Developing a flexible, high-efficiency Agrobacterium-mediated sorghum transformation system with broad application

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Summary

Sorghum is the fifth most widely planted cereal crop in the world and is commonly cultivated in arid and semi-arid regions such as Africa. Despite its importance as a food source, sorghum genetic improvement through transgenic approaches has been limited because of an inefficient transformation system. Here, we report a ternary vector (also known as cohabitating vector) system using a recently described pVIR accessory plasmid that facilitates efficient Agrobacterium-mediated transformation of sorghum. We report regeneration frequencies ranging from 6% to 29% in Tx430 using different selectable markers and single copy, backbone free ‘quality events’ ranging from 45% to 66% of the total events produced. Furthermore, we successfully applied this ternary system to develop transformation protocols for popular but recalcitrant African varieties including Macia, Malisor 84-7 and Tegemeo. In addition, we report the use of this technology to develop the first stable CRISPR/Cas9-mediated gene knockouts in Tx430.

Introduction

Tolerant to hot and dry conditions, sorghum (Sorghum bicolor L.) is the fifth most widely planted cereal crop in the world and the second most important cereal (after maize) in sub-Saharan Africa (Awika and Rooney, 2004; Hei et al., 2014; Ji et al., 2013; Liu and Godwin, 2012). It is estimated that about 500 million people worldwide rely on sorghum as their daily staple food source (Awika and Rooney, 2004; Che et al., 2016; Hei et al., 2014; Liu and Godwin, 2012). However, sorghum grain is seriously micronutrient deficient in pro-vitamin A (β-carotene), in the bioavailability of iron and zinc, and is poor in protein digestibility (Che et al., 2016). Due to the limitation of traditional breeding, plant biotechnology has been demonstrated as an essential component for crop biofortification and improving crop agro-nomic performance. However, the lack of an efficient sorghum transformation system for genetic modification, especially for the important African varieties, has impeded sorghum research aimed at elucidating mechanisms for heat and drought resistance, increasing yield and improving grain quality.

In principle, a successful transformation system relies on a combination of factors including a selectable marker, efficient gene delivery and responsive tissue culture. Although significant progress has been made in recent years and a transformation efficiency of ~21% can be achieved with microprojectile bombardment (Liu and Godwin, 2012), the event quality from particle bombardment-derived plants is typically low and rarely results in precise single-copy insertions of only the desired DNA sequence or ‘quality events’ (QE) (see Experimental Procedures for ‘QE’ definition). While Agrobacterium-mediated transformation (AGMT) is more amenable to low and single-copy insertions of precise DNA sequence, optimized tissue culture protocols are limited to Tx430 and transformation efficiencies of total event production are only 10% (Wu et al., 2014). More virulent Agrobacterium strains such as AGL1 have achieved higher transformation frequencies; however, the event quality decreased even more significantly, resulting in fewer overall usable quality events (Wu et al., 2014; Zhi et al., 2015). When compared with transformation methods for other cereal crops, such as corn and rice, sorghum transformation lags far behind (Ji et al., 2013).

Binary vectors are ideal for introducing genes into crop plants because of their ability to integrate transgenes in low and single copy. The broader application of AGMT has been achieved by implementing so-called super-binary, co-integrate vectors (CIV) resulting in vastly improved crop transformation, especially for cereals (Ishida et al., 1996). However, the super-binary plasmid pSB1 (Figure 1a) has limited number of vir genes (B, G and part of C) included on the plasmid and requires a co-integration step to generate the final construct. In addition, the large plasmid size also presents a challenge for high-throughput vector construction (Ishida et al., 1996). To address these issues and improve plant transformation, Anand et al. (2017a) designed a series of pVIR vectors [pHP70298, pHP71539 (Figure 1b) and pHP79761] containing an optimal set of vir genes. These genes were assembled into ternary vector (also known as cohabitating vector) (Anand et al., 2017a,b) in which a T-DNA-less accessory pVIR plasmid and a T-DNA transfer competent binary plasmid are cotransformed into an Agrobacterium cell and used for plant transformation (Figure 1b). These pVIR vectors have many desirable features, including smaller vector sizes, enhanced vector stability and amended vir genes for enhanced T-DNA gene delivery. Furthermore, the ternary vector system was demonstrated to improve maize transformation (Anand et al., 2017b). This encouraged us to assess it for enhancing sorghum
transformation in combination with the media optimization previously established (Wu et al., 2014).

Using surfactants and the ternary vector system, we developed a highly efficient and broadly applicable sorghum transformation system that works with a range of selectable markers for Tx430 as well as the recalcitrant Africa varieties including Macia, Maisor 84-7 and Tegemo. We applied our sorghum transformation system to generate high-quality transgenic events and demonstrated its potential to support efficient genome editing by creating the first stable knockouts in sorghum using CRISPR/Cas gene editing technology.

Results

Enhancing Agrobacterium-mediated sorghum transformation frequency with surfactant

We chose to investigate whether the surfactant Silwet-70 could enhance sorghum transformation as has been demonstrated transiently for both corn and wheat transformation (Cao et al., 2014; Wu et al., 2003). We tested the effects of Silwet-70 application on Agrobacterium-mediated immature embryo transformation of Tx430 using the transformation protocol published by Wu et al. (2014). Plasmid pPHP38332 (Figure 1a) was constructed as a CIV vector from super-binary vector pSB1 (Figure 1a) and transferred into Agrobacterium auxotrophic strain LBA4404 Thy- (thymidine mutant), a strain for better control of bacterial overgrowth during tissue culture compared to strain LBA4404 Thy- minus pPHP71539 plasmid. To enhance sorghum transformation as has been demonstrated previously (Wu et al., 2014), we designed two ternary donor constructs derived from pPHP38332(CIV). This observation corroborated the maize results demonstrating superior T-DNA delivery with the ternary construct, compared to the CIV construct (Anand et al., 2017b), a preferred design for sorghum transformation. The ternary design also offers simple and versatile vector assembly requiring no co-integration step, which can occasionally result in vector integrity, complicating vector assembly and mobilization into multiple Agrobacterium strains.

Having confirmed that the ternary vector enhanced transient T-DNA delivery in sorghum, we investigated whether this translated into improved stable transformation in sorghum. To this end, we designed two ternary donor constructs derived from pPHP82637 (Figure 1e), pPHP78152 and pPHP78233 with T-DNA sizes of 17–18 kb, respectively. The T-DNA harbours a PMI selectable marker and three different proprietary genes for transformation efficiencies for the two ternary constructs were 29% and 25%, respectively (Table 2). The stable transformation frequency observed with the larger size T-DNA (17–18 kb) in the

Figures 1 Schematic representation of the molecular components of (a) CIV and (b) ternary vector systems. pPHP71539 works as the accessory plasmid for the ternary vector system.
ternary vectors was considerably higher compared to transformation frequency (15%) observed with the CIV construct pPHP38332 (T-DNA, 8 kb) (Table 1).

Next, we evaluated the event quality of the transgenic plants produced with the ternary vector system. Higher transformation frequencies can often be accompanied by an undesirable increase in copy number of T-DNA insertions and backbone integration (Oltmanns et al., 2010; Zhi et al., 2015). Multicopy events have the potential to affect transgene expression and stability as well as impede regulatory assessment (Oltmanns et al., 2010; Zhi et al., 2015).

To assess event quality, T0 plants were generated using pPHP38332(CIV), pPHP78152(ternary) or pPHP78233(ternary) as detailed in Experimental Procedures. T0 plants were subjected to detailed molecular event characterization as previously reported (Wu et al., 2014; Zhi et al., 2015). As represented in Table 2, 54% and 65% of the events produced with the ternary constructs were determined to be single-copy insert, backbone-minus quality events, which was comparable to quality event frequency determined for the CIV construct pPHP38332 (66%) with a relatively smaller T-DNA (Table 1). Based on the above observation, it was concluded that the ternary vector results in a higher transformation efficiency with minimal to no impact on the quality of events produced in sorghum. In practical terms, this means fewer immature embryos are required to produce the same number of quality events compared to the CIV transformation system.

Transformation for different sorghum varieties

Genotype dependence is a major limitation of an immature embryo-based transformation system currently observed in the cereals, corn, rice, wheat and sorghum (Harwood, 2012; Hiei et al., 2014; Que et al., 2014). Although sorghum transformation has been improved for several model varieties in the past decade (Ji et al., 2013), no transformation has ever been carried out in the background of native African varieties. To broaden the application of the sorghum transformation technology and to facilitate sorghum research for yield and quality improvement in African countries, we further evaluated transformability of three popular Africa varieties, Macia, Malisor 84-7 and Tegemeo using the ternary vector design. We successfully transformed both Malisor 84-7 and Tegemeo using the CIV construct pPHP38332 with transformation frequencies of 9.3% and 6.4%, respectively (Table 1). However, with the CIV vector, Macia was found to be highly recalcitrant to transformation and no events were generated from 5000 embryos infected under the same culture conditions (Table 1). In contrast, using the ternary vector design with the accessory plasmid pPHP71539 and binary vectors pPHP71852 and pPHP78233, we demonstrated successful transformation of all three African varieties (Table 2). The variety

![Figure 2](image1.png) Effect of surfactant Silwet-70 on transformation frequency in Tx430. Error bars indicate the ±SD from three treatments with more than 100 embryos per treatment.

![Figure 3](image2.png) T-DNA delivery determined by YFP transient assay. Sorghum Tx430 immature embryos were infected with *Agrobacterium* carrying either pPHP38331(CIV) or pPHP45981(ternary) with pPHP71539 as accessory plasmid. T-DNA delivery was represented by the number of transgenic cells exhibiting YFP fluorescence and the fluorescence intensity on the surface of sorghum embryos.

![Table 1](image3.png) Transformation efficiency and event quality using CIV transformation system and PMI as selectable marker

| Construct | Variety      | Transformation efficiency % | Quality events % |
|-----------|--------------|-----------------------------|------------------|
| pPHP38332 | Malisor 84-7 | 9.3 ± 2.8                   | 61%              |
| (4.6 kb T-DNA) | Tegemeo | 6.4 ± 2.7                   | NA               |
| pPHP38332 | Macia        | 0.0 ± 0.0                   | NA               |
| pPHP38332 | Tx430        | 15.1 ± 1.7                  | 66%              |

Data were presented as the average ± SD of three biological replications. Thirty embryos were used for each biological replication. NA: Not available.
selectable markers (Miki and McHugh, 2004), we further explored and quality event production efficiencies (54% described by Wu et al., 2014). Given the concerns of using antibiotic and herbicide genes as selectable marker

Table 2 Transformation efficiency and event quality using ternary transformation system and PMI as selectable marker

| Construct     | Variety       | # of embryos | # of events | Transformation efficiency % | Quality events % |
|---------------|---------------|--------------|-------------|-----------------------------|-----------------|
| pPHP78152     | Malisor       | 561          | 53          | 9.4%                        | 47%             |
| (17 kb T-DNA) | Tegemeo       | 420          | 8           | 1.9%                        | NA              |
|               | Macia         | 440          | 3           | 0.7%                        | NA              |
|               | Tx430         | 350          | 103         | 29%                         | 54%             |
| pPHP78233     | Malisor       | 599          | 33          | 5.5%                        | 51%             |
| (18 kb T-DNA) | Tegemeo       | 450          | 5           | 1.1%                        | NA              |
|               | Macia         | 521          | 7           | 1.3%                        | NA              |
|               | Tx430         | 400          | 99          | 25%                         | 65%             |

NA, Not available.

Tegemeo exhibited a lower transformation frequency with the ternary vector compared to the CIV construct (Tables 1 and 2) which we speculate could be due to the larger size of the T-DNA in the ternary (17 and 18 kb) vs the CIV vector (4.6 kb). Nonetheless, these results strengthen our finding that enhanced T-DNA delivery using the accessory plasmid pPHP71539 can broaden the transformatibility for various sorghum varieties. This to our knowledge is the first successful demonstration of AGMT in African sorghum varieties.

Transformation using alternative selection markers

To broaden the applications of the ternary vector for transformation technology and provide extra flexibility for academic and industrial application, we tested alternate selectable markers for sorghum transformation. Two different selectable markers neomycin phosphotransferase II (NPTII)/G418 and a newly described Phosphonate dehydrogenase (PTXD)/phosphite (Phi) were evaluated for sorghum transformation.

NPTII is a widely used selectable marker gene that has been utilized successfully in corn transformation and previously described for sorghum transformation using particle bombardment (Ji et al., 2013; Liu and Godwin, 2012). Using the ternary construct with a T-DNA vector (pPHP81561, Figure S1c) containing the NPTII gene and following a transformation procedure described by Wu et al. (2014), we optimized the concentration of G418 at 250 mg/l and achieved a transformation efficiency as high as 26% in Tx430 (Table 3), of which about 45% of the events were quality events. This is comparable to the PMI selectable marker system in terms of transformation (25%–29%) and quality event production efficiencies (54%–65%) (Table 2).

Given the concerns of using antibiotic and herbicide genes as selectable markers (Miki and McHugh, 2004), we further explored the possibility of using the bacterial PTXD gene as a positive selectable marker for Agrobacterium-mediated sorghum transformation. Phosphite (Phi) has a similar molecular structure as phosphate (Pi). Unlike Pi, however, which can be used directly by plant cells as bioavailable phosphorus, Phi cannot be metabolized and plants that are grown on phosphite-containing media are starved of phosphorus. It is reported that PTXD catalyses the conversion of Phi into inorganic Pi that can then be used by plant cells as a phosphorus source (Lopez-Arredondo and Herrera-Estrella, 2013). This enables transgenic plant cells carrying the PTXD gene to survive on the medium contain Phi as the sole phosphorus source. This selection system (PTXD/Phi) has been reported in corn, tobacco and Arabidopsis transformation (Lopez-Arredondo and Herrera-Estrella, 2013; Nahampun et al., 2016).

Table 3 Transformation efficiency and event quality of five independent experiments using ternary transformation system and NPTII as selectable marker

| Construct | Experiment | # of embryos | # of calus | # of calus with shoots | Callus % | Transformation efficiency % | Quality events % |
|-----------|------------|--------------|------------|------------------------|----------|------------------------------|-----------------|
| pPHP81561 | 1          | 209          | 62         | 44                     | 30%      | 21%                          | 45%             |
|           | 2          | 115          | 30         | 25                     | 26%      | 22%                          |                 |
|           | 3          | 115          | 29         | 24                     | 25%      | 21%                          |                 |
|           | 4          | 121          | 40         | 32                     | 33%      | 26%                          |                 |
|           | 5          | 117          | 46         | 29                     | 39%      | 25%                          |                 |
pyogenes Cas9 (spCas9) and their preceding 20 nucleotides in the Sb-CENH3 gene. Three targets sites were selected based on their location and orthogonality of cognate gRNAs engineered using guanine to pair with the 20th and most distal nucleotide to facilitate expression from the U6 promoter (Figure 5).

Fewer than 300 sorghum immature embryos were utilized for AGMT for each ternary construct and subsequent tissue culture to generate stable genome edited events using G418 selection. The overall regeneration efficiency ranged from 8% to 16% for the three constructs targeted to the essential Sb-CENH3 gene (Table 5) which is much lower than that using pPHP81561 (Table 3) for conventional transformation and the same selectable marker system. We expect the lower efficiency could be due to potential lethality of biallelic Sb-CENH3 knockouts (see footnote for the definition in Table 6) as has been observed in maize and Arabidopsis (Kelliher et al., 2016; Ravi and Chan, 2010). All the T0 plants were analysed by deep sequencing to identify mutations at the target sites (see Experimental Procedures for detail). As shown in Table 6 and Table S2, the targeted editing efficiency (see footnote for the definition in Table 6) was 37%–40% based on the transgenic plants analysed and targeted knockout efficiency (see footnote for the definition in Table 6) was in the range of 20%–37%. No biallelic knockouts were ever identified for all three constructs. This is consistent with a hypothesis of lethality for biallelic Sb-CENH3 mutations. Based on the information described above, the knockout efficiency per embryo transformed is about 1%–5% (Table 6 and Table S2). We expect that the overall knockout efficiency would be higher if editing were conducted with genes that were nonlethal as biallelic mutations.

**Discussion**

Sorghum has long been considered one of the most recalcitrant crops for Agrobacterium-mediated gene delivery, tissue culture, regeneration and genetic transformation. This situation has been significantly improved through efforts on tissue culture media optimization, as described by Wu et al. (2014) and through gene delivery system improvements as described in this report. The use of a ternary vector system containing the T-DNA binary vector and the optimized pVIR accessory plasmid significantly improved the efficiency of sorghum transformation compared to the previously described CIV vectors. The ternary vector not only

![Figure 4](image-url) Stringent PTXD/Phi selectable system. Picture represents the development of 6 weeks old transgenic and nontransgenic callus on callus induction medium containing 300 mg/L Phi. The transgenic callus is indicated by the arrow.

![Figure 5](image-url) Diagram of the DNA sequence of the target region of Sb-CENH3 gene. The three-nucleotide sequences highlighted in grey indicate the corresponding PAM motifs recognized by the Cas9 protein. crRNA hybridization targets for three different gRNAs are underlined. Exons and introns are represented by the uppercase and lowercase letters, respectively.

| Construct | Experiment | # of embryos | # of event with shoots | Transformation efficiency % | Quality events % |
|-----------|------------|--------------|------------------------|-----------------------------|-----------------|
| pPHP70444 | 1          | 100          | 4                      | 4%                          | 47%             |
|           | 2          | 100          | 3                      | 3%                          |                 |
|           | 3          | 100          | 4                      | 4%                          |                 |
|           | 4          | 100          | 6                      | 6%                          |                 |

**Table 4** Transformation efficiency and event quality of four independent experiments using ternary transformation system and PTXD as selectable marker

**Exon 1**

Sb-CENH3-CR1

5’-ATGGCTCGAAACCAAGCACCCAGCCGTAGGAGCTGCCGAGCAAGCCCAAGAAGAAGCTCCAGTTCGAGCGCGCAG-3’

3’-TACCGAGCTTGTGACGAGCTGCCGACGGCGTGTTCTGAGTCAAGCCTTGTGCAGAGTCAGCAGTGCCGTCGTC-5’

**Exon 2**

Sb-CENH3-CR2

5’-gtagGTGGGGGCGAGTAGTTGTGCTGCCGACGGCG-3’

3’-acgtcCACCCCGCTCATGAGCCGCGGTCGTC-5’

**Exon 5**

Sb-CENH3-CR3

5’-GTCAAGATTTTGACGAGCAGATGACGAGCTATACACCCCTGAGCAGCCCTATTGCTGCAGAGAC-3’

3’-CAGTTTCTCAATTGAGCTAGATTGTGACGAGCAGTTGATGCTGAGGAGGAAGAGCGACGCGGCGTGC-5’
The success of targeted genome modification in sorghum provides a biallelic lethal gene, Sb-CENH3, with a knockout efficiency ranging from 1% to 5% for genome modification using CRISPR/Cas genome editing technology. A plasmid pPHP71539 made it possible to generate targeted drag (Mumm, 2013).

Together with surfactant application for the immature embryo infection, the ternary vector was able to achieve 29% transformation frequency with the PMI/mannose selection system (Table 2) and around 26% based on the NPTII/G418 selection system (Table 3) in inbred line Tx430. In addition, the ternary vector system allowed stable transformation of large size T-DNAs containing four different proprietary genes (T-DNA up to 18 kb) without adversely affecting transformation frequency and quality event frequency (Tables 1–3), thus making the ternary vector system even more attractive for genome engineering.

A new nonantibiotic and nonherbicide system was developed for sorghum transformation based on PTXD as the selectable gene. Despite somewhat lower transformation frequency (Table 4), PTXD/Phi selection system is nonetheless an attractive transformation system that can be further improved, for example through monocot codon optimization of the PTXD gene to enhance the positive selection efficiency.

To further explore the transformation potential mediated for different sorghum varieties, we further applied the ternary vector system to three African varieties, namely Malisor 84-7, Tegemeo and Malisor B4-7, and achieved efficient transformation frequencies as well (Table 2). The optimized ternary vector system allows us to introduce transgenes directly into these Africa varieties without trait introgression backcrossing, a time-consuming process that typically takes many years to achieve and often results in yield drag (Mumm, 2013).

Furthermore, the ternary vector system containing the accessory plasmid pPHP71539 made it possible to generate targeted genome modification using CRISPR/Cas genome editing technology with a knockout efficiency ranging from 1% to 5% for the biallelic lethal gene, Sb-CENH3 (Table 6 and Table S2). The success of targeted genome modification in sorghum provides a powerful tool for studying sorghum genetics which will contribute to elucidating sorghum heat- and drought-resistant mechanisms and improving its yield and grain quality in sorghum in the near future.

Overall, the broad applications of the ternary system with pPHP71539 as the accessory plasmid described in this study makes Agrobacterium-mediated sorghum transformation become more practicable and can easily be implemented by most laboratories.

### Experimental procedures

#### Constructs for transformation

The CIV research construct pPHP38332 carrying the PMI selectable marker gene and YFP reporter gene has been previously described by Wu et al. (2014) (Figure S1a). The pVIR accessory plasmid pPHP71539, described by Anand et al. (2017a), comprises a pVS1 ORI and gentamycin as the bacterial selectable marker. The binary plasmids pPHP45981 (Figure S1b), pPHP81561 (Figure S1c) and pPHP70444 (Figure S1d) contain the repABC ORI, the spectinomycin bacterial selectable marker, the YFP reporter gene and PMI, NPTII or PTX2, respectively, as plant selectable marker genes, respectively. pPHP78152 and pPHP78233 are production constructs derived from pPHP82637 (Figure S1e) with proprietary genes with varying size of T-DNA (17 and 18 kb, respectively). The ternary design was assembled by first mobilizing the accessory plasmid pPHP71539 in the Agrobacterium auxotrophic strain LBAA4404 Thy- and selected on media supplemented with gentamycin (25 mg/L). Subsequently, the binary constructs were electroporated into Agrobacterium strain LBAA4404 Thy-containing the accessory plasmid and recombinant colonies were selected on media supplemented with both gentamycin and spectinomycin. All constructs were then subjected to next-generation sequencing and sequence confirmed before next transformation experiments.

CRISPR/Cas gene editing was achieved using the LBAA4404 Thy-Agrobacterium strain and the pPHP71539 pVIR accessory system described by Anand et al. (2017a). The spCas9 and sgRNA editing machinery and the NPTII selectable marker were expressed on a TDNA expressing binary vector (pPHP82151) (Figure S3). The N20 region labelled as the crRNA DNA-hybridization region represents RNA sequence used to target genomic sequence in the located upstream of the DNA triplet ‘NGG’ sequence in the Sb-CENH3 gene that is recognized by the spCas9 enzyme.

Materials reported in this article may contain components subject to third-party ownership (e.g. PTXD, moPAT, PMI). Transgenic and genome edited materials may be subject to governmental regulations. Availability of materials described in this article to academic investigators for noncommercial research purposes

### Table 5 Regeneration efficiency for CRISPR/Cas9-mediated Sb-CENH3 gene editing using ternary transformation system

| gRNA       | # of embryos | # of regenerated T0 plants | Regeneration % |
|------------|--------------|-----------------------------|----------------|
| Sb-CENH3-CR1 | 298          | 39                          | 13%            |
| Sb-CENH3-CR2 | 285          | 23                          | 8%             |
| Sb-CENH3-CR3 | 257          | 42                          | 16%            |

### Table 6 CRISPR/Cas9-mediated gene editing efficiency for Sb-CENH3

| gRNA        | # T0 plants analyzed | % T0 plants with edit (biallelic) | % T0 plants with a knockout (biallelic) | % T0 plants with a knockout (biallelic) | Knockout frequency per embryo transformed |
|-------------|----------------------|----------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Sb-CENH3-CR1 | 31                   | 12 (5)                           | 39% (16%)                              | 9 (0)                                  | 29% (0%)                              | 3%                                     |
| Sb-CENH3-CR2 | 15                   | 6 (5)                            | 40% (33%)                              | 3 (0)                                  | 20% (0%)                              | 1%                                     |
| Sb-CENH3-CR3 | 35                   | 13 (0)                           | 37% (0%)                               | 13 (0)                                 | 37% (0%)                              | 5%                                     |

*Regenerated T0 plants (Table 5) that survived transplanting to soil were analysed for edits.

1Edits are defined as targeted mutagenesis and include both in-frame and frameshift mutations.

2A knockout is defined as targeted mutagenesis that results in a frameshift mutation.

3See Table 5 for the number of embryos transformed per gRNA experiment.
under an applicable material transfer agreement will be subject to proof of permission from any third-party owners of all or parts of the material and to governmental regulation considerations. Obtaining the applicable permission from such third-party owners will be the responsibility of the requestor. Transgenic materials reported in this article may only be made available if in full accordance with all applicable governmental regulations.

**Sorghum transformation and transgenic event quality analysis**

Sorghum genotype Tx430 and African varieties (Maica, Malisor 84-7 and Tegemeo) grown in a greenhouse were used in this study. Malisor 84-7 (PI 656048) and Maica (PI 565121) were requested from USDA GRIN. Tegemeo is a public line obtained from Mycrogen Seeds. Immature embryo explants isolated from those sorghum plants were transformed with Agrobacterium auxotrophic strain LBA4404 Thy- carrying above vectors to generate transgenic sorghum plants. The same Agrobacterium transformation methods were performed as previously described by Wu et al. (2014), with the exception that the restring step was skipped for PTX/Phi selection system and KH2PO4 in the callus development medium was replaced with 300 mg/L Phi.

The integrated copy number of the T-DNA and the vector backbone in these transgenic plants were determined by a series of qPCR analyses (Wu et al., 2014; Zhi et al., 2015). The transgenic plants carrying a single copy of the intact T-DNA integrations without vector backbone were defined as ‘quality events’.

**Amplicon deep sequence**

CRISPR/Cas9 edits were characterized by amplicon sequencing from DNA extracted from a single fresh leaf punch from each plant as per manufacturer’s recommendations via the beadex™ tissue extraction system (LGC Limited, UK). DNA was normalized to 10 ng/μL, and twenty cycle target region PCR was performed on 50 ng of genomic DNA with Phusion™ Flash 2× Master Mix (Thermo Scientific, Waltham, MA) as per the manufacturer’s recommendations. Five microlitres of primary PCR product were transferred to the twenty cycles secondary amplification containing primers to attach individual sample indexes and sequencing components, again with Phusion™ Flash 2× Master Mix. Sequencing was performed on Illumina MiSeq®, paired-end 150 cycles per read according to Illumina standard operating procedure. Sequence reads were aligned to the wild-type reference sequence via Bowtie2. The primers used to amplify Sb-CENH3 genomic loci are sites in Table S1. Edited sequences are reported in Table S2.

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**Author contributions**

P.C., A.A., E.W., J.S., M.S., A.S., M.M., D.L., S.L., Z.Z., M.A. and T.J. designed research; W.Z. conducted sorghum transformation; P.C., A.A., E.W., J.S., M.S. and G.Z. analysed data; P.C., A.A., E.W., J.S. and T.J. wrote the manuscript.

**Conflict of interest**

The authors have no conflict of interest to declare.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** Schematic representation of the molecular components of constructs used in this study (see Experimental Procedures for details).

**Figure S2** Tx430 Sb-CENH3 genomic structure. The 5' UTR is highlighted in green. The exons are highlighted in yellow and the 3' UTR is highlighted in grey. Introns are represented by lowercase letters.

**Figure S3** Schematic representation of the molecular components used for gene editing in this study.

**Table S1** Primers for CRISPR/Cas target sites.

**Table S2** Sequences changes from Cas9 edited plants.