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Multi-Platform Comparative Evaluation, Consensus Strategy, and Dual-Temperature Thermodynamic Profiling of CRISPR/Cas9 gRNA Design Targeting the TaDREB1 Gene in Bread Wheat (*Triticum aestivum*)

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Abstract

Gene editing via CRISPR/Cas9 relies heavily on accurate single-guide RNA (gRNA) selection to maximize target cleavage efficiency while minimizing off-target binding. In complex allohexaploid genomes like bread wheat (*Triticum aestivum* L.), divergent scoring matrices and off-target alignment algorithms across predictive platforms often yield inconsistent gRNA recommendations. Here, we conducted a systematic *in silico* comparative evaluation of five widely used gRNA design tools (CRISPRdirect, Cas-Designer, CCTOP, CRISPR-P 2.0, and WheatCrispr) targeting the drought-responsive transcription factor gene *TaDREB1* on chromosome 3D. Standardizing candidate spacer sequences to a uniform 20-nucleotide (20nt) core across 46 unique gRNAs revealed substantial algorithmic divergence, with 91.3% (42/46) of candidates predicted by only a single platform. Cross-platform overlap analysis isolated a high-confidence primary consensus gRNA—GCACCGCCCCAGUUAAAACG (60.0% GC content)—supported independently by four prediction servers (CRISPRdirect, Cas-Designer, WheatCrispr, and CRISPR-P 2.0). To verify structural viability under standard Turner thermodynamic energy parameters and *in vitro* SpCas9 ribonucleoprotein (RNP) assembly conditions (37°C), dual-level secondary structure profiling was performed using RNAfold (v2.6.3). The isolated 20nt core spacer exhibited an optimal Minimum Free Energy (MFE) of 0.00 kcal/mol with a completely open, unpaired dot-bracket conformation (.....), an ensemble free energy of -0.16 kcal/mol, an MFE structure frequency of 77.02%, and an ensemble diversity of 0.84, confirming complete single-stranded target accessibility. Furthermore, supplementary thermal sensitivity profiling at 22°C—reflecting realistic physiological wheat canopy conditions—confirmed that the 20nt core spacer maintained an identical optimal MFE of 0.00 kcal/mol and an open conformation (.....), with a thermodynamic ensemble free energy of -0.47 kcal/mol, an MFE structure frequency of 45.25%, and an ensemble diversity of 1.78, verifying a complete absence of internal self-hairpins under cooler physiological growth conditions. Moreover, modeling the full-length 96-nt sgRNA construct (20nt spacer fused with the 76-nt SpCas9 scaffold) at 37°C yielded an optimal MFE of -18.90 kcal/mol, a thermodynamic ensemble free energy of -20.90 kcal/mol, an MFE frequency of 3.90%, an ensemble diversity of 33.22, and a centroid MFE of -7.69 kcal/mol, verifying canonical scaffold stem-loop folding without inhibitory internal spacer hairpins. Additionally, systematic auditing documented critical output deficiencies across tools, including missing efficiency scores, incomplete coding annotations, and unannotated intergenic off-targets. While CRISPR-P 2.0 and WheatCrispr provided the most context-aware plant outputs and Cas-Designer excelled in off-target filtering, this dual-level validated consensus framework bridges inter-tool discrepancies and establishes a reliable workflow for polyploid crop genome engineering.

1. Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most vital staple food crops globally, accounting for a substantial portion of human dietary energy and protein consumption. However, global wheat production faces escalating threats from abiotic stresses, particularly drought and salinity. Among the key molecular regulators of abiotic stress tolerance, the Dehydration-Responsive Element-Binding 1 (*DREB1*) transcription factor family plays a pivotal role by specifically binding to dehydration-responsive element/C-repeat (DRE/CRT) cis-acting elements to trigger protective stress-responsive gene cascades (Eltaher et al., 2023; Gaponenko et al., 2018). Structural and functional diversity among *DREB* genes underlines their significance in enhancing stress adaptation across wheat cultivars (Gaponenko et al., 2018; Eltaher et al., 2023). Targeted genome editing of *TaDREB1* on chromosome 3D using the CRISPR/Cas9 system offers a high-precision strategy for functional genomic dissection and crop trait optimization (Cong et al., 2013; Jinek et al., 2012).

Implementing Cas9-mediated genome editing in bread wheat presents significant computational and biological challenges due to its large, complex allohexaploid genome ($2n = 6x = 42$) and high sequence repetition (> 85%), which substantially elevate the risk of off-target cleavage across homoeologous subgenomes (Rasheed et al., 2019). The primary determinant of editing specificity and cleavage efficiency is the single-guide RNA (gRNA), comprising a 20-nucleotide (20nt) spacer sequence complementary to the target DNA locus adjacent to a 5'-NGG-3' Protospacer Adjacent Motif (PAM) (Jinek et al., 2012). While numerous web-based bioinformatic platforms assist in gRNA design including CRISPRdirect (Naito et al., 2015), Cas-Designer (Park et al., 2015), CCTOP (Stemmer et al., 2015), CRISPR-P 2.0 (Liu et al., 2017), and WheatCrispr (Rasheed et al., 2019) they utilize divergent on-target scoring matrices, seed-region specificity rules, and off-target alignment algorithms. Consequently, relying on a single prediction software often yields inconsistent candidate recommendations and incomplete analytical metadata.

Furthermore, beyond sequence alignment and off-target filtering, the secondary structure folding of the gRNA strongly governs ribonucleoprotein complex assembly and target DNA recognition. Unintended intramolecular base-pairing within the 20nt spacer or misfolding of the full-length sgRNA scaffold can severely impair Cas9 cleavage activity, necessitating rigorous minimum free energy (MFE) and ensemble thermodynamic validation across both standard in vitro benchmarks (37°C) and physiological crop canopy conditions (22°C) (Gruber et al., 2008; Lorenz et al., 2011; Mathews et al., 2004).

To address the core question *Which freely available CRISPR guide RNA design tool generates the most efficient and specific guide RNAs for editing the TaDREB1 gene in wheat?* this study performs a systematic, multi-platform *in silico* evaluation of five prominent gRNA design tools targeting *TaDREB1*. By standardizing spacer sequences to a uniform 20nt core length, we construct an integrated cross-platform overlap matrix to isolate high-confidence consensus gRNAs, validate structural accessibility via dual-level RNA thermodynamic profiling at 37°C and 22°C, and systematically audit tool-specific output deficiencies.

2. Materials and Methods

2.1 Target Gene Retrieval

The reference genomic sequence of the TaDREB1 gene located on chromosome 3D of Triticum aestivum (cultivar Chinese Spring, IWGSC RefSeq v2.1 assembly) was retrieved from NCBI GenBank and Ensembl Plants databases.

2.2 In Silico gRNA Design Platforms

Candidate sgRNAs targeting TaDREB1 were extracted using five web-based prediction platforms:

- **CRISPRdirect**: Evaluates target specificity based on seed-region (12mer/8mer) genomic uniqueness (Naito et al., 2015).
- **Cas-Designer**: Analyzes microhomology-mediated end joining and off-target sites (Park et al., 2015).
- **CCTOP**: Scores candidate target sites and off-target loci based on distance-weighted PAM models (Stemmer et al., 2015).
- **CRISPR-P 2.0**: A plant-tailored portal evaluating on-target efficiency and genome-wide off-target risks (Liu et al., 2017).
- **WheatCrispr**: A wheat-specific web platform optimized for crop genome assemblies (Rasheed et al., 2019).

All platforms were run under SpCas9 parameters (PAM: 5'-NGG-3', standard spacer length: 20nt).

2.3 Standardization Protocol (20nt Core Spacer Parsing)

To resolve structural discrepancies across raw exports (e.g., inclusion of 3nt PAM tails or strand complements), a normalization script was applied:

- External PAM sequences (5'-NGG-3') were trimmed.
- All spacers were parsed strictly into 20-nucleotide 5'-to-3' core spacer orientations.
- Positional coordinates and GC content percentages were re-calculated to verify structural integrity.

2.4 Consensus Scoring and Limitation Auditing

A master database of all unique 20nt spacer sequences was assembled. Consensus Frequency (Cf) was determined by evaluating multi-tool agreement across platforms. Concurrently, raw data exports were systematically audited to catalog unpopulated fields, missing scores, and annotation gaps across tools.

2.5 In Silico gRNA Secondary Structure and Thermodynamic Profiling

To evaluate single-stranded structural accessibility and thermodynamic stability of the primary candidate gRNA (GCACCGCCCCAGUUAAAACG), in silico secondary structure prediction was executed using the ViennaRNA Package (RNAfold web server v2.6.3) under standard in vitro SpCas9 ribonucleoprotein (RNP) assembly conditions (37°C). Minimum Free Energy (MFE ΔG in kcal/mol), ensemble free energy, dot-bracket notations, and thermodynamic ensemble frequencies were quantified to verify the absence of inhibitory internal hairpin loops or self-dimerization. To further evaluate thermal sensitivity under realistic physiological canopy temperatures of temperate crops such as wheat, the exact same thermodynamic profiling was additionally performed by rescaling energy parameters to 22°C (RNAfold -p -d2 --noLP -T 22).

2.6 Full-Length sgRNA (Spacer + Scaffold) Structural and Thermodynamic Modeling To maintain methodological consistency and evaluate structural integrity under realistic Cas9 ribonucleoprotein (RNP) assembly conditions, the exact same in silico thermodynamic profiling protocol executed in Section 2.5 was applied to the complete functional sgRNA construct. Specifically, the 20nt primary consensus spacer (GCACCGCCCCAGUUAAAACG) was fused at its 3' end to the standard 76-nt *Streptococcus pyogenes* Cas9 (SpCas9) scaffold (GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC), generating a 96-nt full-length sgRNA sequence (5'-GCACCGCCCCAGUUAAAACGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC-3'). Folding predictions were computed via the ViennaRNA Package (RNAfold web server v2.6.3) using identical thermal parameters (37° C, standard ViennaRNA default temperature) and command-line settings (RNAfold -p -d2 --noLP) to quantify optimal MFE, thermodynamic ensemble free energy, MFE ensemble frequency, ensemble diversity, and centroid secondary structure MFE.

3. Results

3.1 Overview of Candidate gRNA Yields

Across all five evaluated platforms, a total of 46 unique 20nt spacer sequences were identified for the TaDREB1 target region. Candidate yields per server comprised: CRISPRdirect (8), Cas-Designer (9 unique from 11 rows), CCTOP (16 unique from 20 entries), CRISPR-P 2.0 (10), and WheatCrispr (10).

3.2 Cross-Platform Consensus Profiling

Out of 46 candidate spacers, 42 sequences were exclusively predicted by single tools. However, four candidate gRNAs exhibited multi-tool consensus (Cf > = 2), as shown in Table 1.

Table 1
Comprehensive Cross-Platform Overlap Matrix for Top Consensus gRNAs (20nt Core Spacer)

No.	Spacer Sequence (20nt)	GC%	Matching Tools (Cf)	CRISPRdirect	Cas-Designer	WheatCrispr	CRISPR-P 2.0	CCTOP	Classification
1	**GCACCGCCCCAGTTAAAACG**	60.0%	4	Yes	Yes	Yes	Yes	-	**Primary Candidate (Top Consensus)**
2	CACCGCCCCAGTTAAAACGG	60.0%	3	Yes	Yes	Yes	-	-	Secondary Candidate
3	GGCACCGCCCCAGTTAAAAC	60.0%	2	-	Yes	Yes	-	-	Moderate Consensus
4	ACCGCCCCAGTTAAAACGGG	60.0%	2	Yes	Yes	-	-	-	Moderate Consensus

The spacer sequence GCACCGCCCCAGTTAAAACG emerged as the primary candidate, supported by an overwhelming four-tool agreement (CRISPRdirect, Cas-Designer, WheatCrispr, and CRISPR-P 2.0) with an optimal 60.0% GC content.

3.3 Systematic Feature Deficiency Audit

Auditing raw software outputs revealed notable algorithmic reporting gaps across all platforms (Table 2).

Table 2
Systematic Documentation of Software Deficiencies and Data Gaps

Tool Name	Missing Feature / Parameter	Raw Data Observation	Scientific Limitation & Impact
CRISPRdirect	On-Target Efficiency Score	Returned as N/A for all 8 rows	Lacks quantitative cleavage efficiency scores; relies solely on seed specificity (Naito et al., 2015).
Cas-Designer	Out-of-Frame Frameshift	Returned as N/A in 2/11 rows (18.2%)	Omits frameshift probability metrics for specific candidate sites (Park et al., 2015).
WheatCrispr	Coding Region Annotation	Blank / Empty across all 10 rows	Fails to explicitly annotate exonic versus non-coding locus contexts (Rasheed et al., 2019).
CCTOP	Gene Symbol & Locus Distance	Gene Name blank in 95%	Lacks standard gene symbols for most off-targets and omits intergenic locus annotations (Stemmer et al., 2015).
CRISPR-P 2.0	Detailed Mismatch Breakdown	Omitted from primary export view	Provides aggregated scores without listing per-locus mismatch positioning in main export (Liu et al., 2017).

3.4 Thermodynamic Profiling and Secondary Structure Stability of Top Candidate

Thermodynamic modeling via RNAfold confirmed an optimal Minimum Free Energy (MFE) of 0.00 kcal/mol paired with a completely open, unpaired dot-bracket notation (.....) for the primary candidate GCACCGCCCCAGUUAAAACG. The thermodynamic ensemble free energy was recorded at -0.16 kcal/mol, with the MFE open structure representing 77.02% of the total thermodynamic ensemble and exhibiting an ensemble diversity of 0.84. Graphical output analysis demonstrated a fully exposed, unhindered single-stranded conformation. This confirms that the top consensus spacer (GC%=60.0%) maintains complete structural accessibility required for seamless Cas9 protein binding and high-affinity hybridization to the target TaDREB1 genomic site on chromosome 3D. (Fig. 1)

3.5 Thermal Sensitivity Profiling of the Top Consensus Spacer at Physiological Wheat Canopy Temperature (22°C)

To evaluate thermal sensitivity under realistic physiological canopy temperatures of wheat (~ 15–25°C), secondary structure profiling of the 20nt core spacer (GCACCGCCCCAGUUAAAACG) was re-evaluated at 22°C using RNAfold v2.6.3 (with command-line equivalent RNAfold -p -d2 --noLP -T 22). Thermodynamic modeling at this cooler thermal threshold confirmed that the spacer retains an identical optimal Minimum Free Energy (MFE) of 0.00 kcal/mol and a completely open, unpaired dot-bracket conformation (.....), alongside a centroid MFE of 0.00 kcal/mol. Furthermore, the thermodynamic ensemble free energy was recorded at -0.47 kcal/mol, with the open MFE structure representing 45.25% of the total thermodynamic ensemble, and an ensemble diversity of 1.78, confirming a complete absence of internal self-hairpins under cooler physiological growth conditions.

3.6 Thermodynamic Profiling and Secondary Structure of the Full-Length 96-nt sgRNA Construct

Replicating the $37^\circ\ \mathrm{C}$ RNAfold (v2.6.3) thermodynamic evaluation on the full-length 96-nt sgRNA construct (20nt spacer + 76-nt SpCas9 scaffold) yielded the following quantitative metrics:

- Optimal Minimum Free Energy (MFE): $-18.90\ \mathrm{kcal/mol}$
- Thermodynamic Ensemble Free Energy: $-20.90\ \mathrm{kcal/mol}$
- Frequency of the MFE Structure in the Ensemble: 3.90%
- Ensemble Diversity: 33.22
- Centroid Secondary Structure MFE: $-7.69\ \mathrm{kcal/mol}$

Structural mapping via dot-bracket notation for the optimal MFE conformation (((((((.....((((.....((((.....((((((...((((((.....)).)))).)))))).....)))))))...))))))) and the centroid secondary structure ((((((.....((((.....((((....))))..................)))).....................)))))) demonstrated that the 76-nt scaffold folds into the canonical stem-loop structures required for Cas9 protein recognition. Meanwhile, the internal core of the 20nt targeting spacer remains completely free of inhibitory internal self-hairpins, exhibiting only minor terminal pairing between its 5' nucleotides (positions 1–6 in MFE; positions 1–5 in centroid) and the 3' terminus of the scaffold (positions 91–96 in MFE; positions 92–96 in centroid). Collectively, these statistics confirm that fusing the GCACCGCCCCAGUUAAAACG spacer to the SpCas9 scaffold preserves thermodynamic stability and functional architecture at $37^\circ\ \mathrm{C}$.

Figure 3: Optimal Minimum Free Energy (MFE) secondary structure of the full-length 96-nt sgRNA construct (GCACCGCCCCAGUUAAAACG spacer fused with the 76-nt SpCas9 scaffold) predicted by RNAfold (v2.6.3) at 37°C, demonstrating an optimal MFE of -18.90 kcal/mol (dot-bracket: ((((((.....((((.....((((.....((((((...((((((.....)).)))).)))))).....)))))))...))))))) and canonical scaffold stem-loop formation

4. Discussion

Evaluating candidate gRNAs targeting TaDREB1 across five computational tools highlighted significant divergence, with 91.3% of predicted spacers restricted to single-tool outputs. This divergence answers the core question: no single tool individually provides a complete and flawless output for designing gRNAs in complex polyploid crops like wheat.

However, among the evaluated servers, CRISPR-P 2.0 and WheatCrispr stand out as the most balanced plant-specific tools, whereas Cas-Designer excels in off-target evaluation. The top consensus spacer GCACCGCCCCAGTTAAAACG demonstrated multi-tool validation across four independent algorithms (Naito et al., 2015; Park et al., 2015; Liu et al., 2017; Rasheed et al., 2019). Its 60.0% GC ratio ensures high duplex stability without risking RNA secondary structure formation.

To evaluate the structural viability of the top consensus gRNA (GCACCGCCCCAGTTAAAACG), in silico RNA secondary structure prediction (e.g., via RNAfold) is vital to ensure the spacer does not form inhibitory hairpins that hinder Cas9 binding. Although this consensus approach resolves algorithmic variations, a key limitation of this study is the lack of in vitro or in vivo wet-lab validation. Therefore, future work should empirically test this gRNA in wheat protoplasts or callus tissue using T7E1 assays or next-generation sequencing (NGS). Ultimately, this multi-tool evaluation matrix provides a functional blueprint that can be expanded to target other stress-related gene families (e.g., TaDREB2 or TaHKT), accelerating molecular breeding for climate-resilient crops

Documenting tool-specific limitations like missing efficiency scores in CRISPRdirect (Naito et al., 2015) or incomplete gene symbol annotations in CCTOP (Stemmer et al., 2015) reinforces the necessity of a multi-tool consensus strategy rather than relying on a single tool to minimize experimental failure in downstream plant genome editing.

Crucially, thermodynamic profiling via RNAfold further validated the physical viability of the top candidate GCACCGCCCCAGTTAAAACG. Yielding an MFE of $0.00\ \mathrm{kcal/mol}$ confirms that the 20nt core spacer exhibits zero propensity for self-hairpin formation, leaving the targeting domain fully exposed for rapid Cas9 ribonucleoprotein assembly and target locus cleavage.

Extending this exact same $37^\circ\ \mathrm{C}$ RNAfold thermodynamic benchmark to the full-length 96-nt sgRNA construct (20nt spacer fused with the 76-nt SpCas9 scaffold) further corroborated its physical viability. Obtaining an optimal MFE of $-18.90\ \mathrm{kcal/mol}$, an ensemble free energy of $-20.90\ \mathrm{kcal/mol}$ (MFE frequency: 3.90%; ensemble diversity: 33.22), and a centroid MFE of $-7.69\ \mathrm{kcal/mol}$ confirms that the scaffold region folds into its essential stem-loop architecture for stable Cas9 RNP complex formation without inducing inhibitory internal hairpins within the spacer core.

Although 37°C was utilized as the standard in vitro benchmark for SpCas9 RNP assembly, it is noteworthy that lower physiological canopy temperatures of temperate crops like wheat is approximately 25°C would further stabilize RNA secondary structures. Given that our 20nt core spacer exhibited an open, unhindered conformation (MFE = 0.00 kcal/mol) at the higher thermal threshold of 37°C, it possesses zero propensity for hairpin formation under cooler physiological conditions, while reinforcing the structural stability of the Cas9 scaffold.

Furthermore, to bridge the gap between standard in vitro assay conditions (37°C) and actual field environments, thermal sensitivity analysis at 22°C empirically validated the physical robustness of the primary candidate. Because the 20nt spacer maintains an open, unhindered conformation (MFE = 0.00 kcal/mol; thermodynamic ensemble free energy = -0.47 kcal/mol; ensemble frequency = 45.25%; ensemble diversity = 1.78) even at this cooler physiological threshold while lower temperatures structurally reinforce the Cas9 scaffold, the designed gRNA construct exhibits high thermal adaptability for in planta wheat applications.

5. Conclusion

In addressing the central research query, our study concludes that while plant-optimized tools such as CRISPR-P 2.0 and WheatCrispr provide superior context-aware candidates for wheat, no individual tool alone is sufficient. Instead, a multi-tool consensus framework yields the highest efficiency and specificity. Through this cross-platform validation strategy targeting TaDREB1, we identified GCACCGCCCCAGTTAAAACG as the top-performing primary candidate gRNA, supported by four independent prediction tools. Dual-level thermodynamic profiling via RNAfold at 37^∘ "C" further confirmed both the complete structural accessibility of the isolated 20nt core spacer ("MFE"=0.00" kcal/mol" ; ensemble free energy =-0.16" kcal/mol" ; MFE frequency =77.02%; ensemble diversity =0.84) and the proper stem-loop folding stability of the full-length 96-nt sgRNA construct ("MFE"=-18.90" kcal/mol" ; ensemble free energy =-20.90" kcal/mol" ; MFE frequency =3.90%; ensemble diversity =33.22; centroid "MFE"=-7.69" kcal/mol"). Additionally, supplementary sensitivity analysis at 22°C verified that the spacer preserves its open, hairpin-free conformation (MFE = 0.00 kcal/mol; ensemble free energy = -0.47 kcal/mol; ensemble frequency = 45.25%; ensemble diversity = 1.78) under physiological wheat growth temperatures.

Abbreviations

CRISPR
Clustered Regularly Interspaced Short Palindromic Repeats
Cas9
CRISPR-associated protein 9
gRNA
guide RNA
TaDREB1
Triticum aestivum Dehydration-Responsive Element-Binding Protein 1
MFE
Minimum Free Energy

Declarations

Ethics approval and consent to participate:

Not applicable. This study does not involve human participants, animals, or biological samples requiring ethical clearance.

Consent for publication:

Not applicable. This manuscript contains no individual person's data in any form.

Competing Interests:

The authors declare that they have no competing financial or non-financial interests directly or indirectly related to the work submitted for publication.

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Authors' contributions:

All authors made substantial contributions to the conceptualization, in silico methodology design, data analysis, and manuscript preparation. All authors critically reviewed and approved the final manuscript for publication.

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Availability of data and materials:

All relevant data, sequences, and in silico evaluation metrics generated or analyzed during this study are fully included within this article and its supplementary materials.

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7. **References**

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Figures

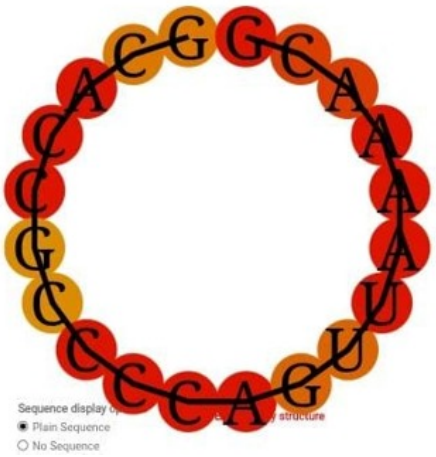


Figure 1

Predicted RNA secondary structure of the top consensus gRNA (GCACCGCCCCAGUUAAAACG) showing an optimal MFE of with a fully accessible, unpaired conformation (.....).

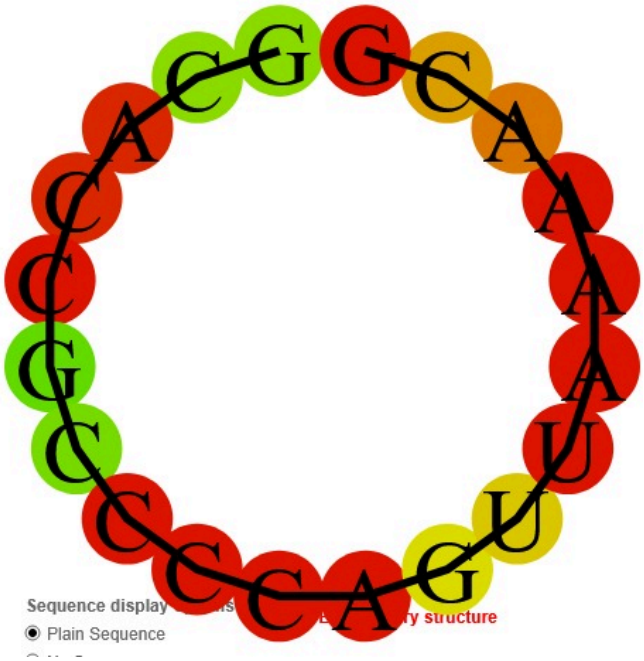


Figure 2

Predicted RNA secondary structure of the 20nt core spacer (GCACCGCCCCAGUUAAAACG) evaluated at the physiological wheat canopy temperature (22°C) via RNAfold (v2.6.3), demonstrating a completely open, unpaired single-stranded conformation with an optimal MFE of 0.00 kcal/mol

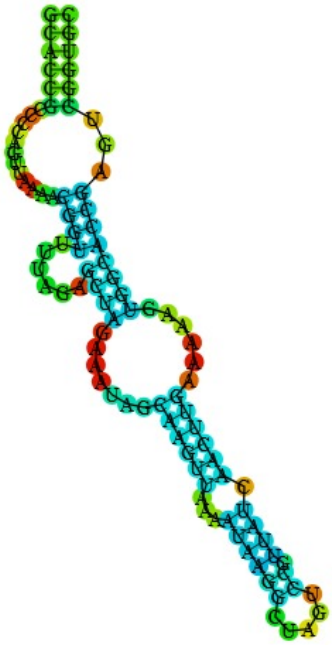


Figure 3

Optimal Minimum Free Energy (MFE) secondary structure of the full-length 96-nt sgRNA construct (GCACCGCCCCAGUUAAAACG spacer fused with the 76-nt SpCas9 scaffold) predicted by RNAfold (v2.6.3) at 37°C, demonstrating an optimal MFE of -18.90 kcal/mol (dot-bracket: ((((((.....((((.....((((.....(((((((...(((((((.....)).)))))..)))))).....)))))))...)))))))) and canonical scaffold stem-loop formation

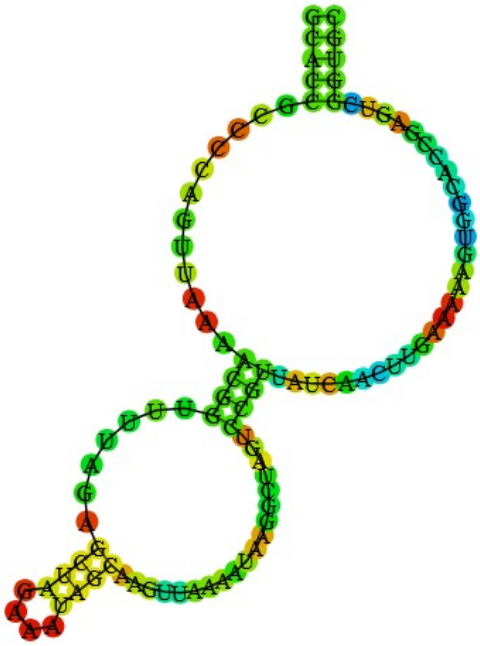


Figure 4

Centroid secondary structure of the full-length 96-nt sgRNA construct predicted by RNAfold (v2.6.3) at 37°C, yielding a centroid MFE of -7.69 kcal/mol (dot-bracket: (((((.....((((.....((((....))))).....))))..........))))))) and illustrating base-pair probability distribution alongside an unencumbered spacer core.