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CRISPR/Cas9 mediated *TaRPK1* root architecture gene mutagenesis confers enhanced wheat yield

Abstract

CRISPR/Cas9 system has been emerged as an efficient tool for sustainable crop improvement. Roots are the "principal hidden organ" that has a crucial function in vascular plants. *Receptor-like protein kinase 1* (*RPK1*) has been reported to regulate root architecture system (RAS), abiotic stress, and yield in *Arabidopsis* and rice. We employed a CRISPR/Cas9-based system, namely LR-1 and LR-2 constructs having double guided RNAs transformed via agrobacterium for targeted mutagenesis of *TaRPK1* genes in order to alter the root architecture and hence yield in *Triticum aestivum*. Sequencing confirmed seven CRISPR/Cas9-based mutated T0 lines of LR-1 constructs and six T0 lines of LR-2 constructs, with an overall mutation efficiency of 41.93%. The T0 plants displayed higher monoallelic mutation compared to the diallelic mutation. 37.5% monoallelic mutation at target site 1 within the D genome by gRNA1 was observed by the LR-1 construct. The LR-2 constructs showed a higher monoallelic mutation frequency of 26.67% at target sites 1 and 2 within A, B, and D genomes. The deletions were mainly short, however longer deletions such as 12d, 17d, 19d, and 20d were detected by gRNA2 of LR-1 construct. Transgenic lines revealed significant alteration in morphology and RSA with a significant increase in number of effective tillers, grain weight, root length, root depth, root volume, and root surface area while reduced root diameter, root angle, and spike length, compared to the wild plants. This significant elevation for tillers and total grain weight proposed that edited lines uplifted grain yield which was caused by to decrease in spike length. The study validates that CRISPR/Cas9 mediated targeted editing of *TaRPK1* is a practical approach for modifying RAS and hence yield enhancement in wheat.

Keywords: *RPK1* editing; Wheat; Agrobacterium-mediated transformation; Root architecture; grain weight.

1. Introduction

Roots are the "principal hidden organ" that has a crucial function in vascular plants. Roots are integral compnenet, mediating the water and nutrient uptake required for the growth of the plant (Ober et al., 2021). Roots distribution and growth in soil affect the rate of transpiration that modulates water fluxes among the atmosphere and soil (Javaux et al., 2013). Root growth is crucial for the acquisition of water and nutrients from the soil. The root's uptake

of nutrients and water modulates the yields of crops which relies on spatial and temporal distribution of roots in soil (Chen et al., 2020). Root system architecture (RSA) encompasses main traits like length, diameter, length density, depth, volume, and surface area that modulate the organization of root structure and are associated with the uptake of nutrients and water (Alahmad et al., 2019, Djanaguiraman et al., 2019, Danakumara et al., 2021). These traits shaping the distribution of roots in soil have been accompanied by the angle of the root (Alahmad et al., 2019). Among several organs of plants studied, drought mainly affects roots because of its direct association with soil. Drought resistance is correlated directly with the diameter of the root, as bulky roots with huge xylem vessels are effective for the nutrients and water acquisition from deep layers of soil during the conditions of rainfall (Maccaferri et al., 2016). The number of root tips and finer roots are the chief elements of the root system which enhances nutrients and water uptake via root volume and surface area (Bishopp and Lynch 2015). In addition, deeper rooting is a vital trait of root that permits water acquisition from deeper profiles of soil which enhances crop productivity (Djanaguiraman et al., 2019, Danakumara et al., 2021). Narrow root seminal angle might enhance the acquisition of residual moisture from deep layers of soil during terminal drought situations and might cause yield improvement (Hamada et al., 2011, Alahmad et al., 2019). Whereas, a wide root seminal angle is accompanied by a root system that is shallow and might explore the top layers of soil for nutrients and water uptake during seasonal rainfall (El Hassouni et al., 2018, Alahmad et al., 2019). The total percentage of plants influenced by drought stress have been doubled in the last 40 years, 12 million hectares of land lost each year to drought and desertification. Worldwide, approximately 79% displayed variability in grain yield in the harvesting region of wheat due to drought stress (Djanaguiraman et al., 2019). Understanding the contribution and variations in features of roots might aid in enhanced productivity and drought-resistant genotypes development (Fradgley et al., 2020).

Monocotyledons and dicotyledons possess different root system architecture. In monocotyledons such as wheat, the root system is comprised of seminal roots (SR) and crown roots (CR), the crown roots arising from shoots basal node (Nehe et al., 2021). The seminal roots infiltrate firstly into the soil and stay functional throughout the plant lifecycle (Maccaferri et al., 2016, Rufo et al., 2020). These function altogether for the nutrients and water intake from the soil. Bread wheat (*Triticum aestivum*) is one of the major grain crops with a production and area of 771.71 million tons and 218.54 million hectares, respectively (FAOSTAT. 2017). Bread wheat is hexaploid (2n = 6x = 42, AABBDD) and domesticated about 8000–25000 years

ago in the Middle East region. Wheat being originated from diploid progenitor species: *Triticum urartu* (A-genome), *Aegilops speltoides-related* grass (B-genome), and *Aegilops tauschii* (D-genome). Owing to hexaploidy and an abundance of both repetitive and transposable elements, bread wheat has become one of the largest crop plant genomes (16 Gb), making it challenging to work with from a genetics, genomics, and breeding perspective (Schilling et al., 2020). Globally, wheat is an essential crop and more than a quarter of the population relies on it for feed (Brandt et al., 2020, Gabay et al., 2023). Since wheat is the main staple crop, production needs to be enhanced by 2050 by approximately 38% in order to feed the rising population (Djanaguiraman et al., 2019). Among the climatic fluctuations predicted in the future, drought is one of the main challenges that restrict growth and hence crop yield across the world. Drought stress might lead to a considerable reduction in the wheat yield and its impact fluctuates with the duration, intensity, and timing of the stress compared to the stages of the growth of the crop (Danakumara et al., 2021). Globally, approximately 79% displayed variability in grain yield in the harvesting region of wheat due to the fluctuations in temperature and precipitation. Therefore, improvement in root architecture traits, drought tolerance, and high-yield maintenance is a major challenge in the improvement of wheat (Djanaguiraman et al., 2019).

Genome editing is the method of creating specific alterations to a known sequence of DNA. These alterations may comprise deletions, insertions, or variations in the sequence of the gene which might result in the desired change in the protein produced (Brandt et al., 2020). Numerous outcomes of editing are attained by sequence-specific engineered nucleases that create targeted double-stranded DNA breaks (DSBs). Sequence-specific nucleases (SSNs) like mega-nucleases, zinc-finger nucleases (ZFN), and transcription activator-like effector nuclease (TALENs) methods of editing genomes were applied broadly in plants. However, recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with Cas nucleases have overtaken these SSNs and mediate the targeting of DNA through guide RNAs (gRNA), which is easy to engineer (Malzahn et al., 2019). CRISPR/Cas9 is considered the most prevalent technology for editing genomes globally, due to its simplicity, versatility, specificity, and flexibility (Li et al., 2019). It has been used in numerous plant species including *Arabidopsis* and *Nicotiana* model-plants, and crop species like maize, rice, and wheat (Feng et al., 2013, Nekrasov et al., 2013, Char et al., 2017, Char et al., 2019, Zafar et al., 2020, Uzair et al., 2021, Ibrahim et al., 2022).

RPK1 is a *Receptor-like protein kinase 1*, belongs to the family of LRR-RLKs, comprises six LRR extracellular motifs, a transmembrane domain (TM), and a single cytoplasmic conserved kinase domain in rice (Rahim et al., 2022). Studies have shown that *OsRPK1* is involved in the architecture of roots by negative modulation of polar auxin transport (*PAT*) and auxin accretion in roots (Zou et al., 2014). Suppression of *RPK1* caused the growth and increased plant height and tiller numbers. Enhanced expression caused a reduction in roots apical meristem and immature adventitious and lateral roots (Zou et al., 2014). *AtRPK1* inhibition, in *Arabidopsis thaliana* exhibited enhanced salt tolerance whereas up-regulated plants displayed lower degrees of salt tolerance in comparison to the normal plants (Shi et al., 2014). The *AtRPK1* level enhanced significantly under less water, ABA, high salt, and less temperature (Hong et al., 1997). Lee et al. studied that *RPK1* inhibition deferred significantly ABA-induced senescence (Lee et al., 2011). *AtRPK1* also plays an essential role during the embryogenesis of the cotyledon primordia (Nodine et al., 2007, Nodine and Tax 2008). *AtRPK1* positively modulates the expression of *the CaM1* gene which further modulates ROS production, senescence of the leaf, and ABA response (Dai et al., 2018).

The CRISPR/Cas9 system has successfully demonstrated precise gene editing in many plant systems (Kim et al., 2023). The first reported use of CRISPR-Cas9 to produce a stably genome-edited wheat plant was the targeted knockout of the Mildew Locus O (Mlo), conferring resistance to the powdery mildew pathogen *Blumeria graminis f.*sp. *tritici* (Bgt) (Wang et al., 2014). Since this first report, wheat genes of agronomical and fundamental scientific interest have been targeted using CRISPR-Cas9 technology, such as *TaGW2* to increase grain weight (Zhang et al., 2018), *TaQsd1* to reduce preharvest sprouting (Abe et al., 2019), *CENH3* for haploid plant induction (Lv et al., 2020), *Sal1* for drought resistance (Mohr et al., 2022). To date, *RPK1* disruption via CRISPR/Cas9 system was not employed for wheat to alter its root architecture and related traits. To address the issue, we synthesized two constructs namely LR-1 and LR-2, with two guide RNAs (gRNAs) each, and developed an *In-planta Agrobacterium-mediated* CRISPR/Cas9 system, hence generating successfully targeted mutations in target sites of *TaRPK1* genes in T0 plants with altered root architecture traits and other agronomic traits. We believe that our study has potential application to produce valuable traits in bread wheat and will aid in the improvement of wheat to guarantee global food security.

2. Material and Methods

2.1 Wheat germplasm and growing conditions

The seeds of both drought-tolerant and high-yielding hexaploid bread wheat cultivar ‘‘Pakistan 2013” were obtained from the National Institute of Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Islamabad, Pakistan. The control and transgenic (T0) plants were grown in glasshouse at NIGAB. The environment of the greenhouse was kept under greenwhouse conditions for day light period of 16 h with 25 ± 3°C temperature and night period of 8 h with 15 ± 3°C temperature. The plant growing pots were filled with soil, peat moss, and sand (1:2:1) soil mixture. Experiments were carried out on complete randomized design (CRD). Three readings were observed for agronomic parameters including number of effective tillers, plant height, spike length, grain related traits, and root architecture traits (root length, depth, diameter, surface area, volume, and root orientation). The main steps involved in CRISPR-based targeted mutagenesis are summarized in Supplementary Figure S1.

2.2 Target selection and guide RNAs designing

The sequences of the *RPK1* genes in the A, B, and D genomes of wheat were retrieved from “Ensemble Plant” through the BLAST of *RPK1* sequences of *Arabidopsis thaliana* and *Oryza sativa* (Shi et al., 2014, Zou et al., 2014). The sequence of *RPK1* with a significant percentage ID and low E-value was selected. For unique gRNA synthesis, the gene target sites were selected from the exon area of *TaRPK1* gene. The gRNAs were designed manually, and the possible off-target sites were identified using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The CRISPR/Cas9 system provided by Dr. Bing Yang (Genetics, Development and Cell Biology department, Ames, Iowa State University, USA) was followed (Char et al., 2019). The gRNA expression cassettes were synthesized according to pENTR4-gRNA expression vector 21–25 bp complementary oligonucleotides were synthesized commercially, with *BtgZ*I restriction site, TGTT and AAAC at the 5´ ends of sense and antisense strand, respectively. Similarly, from the 2nd exon, another four oligos were designed with *Bsa*I restriction site, GTGT and AAAC at the 5´ ends of sense and antisense strand, respectively (Table 1).

Table 1. Sequences of guide RNA used for expression cassettes construction.

Gene name and gRNA	Guide RNA sequence (5´ to 3´)
TaRPK-gRNA1-F	TGTTCTTGTTGTTCTTGGTGTTGC
TaRPK-gRNA1-R	AAACGCAACACCAAGAACAACAAG
TaRPK-gRNA2-F	GTGTCCTATGCTTTCCTTGCTGAT
TaRPK-gRNA2-R	AAACATCAGCAAGGAAAGCATAGG
TaRPK-gRNA3-F	GTGTATGGATGTGTTTGATGTCAG

TaRPK-gRNA3-R	AAACCTGACATCAAACACATCCAT

2.3 CRISPR/Cas9 based Vector construction

CRISPR/Cas9 system was used for *TaRPK1* gene disruption, in order to unveil its role in wheat. Two cassettes containing two single guide RNAs i.e., gRNA1 and gRNA2, and gRNA1 and gRNA3 along with PAM were synthesized from the first and second exon coding region of *TaRPK1*. The pOs-Cas9 and pENTR4 gateway vector system was used for the purpose of editing. The constructs were synthesized following Bing Yang's method (Char et al., 2019), and the Pakistan-13 wheat cultivar was used for transformation. Both the guide RNA cassettes, with a combination of gRNA1 and gRNA2, and gRNA1 and gRNA3 were ligated into the pENTR4 vector separately, using *BtgZ*I and *Bsa*I sites. Finally, through an LR-recombination reaction, each cassette was inserted into the final destination pOsCas9 vector, thus giving rise to LR-1 and LR2 constructs. The CRISPR/Cas9 constructed vectors comprising both the gRNAs were then delivered into the *Agrobacterium tumefaciens* LBA4404 strain.

2.4 Transformation and screening of edited wheat plants

The seeds of the Pakistan-2013 wheat cultivar were used following the method of *in-planta* transformation (Supartana et al., 2005, Abdul et al., 2011). For in-planta transformation, the seeds were first soaked in 70% v/v solution of analytical grade ethanol for 1 minute with constant shaking followed by washing thrice with autoclaved ddH_2O. Seeds were then washed with 50% Clorox for 15 minutes and were allowed to completely dry. Sowing of seeds was done on Murashige and Skoog(MS) solid medium (Murashige and Skoog 1962).

The overnight grew liquid cultures of *A. tumefaciens* strain LBA4404 in LB liquid media were centrifuged at 85000rpm/10 min, and the pellets were separately resuspended in liquid MS media to attain an OD600 of 0.6. These harvested cells resuspended with MS medium were supplemented with 200μM acetosyringone and mixed gently. For the three-day-old seedlings, the apical meristem part was pierced up to 1mm depth, and agrobacterium inoculum was injected into it. After three days of co-cultivation, seedlings were washed with ddH_2O thrice followed by washing with 500 mg/L cefotaxime antibiotic for 15 min to remove excess bacteria that have grown on co-cultivated seedlings. The seedlings were then shifted to the selection and regeneration medium containing MS salts with vitamins; Sucrose 30g/L; cefotaxime 250µg/mL; BAP 1.5mg/L, kanamycin, and hygromycin. The seedlings were then kept at 28°C with the standard light and dark cycle for four weeks. After one month, the

seedlings were transplanted for acclimatization into the growth room, covered with a transparent lid for humidity maintenance. The transformed seedlings were then moved into pots and placed in a greenhouse under a controlled environment (22/16ºC with day/night 16/8h photoperiod and 70% humidity) till maturity.

For the detection of gRNAs efficiency in wheat plants, a T7 endonuclease I assay was performed (Kim et al., 2018). To confirm the DNA double-strand break, PCR amplicons were gel purified, denatured, reannealed, and treated with T7EI. The PCR amplicons were sequenced to determine the mutation frequency.

2.5 Root architecture traits and morphological parameters determination

The root architecture traits including root length, depth, diameter, volume, surface area, and root orientation were observed in T0 transgenic lines and wild-type plants. For this purpose, pictures from four-week-old wheat seedlings were captured and analyzed with the help of RhizoVision software (Seethepalli et al., 2021). Morphological data measured included the number of effective tillers, plant height, spike length, and 1000-grain weight at the maturity stage. A minimum of three replicates from T0 transgenic lines and control plants were recorded for each trait.

2.6 Statistical analysis

All the recorded data was statistically analyzed by using Microsoft Excel 2019. Student *t*-test was performed to determine the significant differences among the samples (n = 3, *= $P < 0.05$, **=$P < 0.01$).

3. Results

3.1 Selection of target homeolog of *TaRPK1* gene

The *RPK1* sequences in the A, B, and D sub-genomes of wheat retrieved by BLAST from “Ensemble Plant”, with the highest percentage ID and low E-value were located on chromosome 2. TraesCS2A02G176500, TraesCS2B02G202900, and TraesCS2D02G183900 wheat sequence Ensemble IDs showed the highest percentage of similarities 86%, 85.5%, and 94.1%, respectively (Supplementary Table S1). The coding sequences were aligned and sgRNAs were synthesized from the conserved region. The sgRNA1 was designed from the conserved region of A and D genomes, sgRNA2 from A, B, and D genomes, and sgRNA3 from

the conserved region of A and B genomes (Supplementary Table S2). These sgRNAs were synthesized from the first exon of the A and B genomes, and the second exon of the D genome (Figure 1).

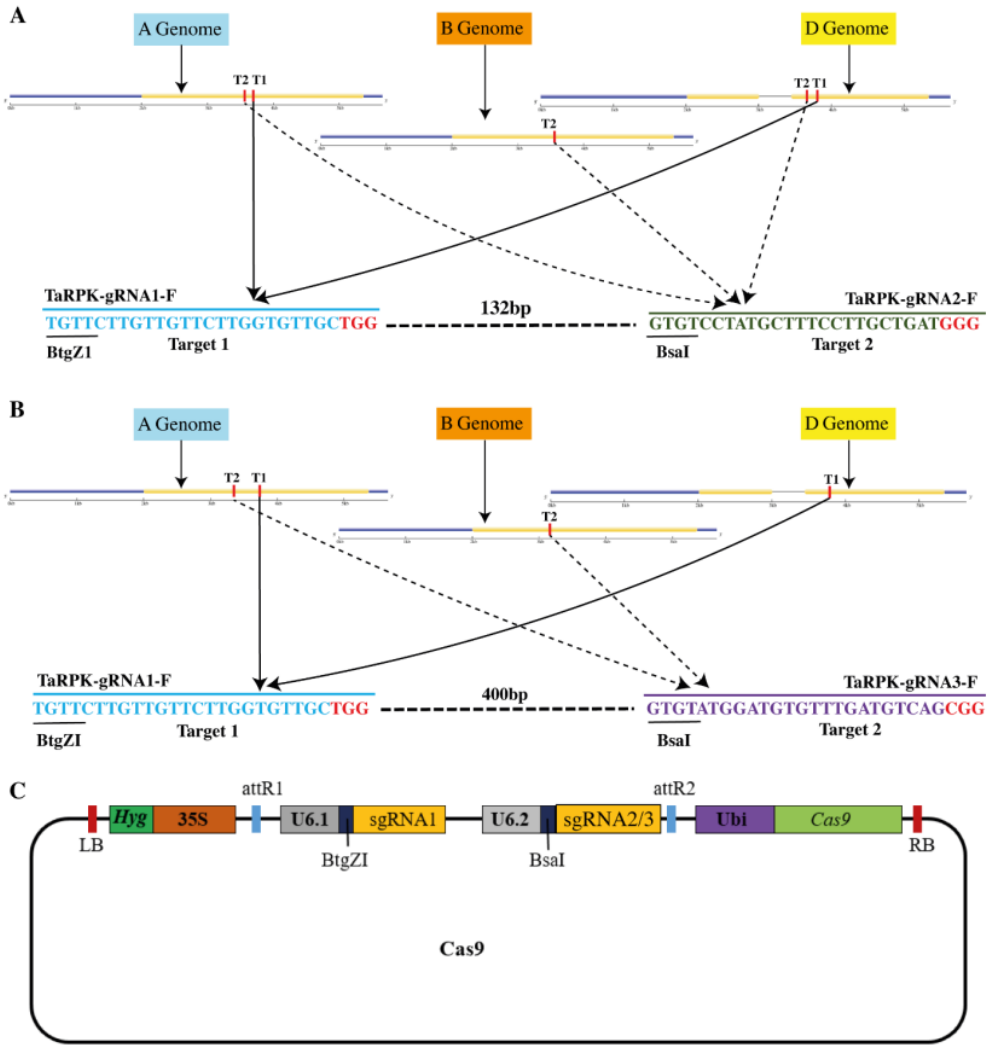


Figure 1: Illustration of sgRNA expression cassette. (A) The representation of *TaRPK1*-gRNA1 (Target 1) and *TaRPK1*-gRNA2 (Target 2) for constructing LR1. **(B)** Indication of *TaRPK1*-gRNA1 (Target 1) and *TaRPK1*-gRNA3 (Target 2) for LR2 construct. PAM sites were shown in red color and restriction endonucleases (*Btg*ZI and *Bsa*I) recognition sequences were underlined. **(C)** Schematic illustration of binary vector transformed into wheat. The constructs *TaRPK1*-gRNA consists of a cassette with Kanamycin resistant gene, flanked by attR1 and attR2 gateway recombination sequence, inserted into the pOs-Cas9 binary vector via LR recombination reaction. The expression cassette comprises gRNA pairs (*TaRPK1*-gRNA1 and *TaRPK1*-gRNA2/3) followed by Cas9 endonuclease regulated by Rice Ubiquitin promoter.

3.2 CRISPR/Cas9 *TaRPK1* Vector and *In-planta* transformation

The binary vector CRISPR/Cas9 containing *TaRPK1*-sgRNAs were constructed accomplished by gateway recombination reaction (Supplementary Figure S2). The first construct with a combination of sgRNA1 and sgRNA2 is termed LR-1, while the LR-2 construct contains the sgRNA1 and sgRNA3 expression cassettes, respectively. In planta apical meristem transformation being an easy, cheap, and efficient method for transformation was adopted for transformation using CRISPR/cas9 targeted mutagenesis (Figure 2). More than 25 independent lines were obtained by using this technique.

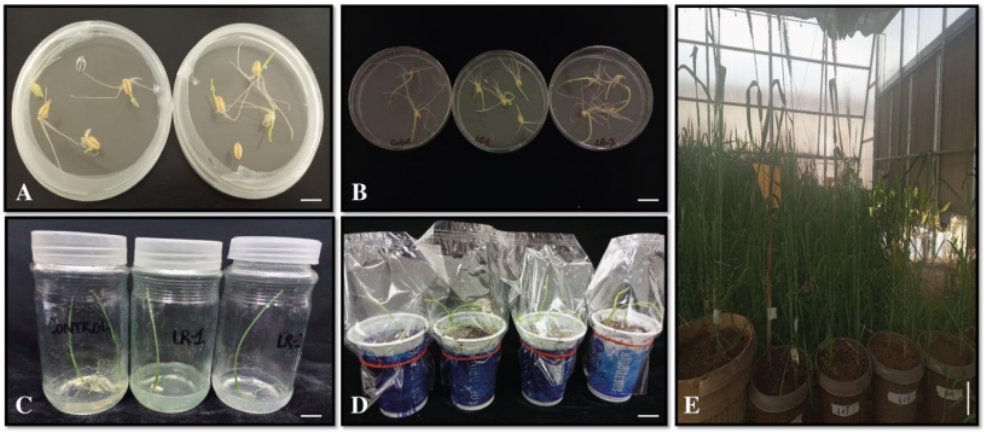


Figure 2: In planta apical meristem transformation CRISPR/Cas9 targeted mutagenesis. **(A)** Wheat seeds germination on MS medium. **(B)** Transfer of ex-plant into co-cultivation media. **(C)** Screening of plants on selection media. Plants with kanamycin and hygromycin resistance survived on selection media. **(D)** Acclimatization of transplanted seedlings in the growth room, by covering the seedlings with a transparent sheet. **(E)** Transformed plants shifted to pots in glass house and grown under controlled conditions.

3.3 Mutation detection in T0 plants

The detection of the edit mutation type was performed after the transformation and regeneration of T0 plants. A total of 31 transgenic plants were obtained from screening of selection antibiotic, 16 of which were of LR-1 constructs and 15 of LR-2 constructs (Table 2). Mutation detection in these transgenic plants was performed by genomic DNA extraction from the leaf tissues of T0 plants (Supplementary Figure S3), followed by designing specific primers flanking target sites of A, B, and D genomes (Supplementary Table S3). The PCR products were digested with T7 endonuclease I (T7EI) assay followed by Sanger sequencing for mutation detection. T7 endonuclease I assay with PCR products did not reveal the expected pattern of digestion for the insertion/ deletion (InDel) mutation. However, Sanger sequencing of the PCR products confirmed seven CRISPR/Cas9-based mutated T0 lines of LR-1 constructs and six CRISPR/Cas9-based mutated T0 lines of LR-2 constructs, with a percentage mutation

frequency of 43.75% (7 out of 16), and 40% (6 of 15) for LR-1 and LR-2 constructs, respectively. The two LR-1 and LR-2 constructs targeting the *RPK1* gene achieved similar mutation frequency. Overall, these results showed a high mutation efficiency of 41.93%.

Table 2. Percentage of mutated plants in the T0 generation.

Vector	Target gene	Construct	sgRNA	No. of plants examined	No. of plants with mutations	% Mutation frequency	sgRNA GC content
CRISPR / Cas9	TaRPK1	LR-1	sgRNA1 sgRNA2	16	07	43.75%	41.67%/ 45.83%
		LR-2	sgRNA1 sgRNA3	15	06	40%	41.67%/ 41.67%
Total				31	13	41.93%	

3.4 Types of mutations caused by CRISPR/Cas9

The sequencing results showed two types of mutations, deletions, and insertions. The insertions of one bp, A, and T were detected at target sites (Figures 3-6). The frequency of insertion was less as compared to that of deletion. Short deletions of one to nine bp were also observed. The deletions were mostly short, however longer deletions such as 12d, 17d, 19d, and 20d (figure 3A) were detected in T0-6, T0-10, T0-14, and T0-16 lines. The longer deletions were observed at target site 2 by sgRNA2 of LR-1 construct, showing high efficiency of sgRNA2 compared to sgRNA1 and sgRNA3 (Figure 5A). The total deletion ratio was higher compared to total insertions and Cas9 cleavage sites usually 3 bp upstream of PAM when all the mutation events were summed. These findings indicate that our CRISPR/Cas9 system did work in the T0-edited plants.

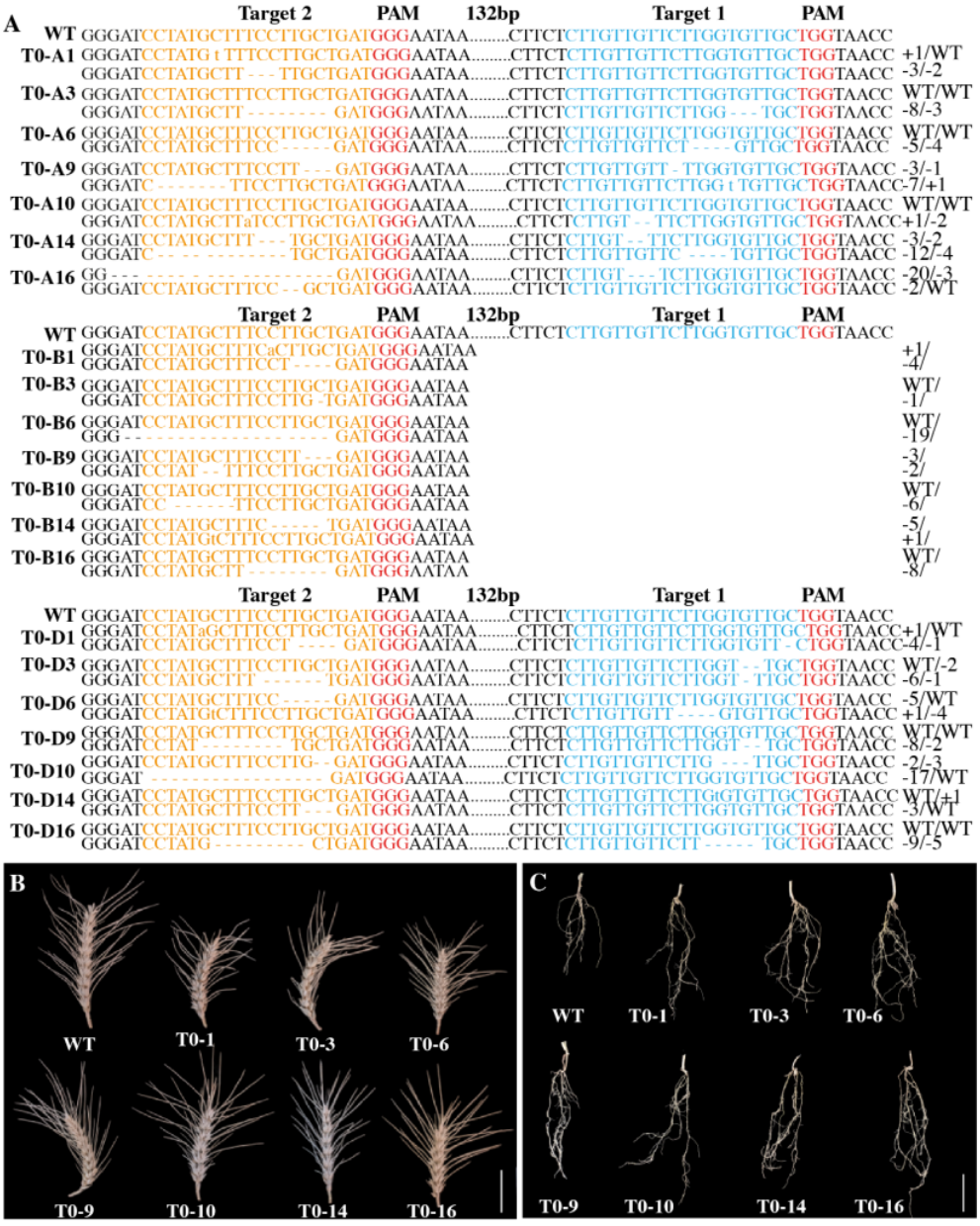


Figure 3: CRISPR/Cas9 induced targeted mutagenesis of the *TaRPK1* target gene by LR-1 construct within A, B, and D genomes of wheat T0 plants (**A**). PAM sites were shown in red, nucleotides in blue denote target site 1 (T1), and nucleotides in orange represent target site 2 (T2). The lowercase letter denotes insertion, dashes represent deletions and dots indicate nucleotides not shown. Spike architecture of wild type and edited transformed LR-1 T0 lines (**B**). Root architecture of wild type and edited transformed LR-1 T0 lines (**C**).

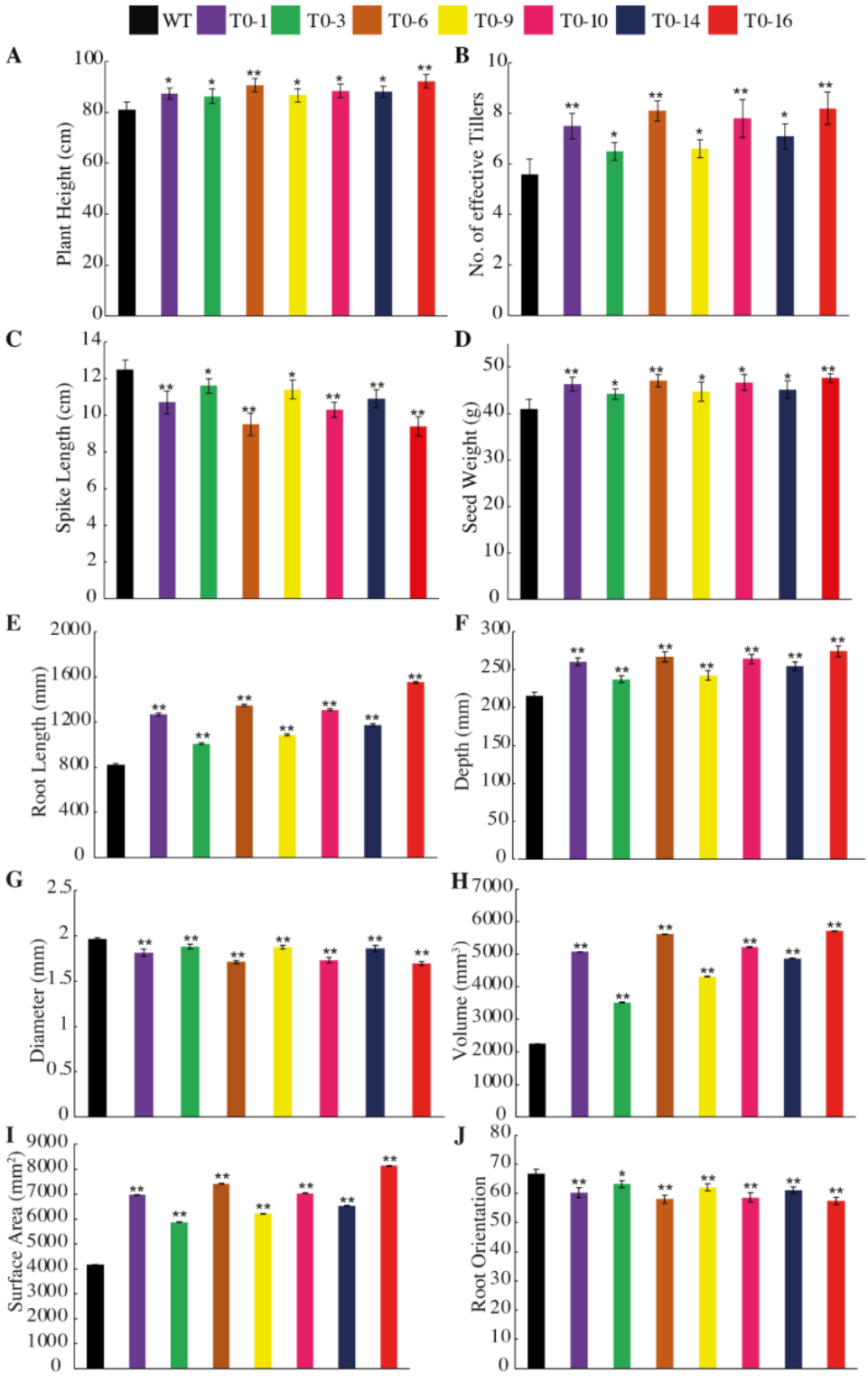
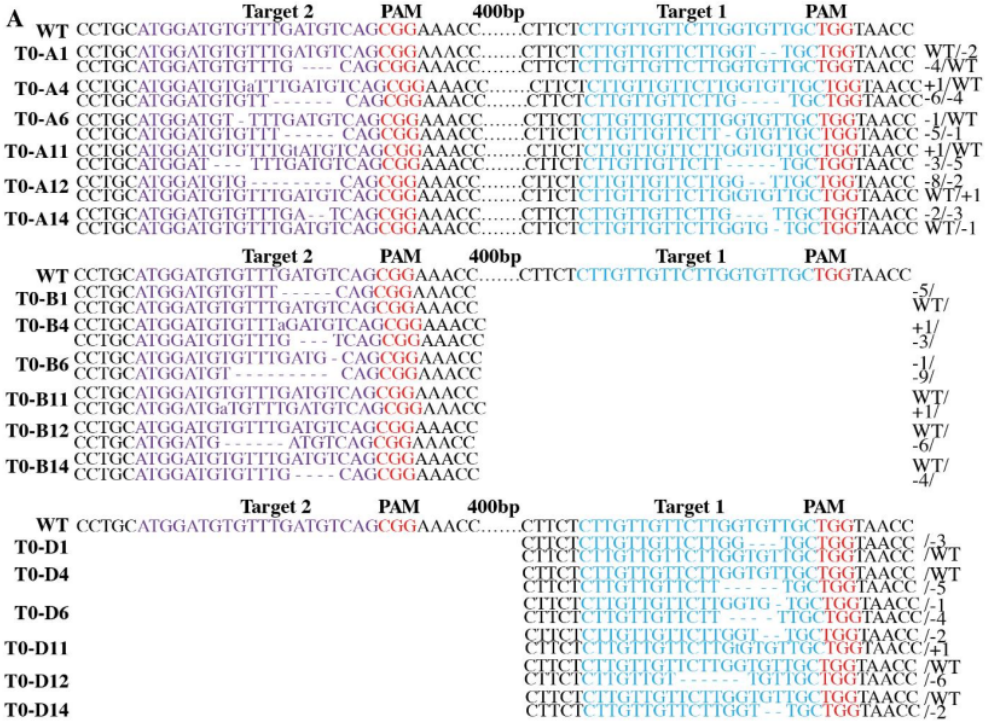

WT
T0-1
T0-3
T0-6
T0-9
T0-10
T0-14
T0-16
A
Plant Height (cm)
B
No. of effective Tillers
C
Spike Length (cm)
D
Seed Weight (g)
E
Root Length (mm)
F
Depth (mm)
G
Diameter (mm)
H
Volume (mm³)
I
Surface Area (mm²)
J
Root Orientation

Figure 4: Phenotypic comparison of LR-1 construct for Plant height (cm, **A**), Number of effective tillers (**B**), Spike length (cm, **C**), Seed weight (g, **D**), Root length (mm, **E**), Root depth (mm, **F**), Root diameter (mm, **G**), Root volume (mm^3, **H**), Root surface area (mm^2, **I**), and Root orientation (**J**) of wild type (WT) and edited T0 lines. The data indicates the mean ± SE of three biological replicates. Student's *t*-test was used (* = $P < 0.05$, and ** = $P < 0.01$).



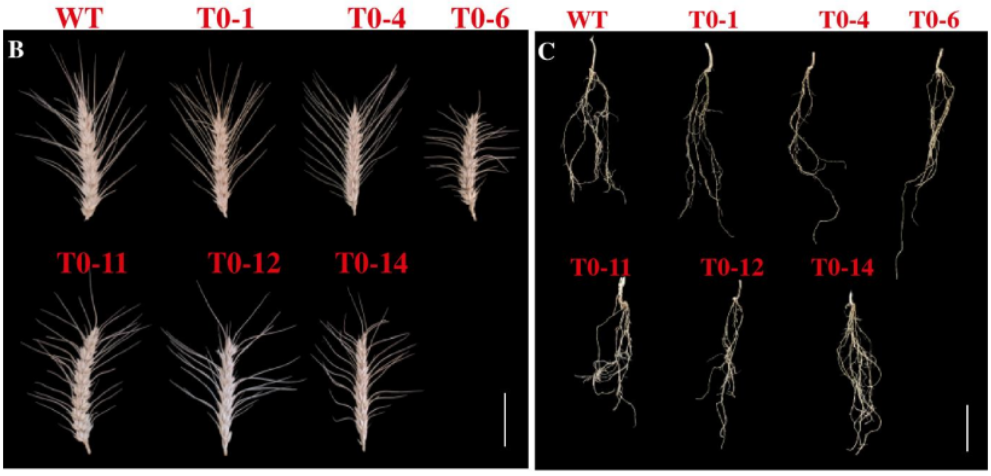


Figure 5: CRISPR/Cas9 induced targeted mutagenesis of the *TaRPK1* target gene by LR-2 construct within A, B, and D genomes of wheat T0 plants (**A**). PAM sites were shown in red, nucleotides in blue denotes target site 1 (T1), and nucleotides in purple represent target site 2 (T2). The lowercase letter denote insertion, dashes represent deletions and dots indicates

nucleotides not shown. Spike architecture of wild type and edited transformed LR-1 T0 lines **(B)**. Root architecture of wild type and edited transformed LR-1 T0 lines **(C)**.

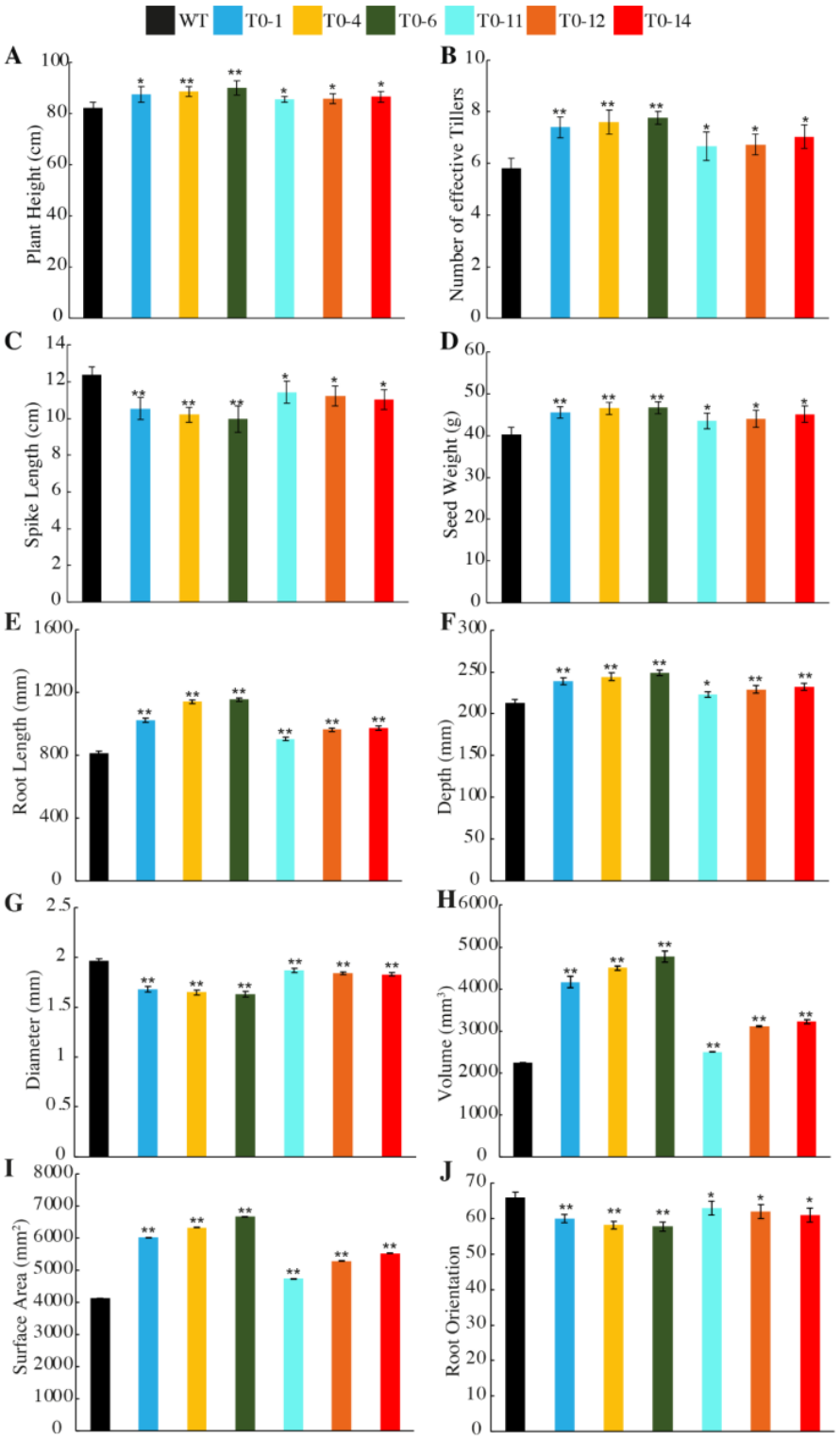

WT
T0-1
T0-4
T0-6
T0-11
T0-12
T0-14
A
Plant Height (cm)
B
Number of effective Tillers
C
Spike Length (cm)
D
Seed Weight (g)
E
Root Length (mm)
F
Depth (mm)
G
Diameter (mm)
H
Volume (mm³)
I
Surface Area (mm²)
J
Root Orientation

Figure 6: Phenotypic comparison of LR-2 construct for Plant height (cm, **A**), Number of effective tillers **(B)**, Spike length (cm, **C**), Seed weight (g, **D**), Root length (mm, **E**), Root depth (mm, **F**), Root diameter (mm, **G**), Root volume (mm^3, **H**), Root surface area (mm^2, **I**), and Root orientation (**J**) of wild type (WT) and edited T0 lines. The data indicates the mean ± SE of three biological replicates. Student's *t*-test was used (* = $P < 0.05$, and ** = $P < 0.01$).

3.5 Monoallelic and Diallelic mutations in T0 generation

Given the complexity of possible results, from zero to three target sites and using 2 different constructs in complex sub-genomes of hexaploid bread wheat, we have adopted the terminology of monoallelic (MA) and Diallelic (DA) mutants to define the allelic status of edited lines. MA mutation means the mutation in one of the homologous copies while DA mutation means the mutation in both alleles of the target gene (Table 3). Results displayed higher monoallelic mutation compared to diallelic mutation. 37.5% monoallelic mutation at target site 1 within the D genome by sgRNA1 was observed by the LR-1 construct (Table 3). The LR-2 constructs showed a higher monoallelic mutation frequency of 26.67% at target sites 1 and 2 within the A, B, and D genomes (Table 3). The highest diallelic mutation of 25% was observed at target site 2, by sgRNA2 of LR-1 construct within A genome of wheat. However, the 20% higher diallelic mutation frequency was detected by sgRNA3 at target site 2 of the LR-2 construct within the A genome (Table 3).

Table 3. Summary of CRISPR/Cas9 mutagenesis frequencies in a generation.

Construct	Genome	sgRNA	No. of plants examined	Target sites on T0 plants	Genotype	
					Monoallelic mutation (MA)	Diallelic mutation (DA)
LR-1	A	sgRNA1	16	T 1	05 (31.25%)	02 (12.5%)
		sgRNA2		T 2	03 (18.75%)	04 (25%)
	B	sgRNA2		T 2	04 (25%)	03 (18.75%)
	D	sgRNA1		T 1	06 (37.5%)	01 (6.25)
		sgRNA2		T 2	04 (25%)	03 (18.75%)
LR-2	A	sgRNA1	15	T 1	04 (26.67%)	02 (13.33%)
		sgRNA3		T 2	03 (20%)	03 (20%)
	B	sgRNA3		T 2	04 (26.67%)	02 (13.33%)
	D	sgRNA1		T 1	04 (26.67%)	02 (13.33%)

3.6 Disruption of *TaRPK1* effects morphological parameters and root system architectural traits

To see if *TaRPK1* gene disruption effects the morphological parameters and root system architecture, we observed and measured these traits in edited lines along with control plants (Figures 3-6). Alteration in spike architecture in the edited plants was observed (Figure 3B).

Measurement of plant height showed that plant height was significantly increased in LR-1 edited T0 lines compared with WT plants (Figure 4A). T0-16 and T0-6 lines showed a significant increase in plant height. A maximum number of effective tillers were observed in the T0-16 line followed by T0-6> T0-10> T0-1 LR-1 edited lines (Figure 4B). The rest of the edited lines also showed significant differences in comparison to the WT plants. Spike lengths were reduced significantly in genome-edited LR-1 lines (Figure 4C). A significant reduction in spike length was observed in T0-16 lines followed by T0-6, T0-10, T0-1, and T0-14 lines, respectively. Data regarding 1000 grain weight was significantly increased in all of the T0-edited LR-1 lines with a maximum in T0-16> T0-6> T0-1 lines compared to the control plants (Figure 4D).

Similarly, we also observed the plants of the LR-2 construct; an altered spike architecture was noticed in LR-2 edited T0 wheat plants (Figure 5B). The morphological traits such as the number of effective tillers and plant height were significantly increased, while the spike length was significantly reduced in LR-2 edited wheat T0-1, T0-4, and T0-6 plants. The other edited lines also showed a significant increase in the height of the plant and the effective number of tillers and decreased spike length compared to WT plants (Figure 6A-C). 1000 grain weight was significantly enhanced in all of T0 edited LR-2 wheat lines (Figure 6D). A significant increase in seed weight was detected in all LR-2 T0 edited lines, with the highest in T0-1, T0-4, and T0-6 lines.

The root architectural traits showed significant effects in LR-1 genome-edited T0 lines (Figure 3C). The root length, root depth, volume, and surface area were significantly increased (Figures 4E-F and H-I), however, the root diameter and root angle were significantly reduced in genome-edited LR-1 (Figure 4G and 4J) T0 lines compared to the WT plants. T0-1, T0-6, T0-10, and T0-16 lines displayed very significant differences in comparison to the control plants. However, in comparison to the wild-type plants, the other T0 lines also exhibited significant differences. An altered architecture of roots was observed in LR-1 edited T0 wheat plants (Figure 3C). Similarly, in the other construct (LR-2), root architectural traits also displayed significant variations in genome-edited T0 lines (Figure 5C). The root length, depth, surface area, and volume were significantly enhanced (Figures 6E-F and H-I). However, the root diameter and root angle were reduced significantly in genome-edited LR-2 T0 lines (Figure 6G and 6J). T0-1, T0-4, and T0-6 lines displayed very significant differences as compared to the WT plants. However, in comparison to the WT plants, the other T0 lines also exhibited significant differences.

Notably, the alterations detected in transgenic edited lines compared to WT plants have occurred due to editing of *TaRPK1* genes in wheat based on specific targeted designed sgRNAs that might have altered the open reading frame and hence the amino acid sequence (Supplementary Figure S4). The observed reduction in spike length, root diameter, and root angle, and enhanced root length, depth, surface area, and volume, results in a significant upsurge in the number of effective tillers and grain weight, compared to WT plants. This increase in grain weight and the number of effective tillers proposed that edited lines recompense the grain yield reduction caused due to decreased spike length. This study reveals that *TaRPK1* editing results in the improvement of root architecture traits and hence yield enhancement.

4. Discussion

Common wheat is an essential crop and has a complex genome comprising of A, B, and D sub-genomes (Zhang et al., 2018). The complexity of the wheat genome in comparison to other plants such as Arabidopsis, rice, and maize makes it a challenging and vital crop for researchers and scientists to study and optimize genome editing systems (Zhang et al., 2019). The CRISPR/Cas9 system has advanced promptly recently (Ma and Liu 2016). In this study, we have targeted *TaRPK1* genes in wheat using CRISPR/Cas9 (Figure 1), which involves all of the steps from the design of sgRNA to agronomic traits analysis across T0 generation.

CRISPR/Cas9 has been used to generate double-strand breaks (DSBs) within the host genome, which is similar to other site-specific nucleases such as meganucleases, ZFNs, and TALENs. The principal repair mechanism is the Non-Homologous End Joining (NHEJ) mechanism. The NHEJ-based small insertion/deletions (indels) mutagenesis is the most important genome editing application (Sander and Joung 2014). The frequency of NHEJ-mediated mutagenesis is influenced by a variety of factors. The major factors affecting the rate of this efficiency are the spatiotemporal expression of sgRNAs and Cas9by promoters, selection of sgRNA, delivery methods of sgRNA /Cas9/ which leads to different numbers of transgenic copies, and, the sequence of a gene and its chromosomal position that affects the target gene accessibility of sgRNA/Cas9 complex to create DSBs (Wu et al., 2014, Horlbeck et al., 2016).

Agrobacterium tumefaciens and biolistic bombardment are the effective and major methods for wheat transformation. As the efficiency of editing is also governed by complex delivery, most of the CRISPR/Cas editing was performed by the biolistic transformation in

wheat (Wang et al., 2014, Liang et al., 2017, Hamada et al., 2018, Wang et al., 2018). The major challenge is that the gene gun transformation involves numerous embryos, and for the editing events that take place during CRISPR/Cas9 transient expression, the CRISPR/Cas9 and selection marker cassette is not incorporated within the genome. This creates several transgene copies with a high gene silencing frequency and low rate of editing. In comparison, Agrobacterium-mediated transformation is less costly as it does not involve expensive apparatus and supplies. This method is the most prevalent because it involves the insertion of single or few copies of transgenes, hence mutations are induced with high efficiency and show stable heritability in wheat (Zhang et al., 2019). This study involves the application of CRISPR/Cas9 by *Agrobacterium tumefaciens*-mediated method for editing the genome at its specific target sites in wheat.

We adopted the two-sgRNAs-for-one-gene approach to enhance presumably double the rate of success or improve the probability of activation of at least one of the gRNA for mutagenesis, as reported in previous studies that not every synthesized sgRNA is highly active or active to create DSBs (Schmitz et al., 2020). This approach would enhance the attainment of targeted mutagenesis and larger deletion mutations (Char et al., 2017).

The target site selection is a crucial step. The gRNAs GC content is essential for the CRISPR/Cas9 efficiency (Wang et al., 2014, Zhang et al., 2018). In our study, the percentage of GC content in sgRNA1 and sgRNA3 was 41.67%, and in gRNA2 was 45.83% (Table 2). The gRNA2 having higher GC% content exhibited a higher efficiency (Zhang et al., 2018). The base composition and position of the gRNA may also affect CRISPR/Cas9 efficiency. Furthermore, chromatin availability is accompanied by the efficiency of CRISPR/Cas9 as reported in the zebrafish and human cells (Daer et al., 2017, Kallimasioti-Pazi et al., 2018, Uusi-Mäkelä et al., 2018). The efficiency of editing may be associated with the target genes' chromatin state (Zhang et al., 2019).

In the present study, we synthesized two constructs namely LR-1 and LR-2, with two gRNAs each, and successfully created mutagenesis in target sites of *TaRPK1* genes, via Agrobacterium-mediated CRISPR/Cas9 in T0 plants. Our results indicated that the primary mutation type was deletion. Similar observations were also reported in other wheat studies (Wang et al., 2014, Liang et al., 2017, Sanchez-Leon et al., 2018, Wang et al., 2018, Zhang et al., 2018, Zhang et al., 2019, Zhang et al., 2019, Ibrahim et al., 2022). However, these observations contradict with rice, where insertion is the dominant mutation type (Zhang et al.,

2014). We reported that the CRISPR/Cas9 system can achieve efficiently specific targeted editing with a mutation frequency of 43.75% and 40% for LR-1 and LR-2 constructs, respectively (Table 2). The stated efficiencies of mutations within different species or even different CRISPR platforms within wheat vary significantly. For instance, a relatively lower editing frequency of 5.6%, to 20.8%–54.2% mutation efficiency was reported in T0 wheat plants (Zhang et al., 2019). 54.17% mutagenesis efficiency which was much higher than earlier reported mutation frequency was also stated in larger and more complex genome cereal crops, and wheat (Zhang et al., 2018). In contrast, a significant rate of editing up to 100% have been reported by several laboratories working on rice (Zhang et al., 2014, Zou et al., 2014, Viana et al., 2019). In dicotyledonous crops, such as soybean and tomato, CRISPR systems generated higher than 50% mutation in T0 plants (Brooks et al., 2014, Li et al., 2015). In maize, 2%–100% editing efficiency was reported in the T0 plants (Liang et al., 2014, Xing et al., 2014, Svitashev et al., 2015, Feng et al., 2016, Zhu et al., 2016, Char et al., 2017).

RPK1 is one of the many genes that modulate the abiotic stresses and root system architecture (RSA), hence affecting yield. Our results are in corroborate the previous study performed on rice, where the knockdown of rice improved the root system architecture, and increased tillering numbers and plant height (Zou et al., 2014). Similar results with alterations in root system architecture, spike architecture, increased numbers of tillers, plant height, and grain weight were observed through CRISPR/Cas9 knockdown of the *TaRPK1* gene in T0 lines of wheat. The LR-1 and LR-2 T0 lines exhibited a significant alteration in morphological and architectural traits compared to WT plants due to modification in amino acid sequence and hence structure and function of TaRPK1 protein due to insertions and deletions caused by CRISPR/Cas9 mediated genome editing. Increased root architectural traits such as root length, depth, volume, and surface area, were exhibited, while decreased root angle and diameter were observed compared to the WT plants in CRISPR/Cas9 knockdown lines. As reported, these variations in the root architecture and their interplay with the shoot system attribute features to face water scarcity during drought stress and maintain yield (Siddiqui et al., 2021). The mechanism of how *RPK1* affects root system architecture might be via negative modulation of polar auxin transport (Figure 7).

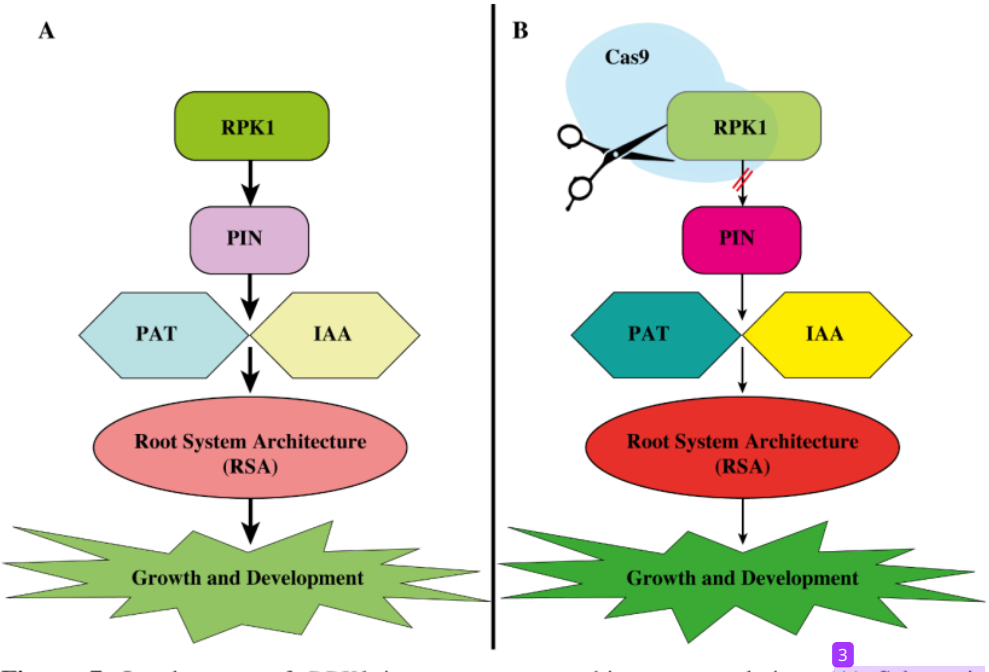


Figure 7. Involvement of *RPK1* in root system architecture regulation. (A) Schematic illustration of normal RPK1 pathway, in which RPK1 is normally expressed and inhibits the expression of other genes such as PIN, IAA, and PAT. This less expression leads to poor root development. (B) Editing of RPK1 by using the Cas9 system leads to less expression of RPK1 and higher expression of PIN, PAT, and IAA. This causes the proper or better root development under drought stress.

5. Conclusion

In summary, the CRISPR/Cas9 system was highly efficient in generating *TaRPK1* gene editing in wheat. Mutant lines harboring the desired modification in *TaRPK1* were obtained. The edited lines displayed altered root architectural and agronomic traits. This study has potential application in raising efforts to produce valuable traits in bread wheat. Our work presents a positive illustration of the CRISPR application, the precision and rapidity with which variations can be attained by this approach will assist in wheat improvement at a rate sufficient to assure food security globally.

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Availability of data and materials: All the data is presented in the main text and supplementary files. Further materials can be demanded from the corresponding author.

Supplementary Material:

Supplementary Table S1: The RPK1 sequences in the A, B, and D sub-genomes of wheat.
Supplementary Table S2. Alignment of A, B, and D CDS genomes.
Supplementary Table S3: Primer sequences used for sequencing of A, B, and D genomes.
Supplementary Figure S1: A flowchart of targeted mutagenesis in bread wheat.
Supplementary Figure S2: Construction of gRNA expression cassettes.
Supplementary Figure S3: Genomic DNA of control (1) and transgenic plants (2-4). The marker was denoted with "M".
Supplementary Figure S4: Multiple sequence alignment of TaRPK1 proteins depicting structural predictions.

Ethics declarations

Ethical approval and consent to participate: Not applicable.

Consent to publish: Not applicable.

Competing interests

The authors declare that they have no competing interests.

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