exon skipping (Reddy et al., 2013). Genome editing has been widely used to inactivate entire genes. However, for studying the function and regulation of AS events, specific silencing of individual mRNA isoforms is highly desired. The canonical GU/AG rule is applicable to most eukaryotic splicing processes of pre-mRNAs (Burset, 2001; Maniatis and Reed, 1987; Shapiro and Senapathy, 1987). Several conserved residues, namely 5′ splice site (GU), 3′ splice site (AG) and the branch point (A), are important for spliceosome assembling and processing (Staiger and Brown, 2013). Recently, genome-editing technologies such as base-editing have offered new ways to introduce precise nucleotide substitutions in endogenous genes. The most widely used base editor, BE3, which consists of the cytidine deaminase rat APOBEC1 fused with a Cas9 nickase, converts targeted C:G to T:A in

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genomic DNA (Chen et al., 2017; Li et al., 2017; Lu and Zhu, 2017; Nishida et al., 2016; Ren et al., 2017; Zong et al., 2017). We reasoned that disrupting the 5′ splice sites by converting them from GT to AT at the DNA level with a Cas9-directed base editor would be an efficient way to manipulate splicing outcomes. In addition, introns from constitutive splicing might also play a key role in gene regulation, such as intron-mediated enhancement (IME) (Chorev and Carmel, 2012; Laxa, 2016). Thus, we further test whether this strategy is also useful for IME studies.

RESULTS

Base editing prevents the generation of *HAB1.1*

We tested this idea in *Arabidopsis*, in which at least 42% of intron-containing genes undergo AS (Filichkin et al., 2010). In plants, IR is the predominant mode of AS (Wang and Brendel, 2006), for example, accounts for more than 30% of AS events in *Arabidopsis* (Ner-Gaon et al., 2004). To check whether disrupting a 5′ splice site could cause constitutive intron retention of alternatively spliced introns, we chose *HAB1* as a target gene. *HAB1* encodes a group A protein type 2C phosphatase (PP2C) with two protein isoforms depending on excision/retention of intron 4 (Wang et al., 2015; Zhan et al., 2015). HAB1.1 contains 511 amino acid residues, encoded by an mRNA lacking intron 4, and is a functional PP2C (Figure 1A)(Wang et al., 2015; Zhan et al., 2015), whereas *HAB1.2* retains intron 4, which results in premature translation arrest and loss of phosphatase activity (Figure 1A)(Wang et al., 2015; Zhan et al., 2015). Previously, to investigate the function of HAB1.1 and HAB1.2, researchers used the 35S promoter to express *HAB1.1* and *HAB1.2* coding sequences in *hab1-1* null mutant (Wang et al., 2015). It should be noted that the 35S promoter and native promoter do not have identical expression levels and patterns, and thus any direct comparison between samples in these assays should be made carefully. We therefore designed an sgRNA to target the 5′ splice site of intron 4 (Figure 1A). By Sanger sequencing of genomic DNA from T1 plants, we identified six heterozygous mutants, each with G-to-A conversions at the desired G of the 5′ splice site of intron 4 and the neighboring G in one allele (Figure 1A and Table S1 in Supporting Information). We harvested seeds from the T1 plants and generated homozygous T2 mutants for further investigation. To see whether disruption of this 5′ splice site completely prevented production of *HAB1.1*, we performed RT-PCR with a forward primer in exon 3 and a reverse primer in exon 5 (Figure 1B and C). This generated a 634 bp fragment from *HAB1.1* mRNA and a 757 bp fragment from *HAB1.2* mRNA from wild type (WT) plants (Figure 1B), whereas only one fragment of the similar size to that from *HAB1.2* was obtained from the mutants (Figure 1B). Sequencing showed that this was an amplicon of *HAB1.2* containing two G-to-A conversions (Figure 1C and Figure S1 in Supporting Information), suggesting that destroying the 5′ splice site leads to the retention of intron 4 and completely prevents production of the AS isoform, *HAB1.1*. Thus, this mutant was designated as *hab1.1* (*HAB1.1* knock-out mutant). Since all of the pre-mRNA was spliced to *HAB1.2* isoform in the mutants, they produced more *HAB1.2* transcripts than the WT (Figure 1B). HAB1.1 and HAB1.2 have been shown to play opposite roles in the abscisic acid (ABA) pathway (Wang et al., 2015). HAB1.1 reduces ABA sensitivity by dephosphorylating the SnRK proteins, which are positive regulators in ABA pathway. HAB1.2 can also interact with SnRK proteins, but has no phosphatase activity (Wang et al., 2015). Therefore, we reasoned that blocking the generation of HAB1.1 would give rise to an ABA hypersensitive phenotype. To test this prediction, we sowed WT and homozygous mutant seeds on half-strength MS medium with or without 0.25 μmol L^{-1} ABA, and found that the *hab1.1* mutants were more sensitive to ABA in terms of both cotyledon greening and true leaf development (Figure 1D–F).

Base editing prevents the generation of *T30G6.16.1*

In *HAB1*, IR affects the coding sequence (CDS). To investigate IR in a UTR (untranslated region) instead, we chose to target *T30G6.16* in *Arabidopsis*. Two transcripts of *T30G6.16* encode the same protein, the only difference between them being due to an IR event in the 5′ UTR (Figure 2A)(https://www.arabidopsis.org/). We designed an sgRNA to target the 5′ splice site of this intron (Figure 2A), and identified five independent T1 heterozygous mutant plants with precise conversion of this splice site GT to AT (Figure 2A and Table S1 in Supporting Information). We then used RT-PCR to examine the transcripts of *T30G6.16* in T2 homozygous mutants and WT plants. A 203 bp and a 303 bp fragment were amplified from *T30G6.16.1* and *T30G6.16.2* cDNA, respectively, in WT plants (Figure 2B), whereas only a fragment of a similar size to the amplicon of *T30G6.16.2* was detected in mutant plants (Figure 2B). Sequencing results showed that this fragment was amplified from an isoform that retained intron 1 as a result of a G-to-A conversion at the 5′ splice site (Figure 2C and Figure S2 in Supporting Information). This mutant was designated as *t30g6.16.1* (*T30G6.16.1* knock-out mutant). Introns in 5′ UTRs could in principle influence gene expression at the transcriptional, post-transcriptional and translational level (Chorev and Carmel, 2012; Shaul, 2017). Since we did not have an antibody, we only examined expression of *T30G6.16* at the mRNA level, using a pair of primers that amplified the same fragment from the *T30G6.16.1* and *T30G6.16.2* isoforms. Figure S3 shows that the point mutation did not in fact alter the absolute level of *T30G6.16* transcripts. We also did not

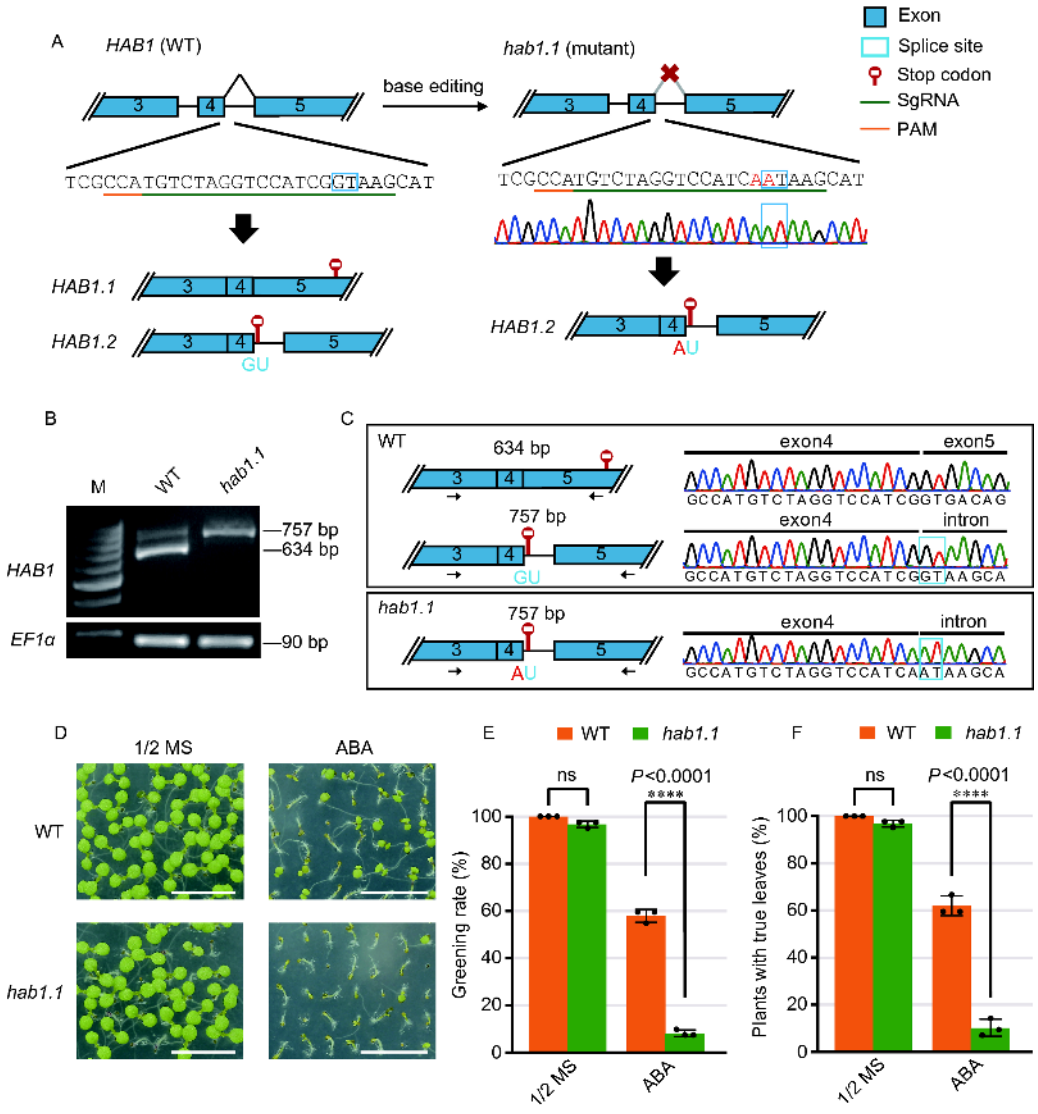

Figure 1 Base editing affects *HAB1* splicing. A, Schematic representations of the influence of base editing on *HAB1* splicing. Left part, the base editor target sequence in *HAB1* and schematic diagram outlining *HAB1* splicing in WT. Right part, the base-edited sequence in *HAB1* and schematic diagram outlining *HAB1* splicing in mutant. B, RT-PCR analysis of *HAB1* mRNA variants in WT and mutant. "M" stands for DNA molecular weight ladder. C, Schematic diagram of *HAB1* mRNA variants in WT and mutant and details of the sequence of RT-PCR amplicons from B. D, E and F, Sensitivity of WT and mutant seed to ABA treatment. Seeds were grown on half-strength MS medium supplemented with or without 0.25 μmol L^{-1} ABA. D, Images were captured 5 d after stratification. Scale bar, 0.2 cm. E and F, Cotyledon greening rates and percentage of plants with true leaves were separately recorded 4 and 7 d after stratification. (n=3 biologically independent experiments). All values represent means±SD. ****, P<0.0001; ns, no significant difference by two-tailed Student's test.

observe any obvious phenotypical effect of the splice mutation. Thus, although we did not discover the function of the AS and its product *T30G6.16.2*, we succeeded in specifically removing the *T30G6.16.1* form in *Arabidopsis*, and the resulting single AS isoform mutant could be of value for further study of the function of *T30G6.16* and this splicing event.

Base editing prevents the generation of *RS31A.2*

In addition to IR, alternative 5′ splicing is another class of AS event that could be influenced by editing 5′ splice sites. To produce such a change, we chose to target the serine/arginine-rich splicing factor, RS31A. In the pre-mRNA of *RS31A*, intron 2 can be spliced at two different 5′ splice sites, or retained, generating three mRNA isoforms, that encode three protein isoforms with different N′-termini (Figure 3A) (https://www.arabidopsis.org/). We designed an sgRNA to target the 5′ splice site of *RS31A.2* of this intron (Figure 3A). In theory, this strategy should specifically prevent the production of *RS31A.2*, leaving *RS31A.1* and *RS31A.3* intact. Sequencing of genomic DNA from T1 plants identified three identical mutants, in which G-to-A conversions had occurred at the desired G of the 5′ splice site of intron 2 and the

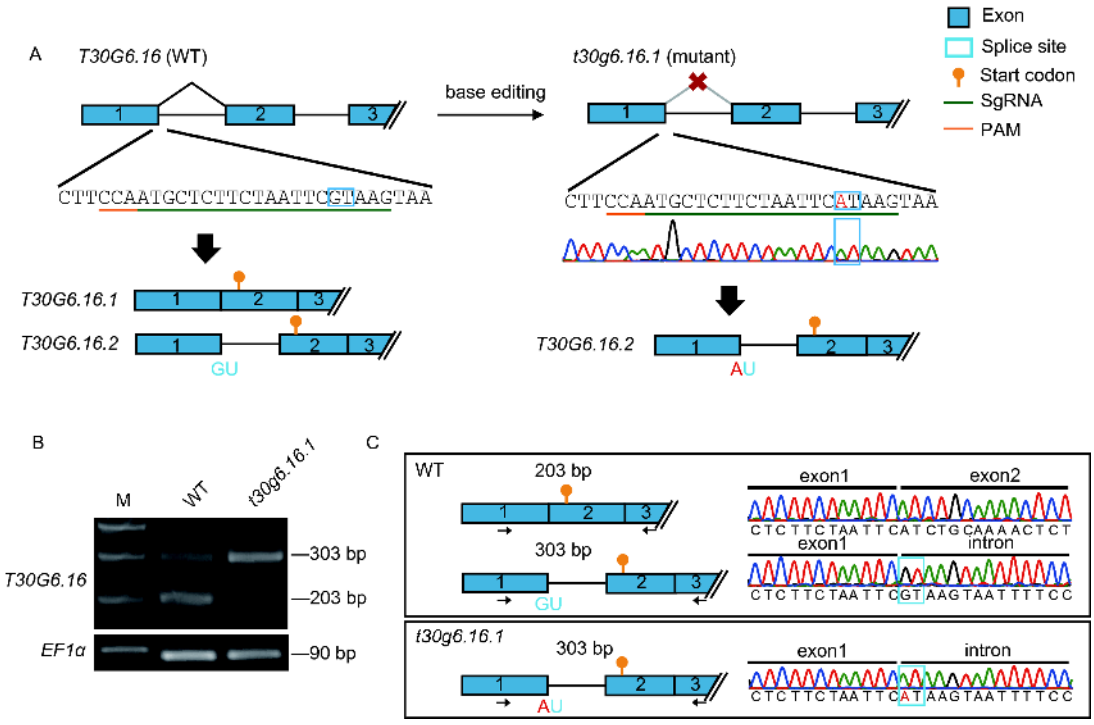

Figure 2 Base editing can alter *T30G6.16* splicing. A, Schematic representations of the influence of base editing on *T30G6.16* splicing. Left, the target sequence in *T30G6.16*, and a schematic diagram of *T30G6.16* splicing in WT. Right, the base-edited sequence in *t30g6.16.1* and a diagram of *t30g6.16.1* splicing in the mutant. B, RT-PCR analysis of *T30G6.16* mRNAs in WT and mutant. "M" is a DNA molecular weight ladder. C, Diagram of *T30G6.16* mRNAs in WT and mutant, and details of the sequence of the RT-PCR amplicons from B.

neighboring G (Figure 3A and Table S1 in Supporting Information). To investigate whether *RS31A.2* was absent from this mutant, we designed a forward primer in exon 2 and a reverse primer in exon 3, and used them to examine the presence of the three mRNA isoforms (Figure 3B and C). In the WT, we detected three fragments, amplified from *RS31A.1*, *RS31A.2* and *RS31A.3*, respectively (Figure 3B), whereas in the mutants we detected, only two fragments of similar sizes to *RS31A.1* and *RS31A.3*, respectively (Figure 3B). Sequencing showed that the shorter amplicon was from *RS31A.1*, and the larger from *RS31A.3*, with the same substituted residues as in genomic DNA (Figure 3B, C and Figure S4 in Supporting Information). This mutant was designated as *rs31a.2* (*RS31A.2* knock-out mutant). From Figure 3B, it can be seen that the level of *RS31A.1* transcript was higher in the mutant than in the WT, while the *RS31A.3* level was lower, suggesting that disruption of this splice site increased splicing on the alternative splice site rather than causing more intron retention. *RS31A* encodes a putative splicing factor (Barta et al., 2010). To investigate the function of *RS31A.2*, we consulted online expression data for *RS31A*, and found that it is significantly upregulated in genotoxic environments caused by bleomycin and mitomycin C treatment (Figure S5 in Supporting Information) (*Arabidopsis* eFP browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We therefore sowed WT and homozygous mutant seeds on half-strength MS medium with or without 40 μmol L^{-1} mitomycin C, and observed that, in terms of both cotyledon greening and true leaf development, the mutant plants were less sensitive to mitomycin C than the WT (Figure 3D–F), indicating that *RS31A.2* plays a significant role in genotoxic responses. In this experiment, we were able to specifically mutate a particular 5′ splice site of *RS31A.2*, erased a specific isoform and then reveal its function. This shows that base editor provides a way to investigate single AS isoforms even when the AS events are very complex.

Base editing changes the splicing of *Act2*

We next attempted to use this approach to investigate constitutive splicing. In some cases, introns near the 5′ region of an mRNA enhance gene expression by a process called intron-mediated enhancement (IME) (Laxa, 2016). IME has been found in plants, animals and even fungi (Laxa, 2016), and its mechanism is complex (Shaul, 2017). For example, splicing is necessary for IME in maize *Adh1*, *Hsp82*, and *Sh1*, but it was not required in *Arabidopsis PAT1* (Shaul, 2017). It has been reported that an intron in the 5′ UTR regulates the expression of *Arabidopsis ACT2* by IME (Jeong et al., 2009). However, the detailed mechanism is unknown. To dissect this mechanism, we attempted to test

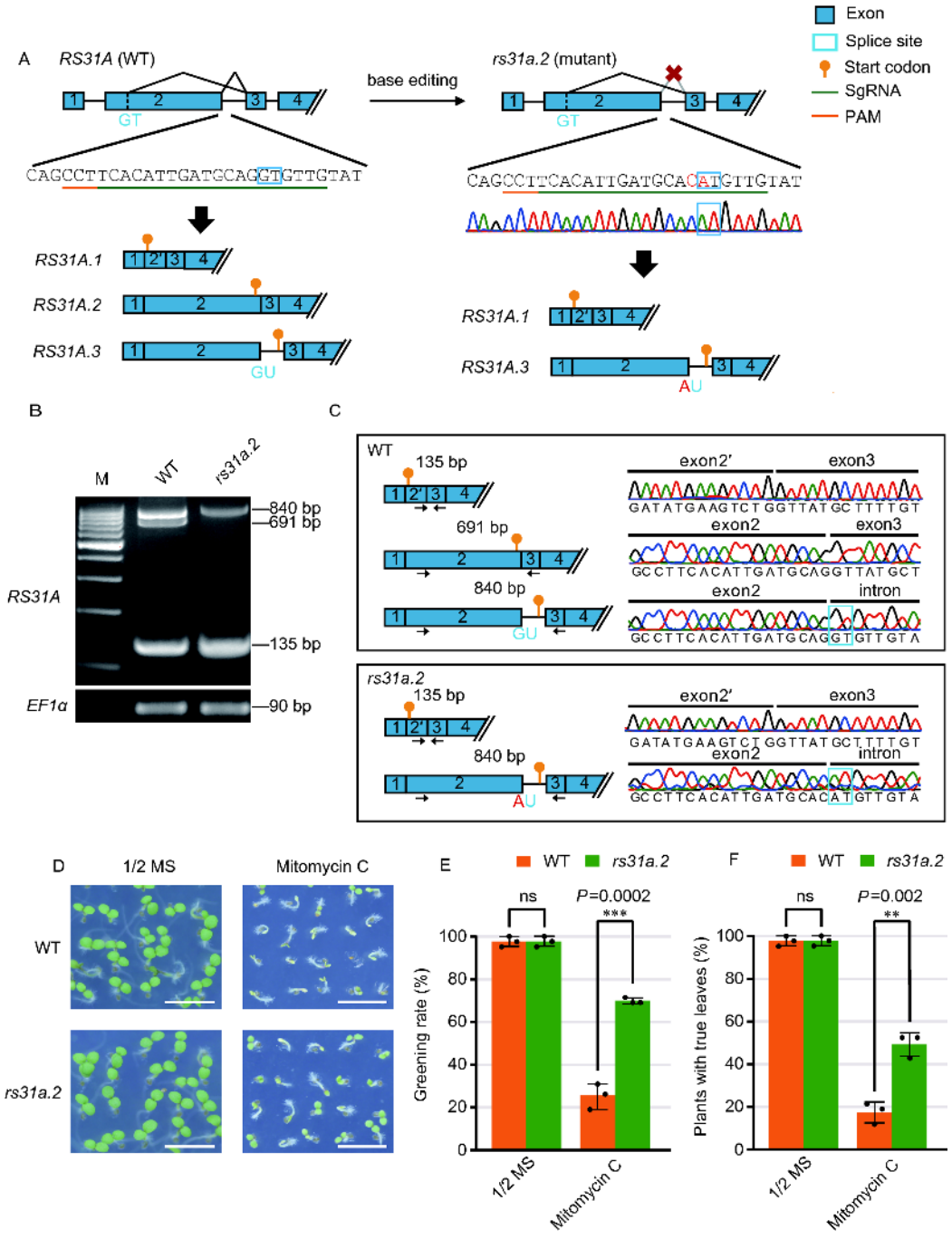

Figure 3 Base editing affects *RS31A* splicing. A, Schematic representations of the influence of base editing on *RS31A* splicing. Left part, the base editor target sequence in *RS31A* and schematic diagram outlining *RS31A* splicing in WT. Right part, the base-edited sequence in *RS31A* and schematic diagram outlining *RS31A* splicing in mutant. B, RT-PCR analysis of *RS31A* mRNA variants in WT and mutant. "M" stands for DNA molecular weight ladder. C, Schematic diagram of *RS31A* mRNA variants in WT and mutant and details of the sequence of RT-PCR amplicons from B. D, E and F, Sensitivity of WT and mutant seed to mitomycin C treatment. Seeds were grown on half-strength MS medium supplemented with or without 40 μmol L^{-1} mitomycin C. D, Images were captured 3 d after stratification. Scale bar, 0.2 cm. E and F, Cotyledon greening rates and percentage of seedling with true leave were separately recorded 3 and 8 d after stratification. (n=3 biologically independent experiments). All values represent means±SD. **, P< 0.01; ***, P<0.001; ns, no significant difference by two-tailed Student's test.

whether splicing was needed for this IME by designing an sgRNA to target the 5′ splice site of this intron (Figure 4A). Sequencing of the genomic DNA of T1 plants identified seven heterozygous mutant lines of the same genotype, each with a G-to-A conversion at the desired G of the 5′ splice site of intron 2 and a G-to-C conversion occurred at the neighboring G (Figure 4A and Table S1 in Supporting Information). To see whether destroying this 5′ splice site blocked this splicing, we designed a pair of primers to amplify the sequence of the 5′ UTR in T2 homozygous mutants and WT

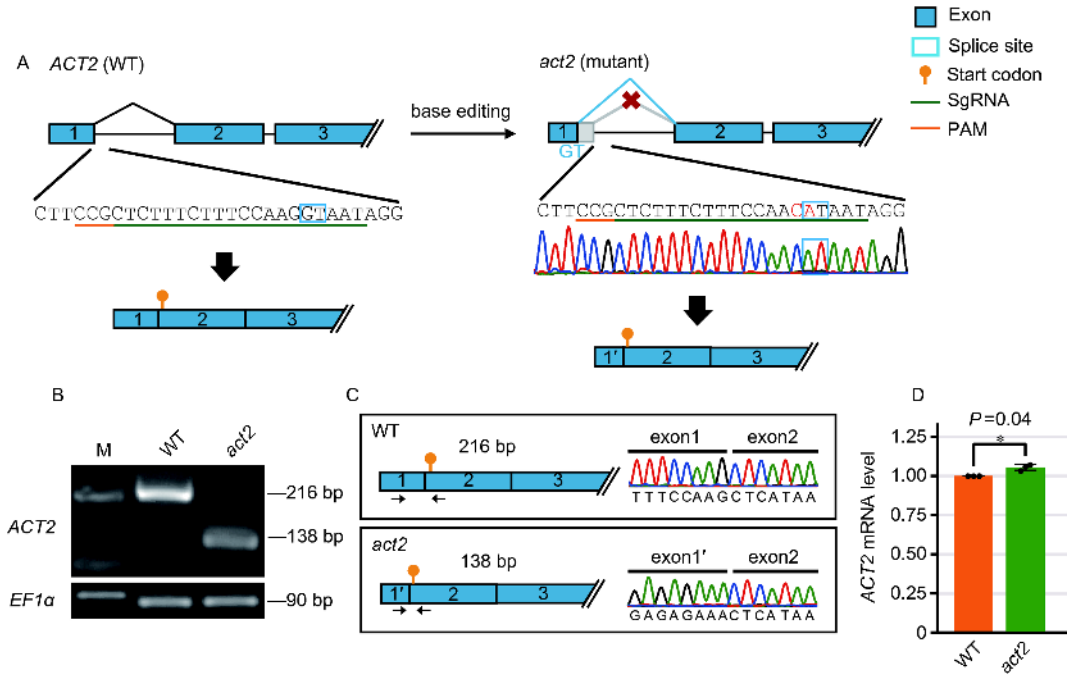

Figure 4 Base editing affects *Act2* splicing. A, Schematic representations of the influence of base editing on *Act2* splicing. Left part, the base editor target sequence in *Act2* and schematic diagram outlining *Act2* splicing in WT. Right part, the base-edited sequence in *Act2* and schematic diagram outlining *Act2* splicing in mutant. B, RT-PCR analysis of *Act2* mRNA variants in WT and mutant. "M" stands for DNA molecular weight ladder. C, Schematic diagram of *Act2* mRNA variants in WT and mutant and details of the sequence of RT-PCR amplicons from B. D, qPCR analysis of total *Act2* transcript level in WT and mutant. All values represent means±SD. *, $P< 0.05$ (n=3 biologically independent experiments).

plants. Interestingly, the mutant amplicon was shorter than the WT form, indicating disrupting the 5′ splice site did not lead to intron retention (Figure 4B and C). Sequencing of the mutant amplicons indicated that the 5′ end of exon 2 had been ligated with another site within exon 1, using an alternative splice site that was not used in WT (Figure 4B, C and Figure S6 in Supporting Information). q-PCR showed that this mutation did not decrease *ACT2* expression at the RNA level (Figure 4D). The above observations hinted that splicing might be important for IME in *ACT2*, and that the alternative 5′ splice site might rescue the IME. To test this idea, we would need to disrupt the alternative 5′ splice site in the mutant background. However, no NGG PAM is present to provide Cas9 recognition. We hope, in the future, we can disrupt this alternative 5′ splice site using the newly developed base editors. There are two possible outcomes: 1) an alternative 5′ splice site will be used for splicing; 2) the intron will be retained. If intron retention occurs, we would be able to test whether splicing is necessary for IME, and we could also investigate the influence of the retained intron on gene expression events, such as translational regulation, because there are several uATGs in the intron. These uATG might initiate translation of uORFs, which are well-known translation-related elements (von Arnim et al., 2014; Zhang et al., 2018). This case indicates that although base editing is also a powerful tool for studying the mechanism and function of constitutive splicing, it needs to be applied more widely.

DISCUSSION

The mRNA splicing plays a fundamental role in gene functional regulation. Very recently, it has been shown that A to G conversions at splice sites can lead to mRNA mis-splicing in *Arabidopsis* (Kang et al., 2018). We demonstrated here that, using a Cas9-directed base editor, G-to-A conversions can be precisely created in the target genes to inactivate the 5′ splice site (GT). The resulting mutants lacked the anticipated AS events and the corresponding mature mRNA forms, and were thus useful for investigating the function of specific AS isoforms. Furthermore, we proved that base editing of the key nucleotides required for constitutive splicing is useful for IME analysis. An accompany study has independently confirmed mRNA mis-splicing and target gene inactivation caused by disruption of splice sites using the same Cas9-base editor. Altogether, our data suggest that base editing provides a powerful tool for investigating the functions of splicing and transcript isoforms. Moreover, off-target mutations were checked in this study. By sanger sequencing, we did not find mutations in the potential off-target sites in the mutant lines of *AtHAB1*, *AtT30G6.16*, *AtRS31A* and *AtAct2* (Table S2 in Supporting Information). As the base editing tools with expanded PAM specificities are becoming available, it is likely that manipulating pre-mRNA splicing via CRISPR-directed base editors will gain extensive applications in gene functional studies in plants and other eukaryotic organisms.

MATERIALS AND METHODS

Plasmid constructs

To obtain mutants of the 5′ splice sites in *AtHAB1*, *AtRS31A*, *AtT30G6.16* and *AtACT2*, PHEE901-*AtHAB1*-sgRNA, PHEE901-*AtRS31A*-sgRNA, PHEE901-*AtT30G6.16*-sgRNA and PHEE901-*AtACT2*-sgRNA were constructed as reported (Chen et al., 2017). The pre-annealed sgRNAs, which are listed in Table S3 in Supporting Information, were inserted into BsaI-digested PHEE901.

Generating mutants of the 5′ splice sites in *AtHAB1*, *AtRS31A*, *AtT30G6.16* and *AtACT2*

5′ splice site (GT) mutants of *AtHAB1* (AT1G72770), *AtRS31A* (AT2G46610), *AtT30G6.16* (AT5G36290) and *AtACT2* (AT3G18780) were generated by genetic transformation by floral dip of *Arabidopsis* (ecotype Col-0) with vectors PHEE901-*AtHAB1*-sgRNA, PHEE901-*AtRS31A*-sgRNA, PHEE901-*AtT30G6.16*-sgRNA and PHEE901-*AtACT2*-sgRNA, respectively (Chen et al., 2017). The transgenic plants were screened on half-strength MS medium with 50 μg mL^{-1} hygromycin and 150 μg mL^{-1} carbenicillin and mutants were identified by Sanger sequencing of PCR products. Primers are listed in Table S4 in Supporting Information.

RNA preparation, RT-PCR and qRT-PCR

Total RNA was extracted from plant samples with an Ultrapure RNA Kit (CW0581M, Cwbiotech, Peking, China). Reverse transcription was performed with M-MLV Reverse Transcriptase (M1701, Promega, Madison, USA). Primers for RT-PCR are listed in Table S4 in Supporting Information. PCR products were cloned into pEasy-B vector (TransGen Biotech, Peking, China), and sequenced. qRT-PCR was performed with a SsoFast EvaGreen Supermix kit (1725204, Bio-Rad, Hercules, USA) following the supplier's instructions. Primers are listed in Table S4 in Supporting Information.

Mitomycin C and ABA treatment

Mitomycin C (AM395, Genview, Jacksonville, USA) and ABA (D8942, Sigma-Aldrich, St. Louis, USA) were used. *Arabidopsis* seeds were surface-sterilized with 70% alcohol for 1 min, following by 10% bleach for 20 min. Then they were washed four times with sterile water, plated on half-strength MS medium with 0, 40 μmol L^{-1} mitomycin C or 0, 0.25 μmol L^{-1} ABA, and grow at 4°C for 3 days for stratification. Plates with mitomycin C need to be wrapped in aluminum foil and kept in the dark. Afterwards, they are unwrapped and placed horizontally in the light at 22°C. Cotyledon greening rate and percentage of plants with true leaves were measured respectively.

Statistical analysis

All numerical values are presented as means±SD. Differences between wild type and relevant mutants were analyzed by two-tailed Student's *t*-tests.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Alignment of the cDNA sequences of *AtHAB1*(AT1G72770) wild type (WT) and mutant.

Figure S2 Alignment of the cDNA sequences of *AtT30G6.16* (AT5G36290) wild type (WT) and mutant.

Figure S3 Comparison of *AtT30G6.16* transcript levels in wild type (WT) and mutant.

Figure S4 Alignment of the cDNA sequences of *AtRS31A* (AT2G46610) wild type (WT) and mutant.

Figure S5 Online expression data for *AtRS31A* in the *Arabidopsis* eFP browser.

Figure S6 Alignment of the cDNA sequences of *AtACT2* (AT3G18780) wild type (WT) and mutant.

Table S1 Molecular and genetic analysis of CRISPR/Cas9-induced splicing site mutations of *AtHAB1*, *AtT30G6.16*, *AtRS31A* and *AtAct2* in T0 generation of *Abadidopsis* and their transmission to T1 generation

Table S2 Potential off-target sites analyzed for *AtHAB1*, *AtT30G6.16*, *AtRS31A* and *AtAct2* in *Arabidopsis*

Table S3 Target sites of the sgRNAs in the 5′ splice site of the four studied genes, and the oligonucleotides to construct each sgRNA

Table S4 List of the PCR primers used in this study

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