

The pVS1-VIR2 helper plasmid reduces regeneration efficiency of GRF-GIF in hexaploid wheat to obtain tri-allelic TaDOG1L4 mutations with reduced seed dormancy

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Abstract

Wheat plays important role as a staple crop and in the supply of brewing ingredients, but its yield has been increasingly threatened by the occurrence of pre-harvest sprouting. While resistance breeding relies on a thorough understanding of seed dormancy genetics and its interaction with the environment, obtaining such knowledge has been hampered by the difficulty of working with wheat directly. *DELAY OF GERMINATION 1* was cloned two decades ago as the first seed dormancy specific gene in *Arabidopsis*. However, its role in regulating cereal grain dormancy remains elusive. This study investigates the role of *TaDOG1L4* in wheat seed dormancy using mutants generated by CRISPR-Cas9. Efficient wheat transformation using immature embryos suggests the usefulness of the GRF4-GIF1 protein chimera in shoot regeneration, while the pVS1-VIR2 helper plasmid seems to interfere with the transformation when combined with the GRF4-GIF1 vector. Phenotyping of *TaDOG1L4* tri-allelic transgenic plants revealed its positive role in seed dormancy, as mutant seeds matured under both cool and warm temperature conditions showed reduced dormancy. These results suggest functional conservation of *DOG1* in regulating seed dormancy.

Introduction

Seed dormancy is a natural postponing mechanism through which seeds arrest germination under otherwise favorable conditions, which maximize the chances of subsequent seedling establishment and completion of the plant life cycle. As an evolutionarily conserved phenomenon, seed dormancy has been widely documented in flowering plants (Linkies et al., 2010). Seed dormancy contributes to environmental adaptation through timing of the right germination window. In turn, the seed maturation environment correlates with seed dormancy levels, as has been consistently documented in association studies conducted under diverse environmental conditions (Donohue et al., 2005; Postma and Ågren, 2015). Among multiple environmental factors examined, temperature, light and nitrate have been shown to play most significant roles in modulating seed dormancy and germination (Ali-Rachedi et al., 2004; Footitt et al., 2013; He et al., 2016). Central to seed dormancy is the plant hormone abscisic acid (ABA), which suppresses seed germination during development and induces desiccation tolerance and seed dormancy at maturation, while also maintains seed dormancy upon imbibition (Chen et al., 2020). Opposite to ABA, gibberellin (GA) promotes germination and the hormonal balance between ABA and GA leads to various levels of seed dormancy in a wide range of species, including cereal crops (Tuan et al., 2018).

DELAY OF GERMINATION 1 (*DOG1*) was identified as the first seed dormancy specific locus in *Arabidopsis* (Bentsink et al., 2006), and has been reported in multiple species spanning both dicots and monocots including cereals (Graeber et al., 2010; Li et al., 2026). Despite the relatively low sequence similarity between *AtDOG1* and cereal *DOG1*Ls genes, ectopic expression of wheat and barley *DOG1*Ls in *Arabidopsis* leads to enhanced seed dormancy (Ashikawa et al., 2010). Overexpression of *TaDOG1L4* in wheat cultivar Fielder increased seed dormancy, while RNAi-mediated knock-down of *TaDOG1L4* decreased the seed dormancy level (Ashikawa et al., 2014). Recent evidence suggests that *TaDOG1*Ls

interact with core ABA signaling components PP2Cs to regulate seed dormancy and germination (Yu et al., 2020; Zhang et al., 2025; Zhang et al., 2026). However, due to the low specificity of RNAi, it remains an open question whether *TaDOG1L4* specifically plays a role in regulating grain dormancy and germination in hexaploid wheat. This study describes the transformation of hexaploid wheat immature embryos using the GRF4-GIF1 vector (Debernardi et al., 2020) and functional characterization of *TaDOG1L4* tri-allelic transgenic lines.

Results

1. Stable wheat transformation using immature embryos

As one of the several species recalcitrant to genetic transformation, hexaploid wheat has been extensively characterized for improved regeneration and transformation efficiency (Hensel, 2020; Liu et al., 2023; Wang et al., 2022). To ensure successful wheat transformation, the recently available JD633 vector harboring a GROWTH-REGULATING FACTOR (GRF) and GRF-INTERACTING FACTOR (GIF) chimeric protein (Debernardi et al., 2020) and the pVS1-VIR2 helper plasmid (Zhang et al., 2019) were tested, as previous studies showed these vectors can improve regeneration and transformation.

When the JD633 construct alone was used, regenerated shoots and transgenic plants were obtained for *TaDOG1L4*, with transformation efficiencies ranging from 5% to 35% (Figure 1, Table 1). However, when combining the JD633 construct with the pVS1-VIR2 helper plasmid, only one shoot regenerated from multiple transformation batches, leading to an overall transformation efficiency of 0.71% (Table 1). This is different from data reported in maize and sorghum, where transformation efficiencies of 8.1% and 38.28% were achieved respectively when GRF4-GIF1 and pVS1-VIR2 were combined (Li et al., 2024; Vandeputte et al., 2024). Such discrepancy was observed across transformation batches handled by different people, strongly reducing the possibilities of unreliable protocol and operational mistakes. Thus, a possible explanation could be that the pVS1-VIR2 vector interferes with the transformation procedure when combined with the GRF4-GIF1 vector.

Table 1, Summary of wheat transformation batches targeting *TaDOG1L4* genes

Batch	Embryos used	Regenerated plants	CAS9 (+) plants	Transformation efficiency	Gene editing events	Vectors used
AV-W2	66	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
EZ-W1	148	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
EZ-W2	89	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
EZ-W3	95	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
EZ-W4	23	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
RQ-W3	104	0	0	0	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
RQ-W5	17	1	0	0.00%	0	<i>TaDOG1L4-JD633+pVS1-VIR2</i>
RQ-W6	19	7	6	31.58%	4	<i>TaDOG1L4-JD633</i>
RQ-W7	20	4	1	5.00%	1	<i>TaDOG1L4-JD633</i>

2. Generation of triple homozygous *TaDOG1L4* mutants in hexaploid wheat

Among *TaDOG1L4* transgenic plants in the T₀ generation, five plants showing independent gene editing events were identified (Table 2). Among the lesions introduced by CRISPR, short insertions and deletions account for the majority, which was consistent with previous observations (Wang *et al.*, 2014). In some cases, more than two lesions were identified in one sub-genome, possibly due to mosaicism or transient expression of the Cas9 machinery (Lorenzo *et al.*, 2022).

These plants were allowed to self-pollinate and screened for tri-allelic transgenic progenies with a single lesion at each sub-genome by Sanger sequencing. Three such plants were recovered and designated MT1, MT2 and MT3 (Figure 2).

The 20ng gRNA sequence was displayed in blue and the PAM sequence was shown in bold. The ATG starting codon was underlined.

Sanger sequencing showed that CRISPR induced lesions differ in the A-genome and B-genome between MT1, MT2 and MT3, while a single nucleotide insertion (insA) in the D-genome was present in all three lines. Specifically, MT1 harbors a single nucleotide insertion in both the A-genome (insG) and the B-genome (insT). MT2 shows a dinucleotide insertion in the A-genome (insGC) and a single nucleotide insertion in the B-genome (insA). In MT3, a single nucleotide insertion in the A-genome (insT) and a dinucleotide deletion in the B-genome (delCG) were identified. These lesions were introduced at the 5'-UTR region and specifically three nucleotides preceding the ATG start codon.

Table 2, CRISPR induced lesions at *TaDOG1L4* genes in the T₀ generation

Sample	Subgenome	Lesion	ICE Indel (%)	ICE Knock-Out Score	Genotype
W6-1	A	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCGG CCATGG ACATGGCACGCTACGTTGCG	97	97	aabbDd
	B	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG CCATGG ACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG TCCA TGGACATGGCACGCTACGTCGCG	98	98	
	D	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTCGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTCGCG	76	76	
W6-2	A	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCGG CCATGG ACATGGCACGCTACGTTGCG	99	99	aabbDd
	B	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG CCATGG ACATGGCACGCTACGTTGCG	98	98	
	D	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG	54	33	
W6-3	A	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG TCCA TGGACATGGCACGCTACGTCGCG	98	98	aabbDd
	B	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG CCATGG ACATGGCACGCTACGTTGCG	98	98	
	D	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG	53	31	
W6-7	A	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCGG CCATGG ACATGGCACGCTACGTTGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG TCCA TGGACATGGCACGCTACGTTGCG	95	95	aabbDd
	B	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG	96	96	
	D	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTCGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG CCATGG ACATGGCACGCTACGTTGCG	98	45	
W7-2	A	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG TCCA TGGACATGGCACGCTACGTCGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG CCCA TGGACATGGCACGCTACGTTGCG	94	94	aabbdd
	B	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG TCCA TGGACATGGCACGCTACGTCGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG	98	98	
	D	TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTCGCG TCCCGTCACTCCCTGTGCGCGCGAGAGACCAAGATCG ACCA TGGACATGGCACGCTACGTTGCG	91	91	

3. Seed dormancy phenotyping of *TaDOG1L4* triple homozygous mutants

The tri-allelic mutants were grown side by side with null-sibling (NS) controls under 16°C and 20°C pre-anthesis temperature, 16h light/8h dark photoperiod conditions. Germination tests were performed using seeds harvested at physiological maturity.

When developed under 20 °C, MT2 and MT3 showed significantly faster germination speed and higher germination rate. Similar results were obtained when seeds were developed under 16 °C (Figure 3). These results support the positive role of *TaDOG1L4* genes in wheat grain dormancy, as previously

shown by RNAi mediated gene knock-down (Ashikawa *et al.*, 2014). It is important to note that the 20nt gRNA sequence spans the 5' prime UTR region and the ATG start codon of *TaDOG1L4* genes (Figure 2), as this guide showed a high overall performance with the lowest off-target score predicted by WheatCrispr (Cram *et al.*, 2019). Thus, the observed phenotypic differences between MT1 and MT2, MT3 may be attributed to the effects of CRISPR lesions on mRNA processing (Kozak, 1986; Neiningner *et al.*, 2019).

Discussion

Stable and efficient transformation of hexaploid wheat

As one of the most important staple crops, wheat has been the focus of a diverse range of investigations for improved yield, disease resistance and grain quality. However, the large and complex genome, long generation time and recalcitrance to genetic transformation have been the major bottlenecks to work with wheat directly. Recent progress revealed the significant boosting effects of morphogenic regulators such as *BABY BOOM* and *WUSCHEL2*, the *pVS1-VIR2* virulence helper plasmid, the GRF4-GIF1 fusion protein and novel transformation protocols such as QuickWheat (Debernardi *et al.*, 2020; Johnson *et al.*, 2023; Zhang *et al.*, 2019). In this study, we combined the *GRF4-GIF1* vector and *pVS1-VIR2* helper plasmid to transform immature embryos of hexaploid wheat cv. Fielder. For multiple batches and gene constructs, the *GRF4-GIF1* vector alone could lead to stable transformation, while the combination of *GRF4-GIF1* and *pVS1-VIR2* vectors led to far lower transformation efficiency. Such results highlight the usefulness of the *GRF4-GIF1* vector to improve the transformation of recalcitrant species like hexaploid wheat, while discrepancy with published results concerning the role of *pVS1-VIR2* may be explained by its interference in transformation. *TaDOG1L4* genes were modified by showing short insertions and deletions introduced by CRISPR, which were faithfully transmitted to the next generations through self-pollination and segregation.

A positive role of *TaDOG1L4* in hexaploid wheat seed dormancy

Previously, RNAi mediated knockdown of *TaDOG1L4* in wheat cv. Fielder decreased the seed dormancy level by several tens of percent (Ashikawa *et al.*, 2014). In this study, three *TaDOG1L4* tri-allelic transgenic plants were generated by CRISPR and subjected to seed dormancy phenotyping. Two mutants showed decreased seed dormancy in comparison with the null-sibling control which underwent the same transformation procedure. This reduction of dormancy can be observed when seeds were matured under either 16°C or 20°C pre-anthesis temperature. Thus, *TaDOG1L4* seems to play positive roles in seed dormancy in wheat cv. Fielder. However, a third mutant behaved similarly as the control, which may be attributed to the different gene editing events that occurred in the mutants (Fig. 2). As previous research already showed that this location is crucial for translation initiation (Kozak, 1984; Kozak, 1986), the observed seed germination phenotype in MT1 and MT2, MT3 may be explained by differential mRNA translation produced by these different lesions.

Material and Methods

Plant material and growth

Wheat cultivar Fielder seeds were sown in 1 liter pots containing 2:1 ratio of soil and vermiculite mixture, supplemented with 1 gram Osmocote per 1.5 liter soil mixture. Plants were grown in a daily cycle of 20 h light / 4 h dark at 20°C in a growth room. Regenerated T₀ wheat plants were grown under the same growth room conditions to allow self-pollination, and seeds were harvested at maturation. Offspring of mutant plants was propagated in an unheated greenhouse and triple homozygous T₁ plants were selected by Sanger sequencing for mutational status. The identified tri-allelic mutants (T₂) were grown at 16 h light/8 h dark photoperiod under 16°C or 20°C in Lovibond growth chambers. Upon flowering, the 16°C scheme was switched to 20°C to accelerate development. Seeds from individual plants were harvested separately and stored in sealed plastic bags under – 80°C to arrest seed dormancy until germination tests.

Single guided RNA design and construct cloning

Previously, homologues of major seed dormancy genes have been identified in wheat (Li and Geuten, 2026). Based on this information, a 20nt sgRNA (Table S1) targeting *TaDOG1L4* in hexaploid wheat was designed using WheatCRISPR (Cram et al., 2019) and synthesized at Integrated DNA Technologies (Leuven, Belgium). This oligo was cloned into the JD633 vector via Golden Gate assembly using AarI and T4 DNA ligase (Debernardi et al., 2020). Products from Golden Gate assembly were transformed into *Escherichia coli* strain DH5α by heat-shock and plated on LB plates containing 50 µg/mL kanamycin. Colonies were randomly picked and verified by PCR, restriction digestion and Sanger sequencing at LGC Genomics (Berlin, Germany).

Plant transformation and molecular analysis

Plant vectors were put into *Agrobacterium tumefaciens* strain AGL1 via electroporation (Figure S1). Immature embryos isolated from developing wheat grains were subject to *Agrobacterium*-mediated transformation (Hayta et al., 2019; Ishida et al., 2015). Around one hundred immature embryos were used per transformation. Regenerated plantlets were selected by 50 mg/ml Hygromycin (Sigma-Aldrich) and acclimated in tubes before transferring to pots. Wheat plants were genotyped by Sanger sequencing the region flanking sgRNA using sub-genome specific primer pairs (Table S2) and data were analyzed using the Inference of CRISPR Edits (ICE) tool (Conant et al., 2022).

Seed germination test

Twenty physiologically mature seeds from one plant were treated as biological replicates and five replicates were used. Seeds were surface sterilized and placed on 0.5% plant agar plates. Plates were incubated at 18°C with daily cycles of 16h light and 8h darkness in a Lovibond growth chamber.

Germination was scored daily for fifteen days and ungerminated seeds were stratified to confirm viability.

Survival analysis of germination data was performed using the `brm()` function in the `brms` R package according to the user manual. Briefly, the raw germination data were first converted into survival format and data from germinated and non-germinated (censored) seeds were combined. Then the data were fit with an accelerated failure time model and the predicted germination curve was returned. Based on the modelling output, a Kaplan–Meier survival curve showing 95% confidence interval and p-value derived from the log-rank test was drawn. The full script is available in the Supplementary Information.

Declarations

Author Contribution

K.G. secured funding and supervised the study. E.Z.A.L. and R.L. conceptualized the study and designed the experiment. E.Z.A.L., R.L., V.M. and M.U.H. performed the experiments. R.L., M.U.H. and K.G. analyzed the data. R.L. wrote the first draft and K.G. revised the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

The authors declare no competing interests.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. CRISPR-Cas9 gRNA sequence was provided in Supplementary Table 1. Primer sequences were provided in Supplementary Table 2. R script for survival analysis was available in the Supplementary Information.

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Figures

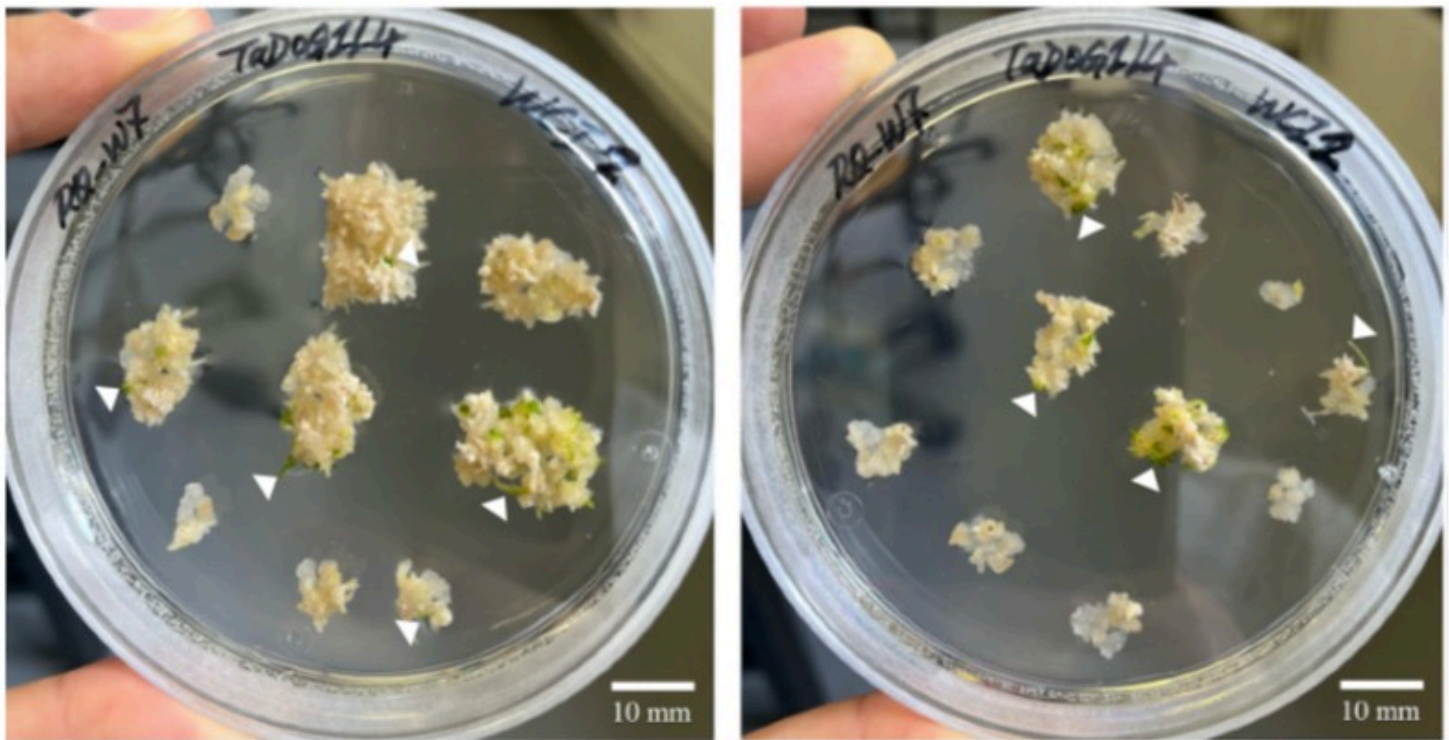


Figure 1

Representative green shoots regenerated from wheat immature embryos transformed with *TaDOG1L4-JD633*vector. The regenerated green shoots were indicated with white arrowheads. Photos of two randomly selected plates were taken around three weeks after the embryos were placed on the callus induction media.

MT1

TaDOG1L4-ABD-WT	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCG CCAT <u>TGG</u> ACATGGCACGCTACGT
TaDOG1L4-A-MT1	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGGCCATGGACATGGCACGCTACGT
TaDOG1L4-B-MT1	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGTCCATGGACATGGCACGCTACGT
TaDOG1L4-D-MT1	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGACCATGGACATGGCACGCTACGT

MT2

TaDOG1L4-ABD-WT	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCG CCAT <u>TGG</u> ACATGGCACGCTACGT
TaDOG1L4-A-MT2	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGGCCATGGACATGGCACGCTACGT
TaDOG1L4-B-MT2	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGA CCATGGACATGGCACGCTACGT
TaDOG1L4-D-MT2	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGA CCATGGACATGGCACGCTACGT

MT3

TaDOG1L4-ABD-WT	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCG CCAT <u>TGG</u> ACATGGCACGCTACGT
TaDOG1L4-A-MT3	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGTCCATGGACATGGCACGCTACGT
TaDOG1L4-B-MT3	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGAT-- CCATGGACATGGCACGCTACGT
TaDOG1L4-D-MT3	TCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGACCATGGACATGGCACGCTACGT

Figure 2

CRISPR induced lesions at TaDOG1L4-ABD sub-genomes in the three tri-allelic plants. Red dashes indicate deleted nucleotides and red letters indicate inserted nucleotides. The 20ng gRNA sequence was displayed in blue and the PAM sequence was shown in bold. The ATG starting codon was underlined.

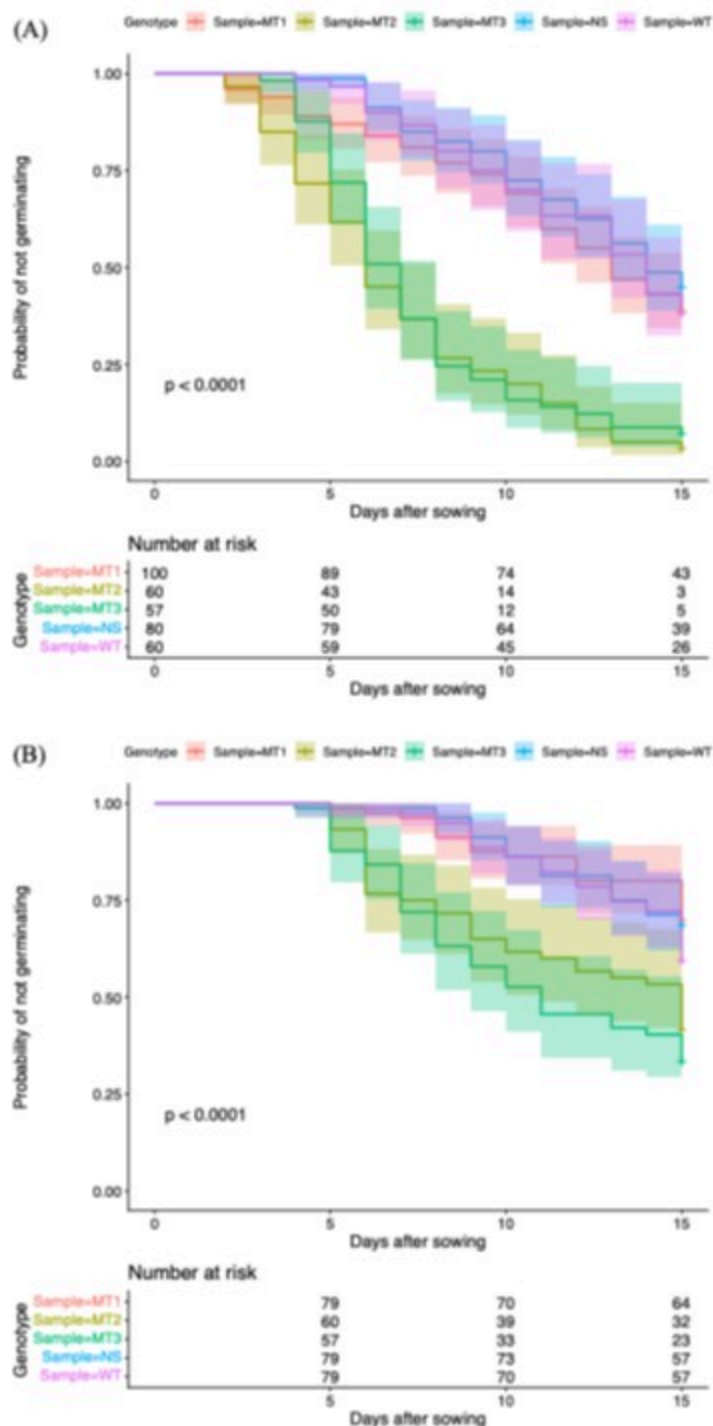


Figure 3

Seeds of the TaDOG1L4 tri-allelic transgenic plants germinated significantly faster than the control. Kaplan – Meier estimation of survival functions for mutants (MT), null-sibling (NS) and wildtype (WT) seeds matured under (A) 20 °C and (B) 16 °C. Point-wise 95% confidence intervals showing seed germination profile were computed using the ggsurvplot () function in the ggsurvfit R package.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryInformation.docx](#)