Establishing genome editing platforms for efficient traits and genetic-gain optimizations in sorghum

Introduction:

The utilization of de novo site-directed variations is an attractive alternative for expanding the genetic base of a crop species, particularly for high-value traits. The development of efficient and reliable ways to incorporate useful genes and to precisely modify plant genomes has been a long-standing goal for crop breeders and biologists.

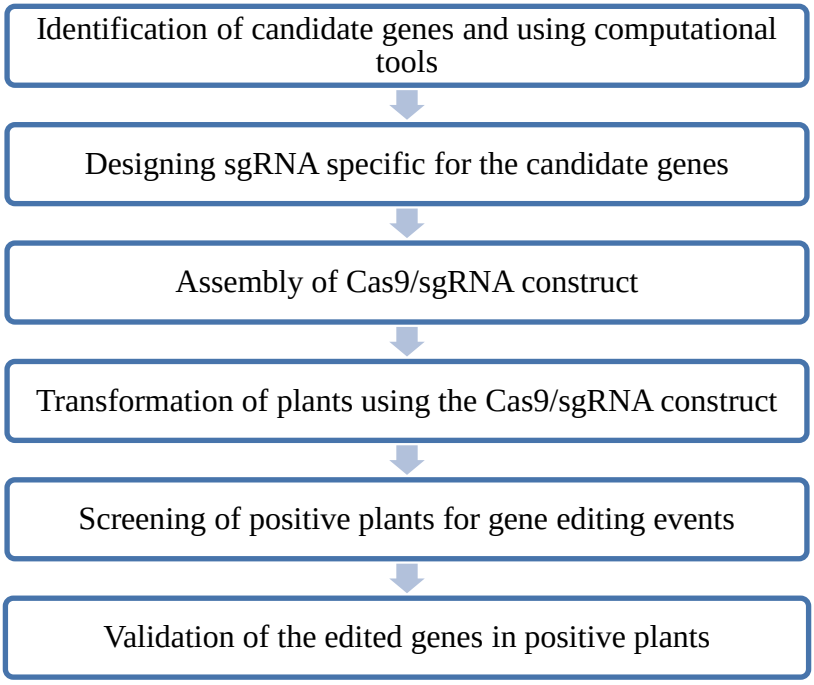
Towards this genome editing innovations could be the "game changers" to speed introduction of genotypes with valuable new traits that are not achievable in reasonable timeframes using conventional/ modern breeding techniques. In this context, precise gene editing will be developed in sorghum applications for inclusion in the breeder's toolbox for crop improvement.

Project Objectives:

1. To carry out optimization of construct components including *Cas9*, Pol III promoters, selectable and phenotypic markers, sgRNA parameters affecting the specificity and efficiency of CRISPR/Cas9 system in Sorghum.
2. To construct binary vectors expressing multiple gRNA's for one step mutation of multiple genes.
3. To verify mutagenesis efficiency, pattern of transmission and off target analysis of the sorghum mutants.

Activities undertaken
Development of stable transgenics using multiple gRNA expression cassettes for 1-2 set of genes

Project Roadmap:



Progress:

During the first year, sets of simple vectors for high-efficiency and precise gene editing in sorghum were developed. Using these gRNA module vectors, multiple gRNA expression cassettes were assembled using either Golden Gate cloning method or the Gibson Assembly method. Employing suitable Pol III promoters and other RNA machinery components, additional gRNA modules were constructed for the assembly of multiple gRNAs. This gRNA module vector set will be extensible and should be easily updatable. Subsequently, in year 2 & 3 these tool kits/ platforms will be tested using several genes followed by their genotypic analysis to reveal the efficiency of the systems in sorghum.

Optimization of guide RNA designing for CRISPR/CAS genome editing:

We have optimized the parameters to be considered while designing guide RNAs which would make the mechanism more efficient and robust. Initially the guide RNA's are selected based on their score values using different softwares like CRISPR-P, CRISPR-W and CAS-DESIGNER. Once shortlisted, 3-4 best suitable ones are selected based on its secondary structure and off-targets scoring. The off-targets for every guide RNA selected were calculated using CAS-OFFFINDER software scoring system. The presence of intact secondary loop structures in the guide RNA scaffold was predicted using Vienna software. The guide RNA selected for the genome editing purpose must have intact RAR (GAAA) Stem loop 2(GAAA) and 3(GAGU) and minimum number of off-targets. Along with these parameters we also check for the presence of self- base pairing within the guide RNA and it should not exceed a score of 6. The GC-Content in the guide RNA has been optimized within the range of 50%- 70%. Along with the self-base pairing we have also considered the presence of repetitive bases in the guide RNA. It is acceptable to have similar bases only up to a score of 3. The above-mentioned parameters have been designed and optimized by combining data from different guide RNA design softwares and previous literature available.

In the case of off-target analysis, after selecting the guide RNA based on its ranking, we have verified and identified the mutation regions for off targets within the genome sequences and have chosen only those guide RNA's which had minimal number of off targets in functionally important genes. Very briefly, some of the parameters that have been considered for designing gRNA's for efficient mutagenesis are included in the table below

Parameter	**Tool used**	**Required score value**
Secondary structure with distinct RAR, STEM Loop1 and 3	Vienna software	RAR- should have GAAA STEM Loop 1-should have GAAA STEM Loop 3-should have GAGU
Off targets	CAS OFFFINDER	Should have minimum scoring value

Self-base pairing	Oligoanalyser	Should be <6
GC- Content	Guide RNA design software	50-70%

Vector construction for expression of CAS-9 and guide RNA by multiplexing:

The vector construction for multiplexing has been done using Golden Gate cloning method. For the precise excision of guide RNA's inside the plant cell, we have inserted the guide RNA's along with alternating tRNA's. These tRNA's would be cleaved during RNA processing which occurs naturally inside the plant cell and thus the guide RNA reaches the target site.

Multiplexing helps in targeting multiple genes at a time and increases the efficiency of gene editing for the development of improved traits in plants. We have designed a vector construct p201N CenH3 for our studies in sorghum. It includes the Cas9 coding gene along with the guide RNA and tRNA scaffold. The following vector map explains the arrangement in detail.

In the case of multiplexing, in order to target multiple genes at a time, we might use many guide RNA's. So their assembly into the vector is done on the principle of golden gate cloning. In golden gate cloning we use type II restriction enzymes, which cleave the DNA outside the restriction sites and create 4 base pair overhangs. The overhang sequence created is not based on restriction enzyme and therefore no scar sequence is introduced. Here we insert *Bsa 1* restriction enzyme sites to the primers designed for the entire guide RNA's. These sites are inserted in such a way that they are overlapping and thus later on during fusion reaction these guide RNA's combine in the desired order and the *Bsa 1* site is completely eliminated.

Sorghum Transformation and analysis: Sorghum (Sorghum bicolor (L.)Moench) variety BTX 623 and M35 were used to develop genome editing line, using Agrobacterium mediated transformation. So far 69 plants using CCD7 construct and 57 plants with ST4A construct were developed and transferred to greenhouse.

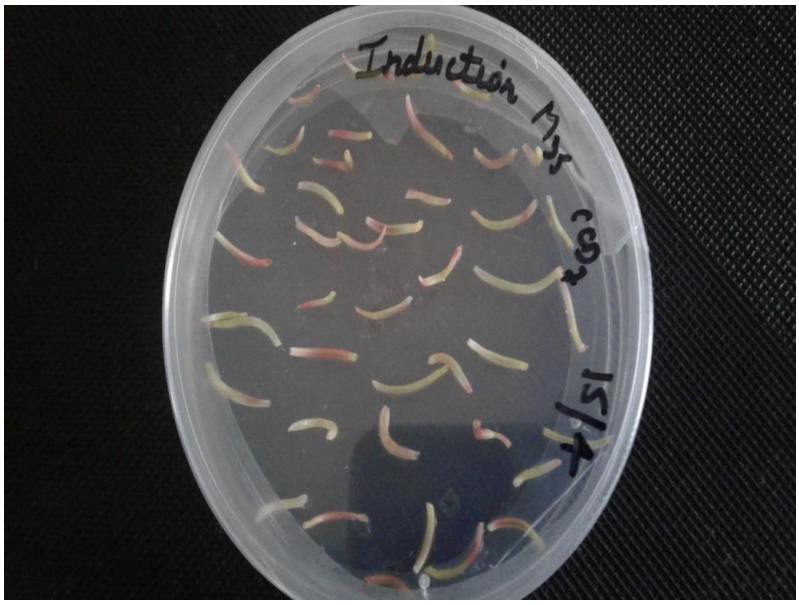


Figure 1: Shoot tips collected from seedlings subjected to Agrobacterium infection are Plated onto shoot induction medium

.Shoot tips are transferred onto shoot induction medium containing cefotaxime after 72 h of infection and are maintained on the medium for 2-3 weeks.

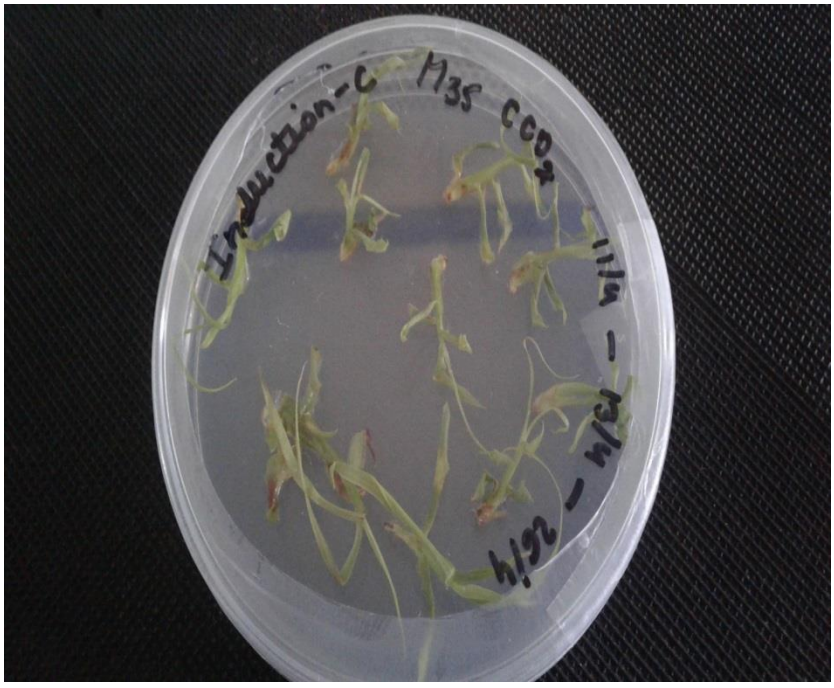


Figure 2: Shoot tips on induction medium after 2-3 weeks.

Shoot tips are then transferred onto multiple shoot induction medium containing 2,4-D for shoot enlargement and are sub cultured after every 10 days.

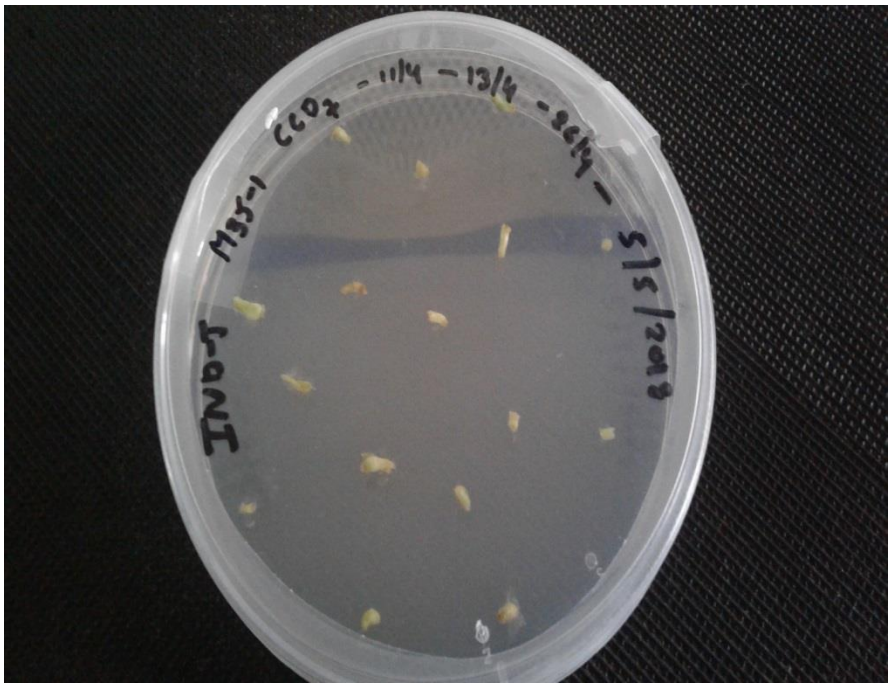
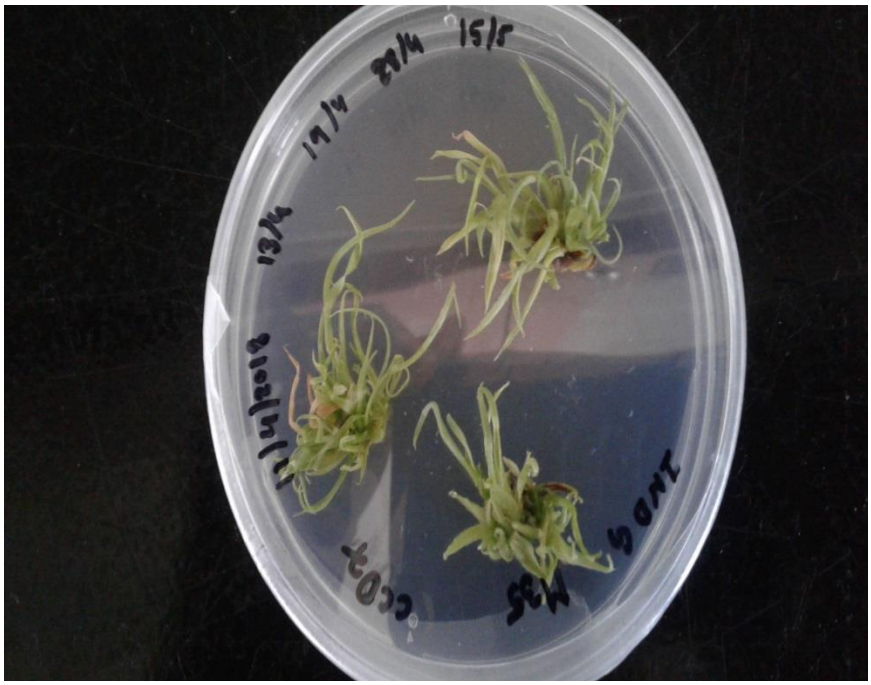


Figure 3: a) Shoot tips sub cultured onto multiple shoot induction medium and b) shoots elongated on the MIM medium.

The elongated shoot tips are transferred onto medium for formation of multiple shoots and the shoots are subjected to antibiotic hygromycin at different concentration levels 2mg/l , 5mg/l and 10 mg/l.



F**igure 4: Multiple shoots formed are subjected to hygromycin selection at 5mg/l and 10 mg/l.**

Multiple shoots formed were transferred onto elongation medium and the individual shoots are separated and transferred into elongation tubes for two weeks. After two weeks the elongated shoots were transferred onto rooting medium and maintained for 3-4 weeks.

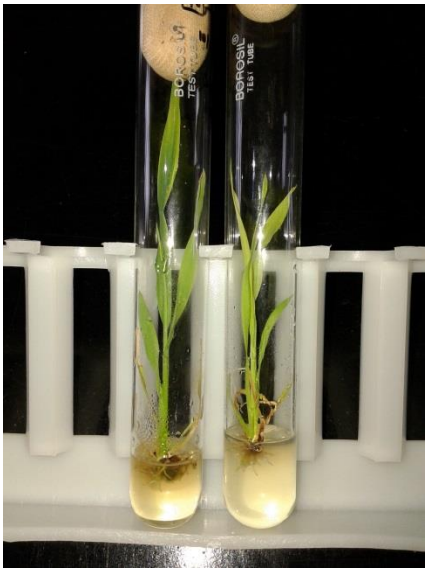


Figure 5: Elongated shoots transferred to rooting medium.

Rooted plants were shifted to ziffy cups and maintained on the culture room for 2 weeks and transferred to greenhouse.

Figure 6: Rooted plants transferred to ziffy cups.

After roots elongation the plants are transferred to greenhouse and observed periodically for hardening of putative transgenic plants.

Figure 7: Hardening and Acclimatization of putative transgenic Sorghum plants in Greenhouse.

Recombinant plasmid p201Ncas9:CCD7 isolated from Agrobacterium strain C58 that was used for co-cultivation of Sorghum in the present study showed the presence of 840bp fragment of ccd7 gene in PCR analysis.