




Generation of a multi-herbicide-tolerant alfalfa by using base editing

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Abstract

Keymessage We present the first report on base editing in alfalfa. Specifically, we showed edited alfalfa with tolerance to both sulfonylurea- and imidazolinone-type herbicides.

Keywords Alfalfa · Base editing · CRISPR/Cas9 · ALS · Herbicides

Alfalfa (*Medicago sativa*) is one of the most important forage legumes worldwide. However, alfalfa-breeding programs are limited by the particular reproductive features of the species itself. Cultivated alfalfa is an allogamous perennial polyploid species that exhibits high levels of self-incompatibility and strong inbreeding depression (Dieterich Mabin et al. 2021). Consequently, the rapid and inexpensive introgression of both transgenes and edited alleles into elite alfalfa germplasm requires the use of dominant traits (Bottero et al. 2021; Jozefkowicz et al. 2018). In this context, the commercial application of full knockout of recessive genes in alfalfa efficiently generated via the CRISPR/Cas9 system (Bottero et al. 2021; Chen et al. 2020; Wolabu et al. 2020).

A major threat to alfalfa production is the presence of weeds. In this context, transgenic glyphosate-tolerant alfalfa varieties have been cost-effective tools to assist farmers in the management of weeds (Samac and Temple 2021). However, the expansion of glyphosate resistance in weeds is an increasing problem that will require a transition to new weed control technologies in the future (Heap and Duke 2018). The recently developed cytidine base editors (CBEs),

which convert a cytosine (C) nucleotide to a thymine (T), offer an alternative to generate rapidly dominant traits, including herbicide-tolerant traits (Mishra et al. 2020). Particularly, CBEs have been used to generate herbicide-tolerant plants via proline (P) to leucine (L) mutations in acetolactate synthase proteins (ALS1 and ALS2) in both dicot and monocot species (Veillet et al. 2019; Zhang et al. 2019).

To expand the benefits of CBEs to alfalfa species, we here produced around 150 independent transgenic events with the pXSE901BG plasmid containing the CRISPR/Cas9-mediated base editor and gRNA against the ALS1 and ALS2 genes from alfalfa (Fig. S1). After a selection process of imazapic-tolerant plants, we identified 17 and 9 edited events mostly showing the expected single C-to-T base conversion in the ALS1 and ALS2 genes at nucleotide sites 530 and 545, respectively (Fig. 1a). Contrary to their parental wild-type regenerative clone C23 (García et al. 2014) and to transgenic (Cas9+) non-edited plants, edited plants displayed tolerance to single and mixed commercial herbicides metsulfuron-methyl, foramsulfuron and iodosulfuron-methyl-Na, and imazapic (Fig. 1b). By using high-resolution melting (HRM) analysis, a method used to identify variations in nucleic acid sequences, we were able to discriminate between wild-type and edited plants quickly and at low cost (Fig. 1c), a critical point for the coexistence of genetically engineered and conventional alfalfa.

While there is a legal framework for the deregulation of transgenic crops, including the glufosinate-tolerant alfalfa variety previously generated by our group (Jozefkowicz et al. 2018), the deregulation of transgenic events is a long-term and costly process. In the case of allogamous species such as alfalfa, the editing machinery can be easily segregated

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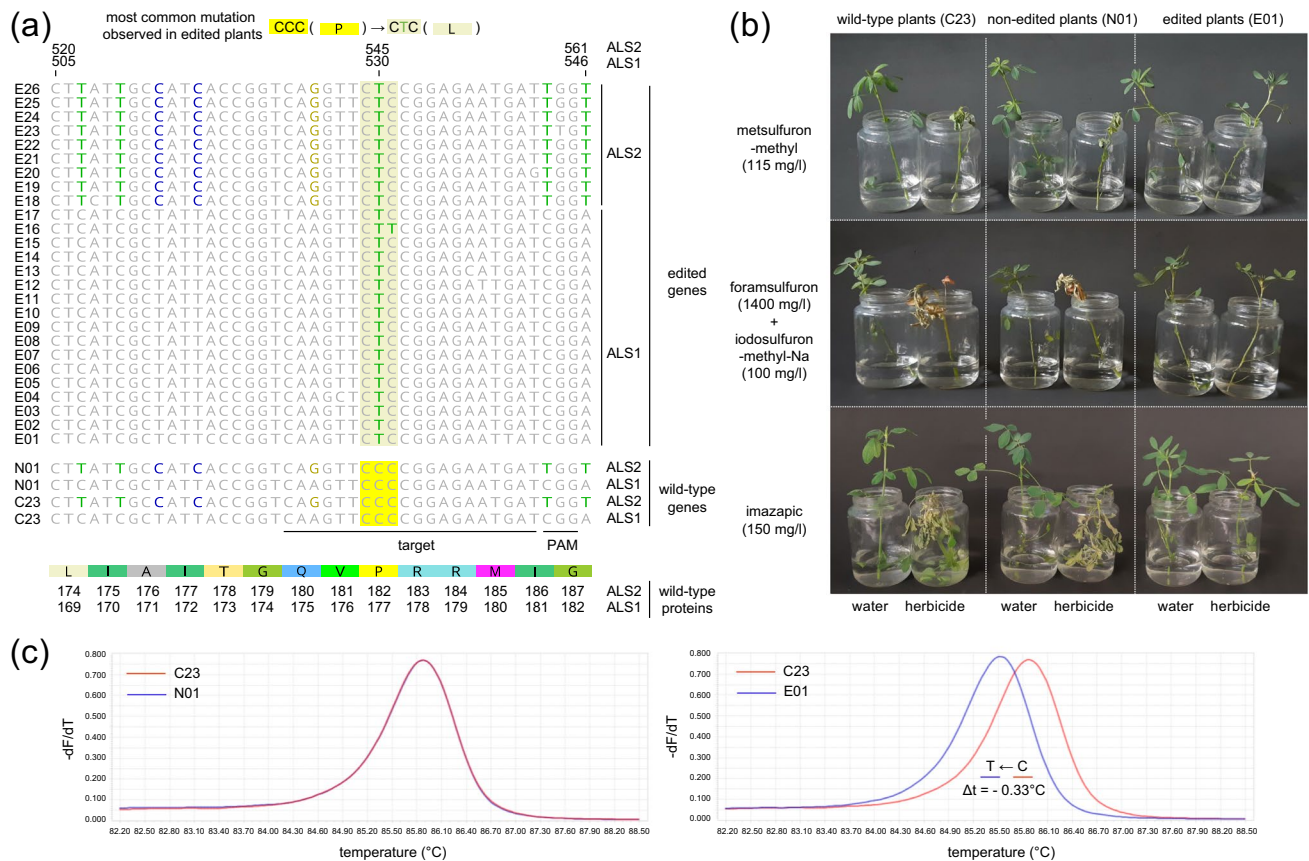


Fig. 1 Generation of alfalfa plants harboring herbicide tolerance mutations that confer tolerance to sulfonylurea- and imidazolinone-type herbicides by base editing the acetolactate synthase genes (ALS1 and ALS2). **a** Sequence analysis of imazapic-tolerant plants revealed 26 edited plants containing single allelic mutations within the ALS1 or ALS2 genes from alfalfa. **b** Representative herbicide tolerance of edited plants (E01). Wild-type regenerative clone used in alfalfa transformation (C23) and transgenic (Cas9+) non-edited plants (N01) were used as sensitive controls. Plants were incubated in tap water supplemented with herbicides for 15 days in a growth cham-

ber, with a 16-/8-h light/dark photoperiod and a constant temperature of 25 °C. Metsulfuron-methyl (DuPont), foramsulfuron and iodosulfuron-methyl-Na (Bayer Crop Science) are sulfonylureas, whereas imazapic (BASF) is an imidazolinone. **c** High-resolution melting (HRM) can efficiently identify the single mutation (C-to-T substitution) which produces herbicide tolerance in alfalfa. Representative melting curves demonstrate distinct patterns between the wild-type (C23 and N01) and mutant (E01) genotypes, where C-to-T conversion reduces melting temperature

during the introgression process (Bottero et al. 2021), and then the *Agrobacterium* transformation does not imply a constraint to generate transgenic-free cultivars. Now, we are introgressing the beneficial C-to-T substitution into elite alfalfa germplasm and have very recently obtained a resolution (NO-2021-54571304-APN-DNB#MAGYP of the Ministry of Agriculture, Argentina) that supports the possible use of this herbicide tolerance trait produced via CBEs as a conventional trait.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00299-021-02827-w>.

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Author contribution Conceived and designed the experiments: MS and GS. Performed the experiments: EB, CG, MS, HT, RF, CP, NA and GS. Analyzed the data: EB, CG, MS, HT, EB, NA and GS. Wrote the paper: GS. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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