Plant Biotechnology Journal

WILEY

REVIEW ARTICLE OPEN ACCESS

Single-Cell CRISPR: An Efficient Strategy for Decoding Plant *Cis*-Regulatory Complexity

Yuanhang Zhang[1] | Xuanxuan Luo[1] | Hongbin Li[2] | Shuangxia Jin[1] | Xianlong Zhang[1] | Maojun Wang[1,2]

[1]National Key Laboratory of Crop Genetic Improvement, Hubei Hongshan Laboratory, Huazhong Agricultural University, Wuhan, China | [2]College of Life Science, Shihezi University, Shihezi, China

Correspondence: Maojun Wang (mjwang@mail.hzau.edu.cn)

Received: 29 December 2025 | **Revised:** 3 March 2026 | **Accepted:** 11 March 2026

Keywords: *cis*-regulatory element | genetic screen | phenotype selection | regulatory network | single-cell CRISPR

ABSTRACT

The generation of complex traits involves the coordinated interplay of multiple gene networks. Elucidating the function of transcriptional *cis*-regulatory elements (CREs) in regulating gene expression is crucial for understanding complex regulatory pathways and improving our ability to modify macro-phenotypes. While traditional bulk sequencing approaches rely on tissue or cell population aggregates, single-cell transcriptomics provides a more precise perspective by capturing cell-type-specific information. The integration of single-cell technology with genome-wide genetic screening, particularly through the single-cell CRISPR (scCRISPR) system, enables the identification of critical regulatory elements and provides novel insights into gene-expression control mechanisms. Here, we summarise recent advances in diverse strategies for functional genome analysis using the scCRISPR system, with an emphasis on its potential to revolutionise single-cell genetic screening of CREs. We also explore the challenges and opportunities for applying these approaches in plant research.

1 | Decoding Plant *Cis*-Regulatory Elements: Traditional Challenges and Single-Cell Opportunities

The emergence of diverse plant cell states and types stems from an intricate regulatory gene network governing cellular differentiation, developmental programming, and environmental responsiveness. Gene expression patterns are subject to differential regulations, exhibiting high cell-type specificity and spatial–temporal dynamics to align with the plant's growth cycle and environmental conditions. This transcriptional regulation is mediated by *cis*-regulatory elements (CREs), the specific genomic non-coding DNA sequences. CREs are broadly classified as promoters, enhancers, silencers, and insulators, alongside various incompletely characterised elements such as tethering elements (Batut et al. 2022).

1.1 | Diversity and Complexity of Plant CREs

CREs play indispensable roles in diverse physiological processes, including growth and development (Xin et al. 2024), morphogenesis (Liu et al. 2024), metabolic homeostasis (Jung et al. 2024) and stress response (Azodi et al. 2020). CREs govern the spatial, temporal, and amplitude-specific expression patterns of target genes by integrating endogenous signalling molecules (e.g., phytohormone gradients; Batut et al. 2022) and exogenous stimuli (e.g., photoperiodic signals; Lanctot et al. 2025; Wang et al. 2024), micronutrient availability (Guo et al. 2024), and temperature fluctuations (Xu et al. 2022) to assemble transcriptional regulatory complexes (Rodríguez-Leal et al. 2017). Some structural and functional characterizations of them have been achieved. Numerous studies have documented regulatory mechanisms governing plant

growth and development, encompassing pivotal processes such as root formation (Franssen et al. 2017), floral initiation (Alvarado et al. 2011), and fruit maturation (Song et al. 2022; Díaz-Valenzuela et al. 2020).

Research on plant genomes has long pursued a comprehensive understanding of genetic regulation (Sun et al. 2022; Xie et al. 2024), with persistent endeavours to annotate CREs within these systems. However, genomic sequences alone are insufficient to characterise CREs activities across plant physiological contexts, as their regulatory functions also manifest through dynamic local chromatin modifications. Specific promoters and enhancers mediate precise spatiotemporal regulation of downstream genes through the formation of transcription complexes via transcription factor (TF) binding. Eukaryotic CREs typically exhibit nucleosome depletion (Marinov and Shipony 2021), a biophysical property leveraged by recent chromatin accessibility profiling techniques to achieve genome-wide functional annotation of regulatory elements. High-throughput chromatin accessibility assays such as Assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) (Buenrostro, Wu, Chang, and Greenleaf 2015) exploit the enzymatic preference of Tn5 transposase for cleaving protein-unprotected genomic regions (Luo et al. 2022). This enables genome-wide mapping of CREs and context-specific chromatin state profiling.

The functional state of CRE is resolved through profiling DNA-protein interactions, including histone modifications (Deb and Kundu 2015) (e.g., H3K4me3 and H3K27ac; Angeloni and Bogdanovic 2019), TF-binding, and chromatin remodeler occupancy. In zebrafish, the H3K27ac-region in the enhancer provides binding sites for stage-specific transcription factors (Bogdanovic et al. 2012). Techniques such as chromatin immunoprecipitation sequencing (ChIP-seq) (Ernst et al. 2011; Park 2009), cleavage and tagging under target (CUT&Tag) (Kaya-Okur et al. 2019), and cleavage and release under target (CUT&RUN) using nuclease (Skene and Henikoff 2017) allow to probe histone modifications on DNA and to differentiate between the inducing or deterring activity of CREs (Wang et al. 2021).

The distinctive features of plant CREs, including their spatially dispersed genomic arrangement and chromatin-mediated responsiveness to environmental signals, have shifted the focus of relevant research from static mapping toward the reconstruction of dynamic regulatory networks. This transition aims to elucidate CRE-regulatory dynamics across specific cell types and defined environmental contexts.

1.2 | Functional Analysis Solution for CREs

Research on plant genomes and CRE has been progressively advancing. Early studies were conducted at the level of tissue, primarily employing mutagenesis protocols to assess the effects of non-coding chromosomal region mutations on reporter gene expression or phenotypic consequences, such as the characterisation of the *cis*-regulatory region governing expression of the floral regulatory gene *FT* in the phloem of *Arabidopsis* leaves (Adrian et al. 2010), and the functional characterisation of upstream silencers regulating the grain yield-associated gene *FZP* in rice (Bai et al. 2017). The reporter gene system described above provides an intuitive functional readout, enabling direct inference of regulatory activity from phenotypic data. However, its utility is constrained by several limitations, including the absence of native chromatin context, low throughput, and a frequent divergence from endogenous regulatory states. These factors collectively limit their broader applicability in functional genomics.

Population-level genetic association studies leveraging omics technologies such as ATAC-seq substantially enhance the experimental scale per study round. Nevertheless, they generally lack cellular resolution and yield correlative rather than causal evidence. Traditional genetic breeding strategies employ tissue culture-based regeneration to generate mutant lines, as exemplified by soybean mutants exhibiting enhanced symbiotic nitrogen-fixing nodule formation (Bai et al. 2020), and the suppression of stomatal regulatory genes in rice (Yin et al. 2017). However, these techniques remain constrained by notable limitations, including protracted in vitro culture periods, technically demanding protocols, and low-throughput phenotypic screening. Employing cell populations directly (e.g., protoplast-based systems or tissue culture platforms) diminishes procedural timelines while marginally enhancing operational efficiency in plant biotechnology workflows. For example, targeting the RY element (CATGCATG) upstream of the *FAD2* gene in peanut increases oleic acid concentration in seed (Neelakandan et al. 2022). Though CRISPR-based genetic screening resolves causal relationships in functional studies, its current lack of population-level implementation thus hampers a systems-level understanding of how CREs operate across distinct cell types and tissues.

1.3 | Single-Cell Opportunities of CRE-Regulatory Function Recognition

A central challenge in exploiting the functional potential of CREs lies in precisely linking sequence variants or perturbations to their molecular and cellular phenotypic outcomes, such as transcriptional shifts, surface protein expression, and cell-state transitions, within specific cell types. Single-cell strategies offer a promising avenue to address this gap. Generated through bulk tissue analysis, this histological dataset provides macroscopic phenotypic; conversely, single-cell resolution is more indispensable for investigating natural selection, evolutionary trajectories, and rare cellular phenotypes within specific genetic lines, given the pivotal roles of cell type-specific genes and regulatory elements in cellular differentiation processes. For example, single-cell transcriptomic profiling of *Arabidopsis* seedlings elucidated cell-type-specific regulatory responses to light, revealing a molecular model of cell development and differentiation during seedling de-etiolation (Han et al. 2023).

High-throughput single-cell epigenomics platform employing microfluidics and combinatorial indexing enables genome-wide chromatin accessibility profiling at single-cell resolution under both steady-state and spatially defined microenvironmental conditions. Deconvolution of epigenetic heterogeneity across cell populations permits precise delineation of cell-type and state-restricted accessible chromatin domains, enabling comprehensive reconstruction of *cis*-regulatory networks that

orchestrate gene expression (Movahedi et al. 2011). For instance, scATAC-seq analysis of rice root tip cells across developmental timepoints revealed spatiotemporal chromatin dynamics during cellular differentiation, achieved through nuclear chromatin profiling (Feng et al. 2022).

Single-cell epigenomic approaches offer distinct technological advantages by resolving phenotypic heterogeneity, delineating cell-type-specific regulatory elements, and ultimately elucidating transcriptional regulatory networks (Jaitin et al. 2014). Despite these advantages, platforms such as scATAC-seq remain observational in nature, thereby impeding mechanistic validation of CRE-mediated gene regulation. Perturbation assays targeting CREs can elucidate causal relationships between regulatory element function and transcriptional changes. Though comparatively understudied, CREs constitute modular chromatin units functionally analogous to genes. This equivalence establishes a conceptual basis for integrating CRE-analysis into functional genomics research frameworks. The CRISPR/Cas screening system has therefore been innovatively leveraged for genome-scale functional characterisation of CREs (Gilbert et al. 2013). The CRISPR/Cas system, which utilises guide RNAs (gRNAs) to direct Cas proteins to target sequences, causes cleavage of DNA strands. Relying on the cell's DNA reparation mechanism (Puchta et al. 1996; Salomon and Puchta 1998), it leads to mutations in specific segments (Wolter and Puchta 2018).

However, to realise scCRISPR screening in plants, several core technical obstacles must be surmounted: (1) A high-throughput perturbation strategy: This necessitates CRISPR libraries with the ability to simultaneously perturb a larger set of CREs; (2) Precise linkage between perturbations and phenotypes: It is essential that the transcriptomic readout of each cell can be accurately traced back to the specific gRNA perturbation it received; (3) Single-cell-level 'one-to-one' delivery: An optimal delivery system must guarantee that each cell receives only a single unique gRNA vector, thereby avoiding interpretive ambiguity arising from multiple perturbations (Shalem et al. 2015; Hsu et al. 2014; Chen et al. 2015). Plasmid-based delivery systems utilising protoplasts may present certain limitations; nevertheless, virus-like vector systems offer a promising approach to overcome this challenge.

2 | CRISPR-Mediated Genetic Screening of Single Cells

CREs modulate transcription of genes critical for plant physiological integrity. Notwithstanding considerable advancements such as screening of plant physiological hormone-specific CREs (Wu et al. 2021) and mapping of upstream effective elements for senescence genes (Vatov et al. 2021), key regulatory paradigms require further elucidation. However, the nonlinear relationship between CREs, transcriptional regulation, and phenotypic outcomes renders current *cis*-regulatory models inherently incomplete. Substantial obstacles, including resolution and identity limitations in perturbation assays and cellular heterogeneity, continue to restrict systemic analysis. These limitations stem from fundamental characteristics of CREs: as non-coding genomic sequences, they lack direct gene products. Their functional output instead depends on dynamic transcription factor binding and the propagation of epigenetic modifications affected by hereditary factors and environmental factors. Conventional molecular biology approaches, such as mRNA quantification with qPCR or protein expression analysis with Western blot, fundamentally assess gene expression outputs rather than regulatory mechanisms. Consequently, these methods cannot directly resolve CRE operational dynamics, necessitating indirect characterisation through proxy measurements of regulatory activity. Cellular heterogeneity presents another fundamental constraint: CRE functionality is intrinsically linked to cell lineage identity, type-specific programming, and dynamic state transitions within native biological contexts (Chiou et al. 2021).

As established, CRISPR/Cas-based genome-wide screening has emerged as a core strategy for unbiased functional characterisation, identifying key genes and regulatory elements underlying specific phenotypes through forward genetics (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). However, traditional screens predominantly measure coarse phenotypic parameters (e.g., cell survival, osmotic stress response, or individual molecular markers), limiting their ability to resolve cascading molecular network effects or cell type-specific regulatory mechanisms arising from genetic perturbations (Shalem et al. 2015; Hsu et al. 2014; Chen et al. 2015). Therefore, we introduce an integrated strategy coupling CRISPR editing with single-cell genomics to simultaneously capture genetic perturbations and multimodal molecular profiles at single-cell resolution (Ma et al. 2020).

Single-cell CRISPR (scCRISPR) screening leverages the cell membrane as a natural compartmentalization barrier, enabling individual cells to serve as discrete units for differential genetic perturbation, isolation and analysis. The experimental workflow comprises three integrated modules: (1) Design and construction of gRNAs (Figure 1A); (2) Vectors delivery and cell sorting (Figure 1B); (3) Single-cell multi-omics sequencing and computational analysis (Figure 1C,D). This platform realises combinatorial barcoding of multiplexed genetic perturbations through collecting information of gRNAs targeting diverse genomic loci, coupled with single-cell identity and phenotype. By correlating gRNA barcodes (perturbation identities) with multimodal molecular readouts (e.g., transcriptomic, Dixit et al. 2016; Frangieh et al. 2021; epigenomic, Rubin et al. 2019; proteomic states, Frangieh et al. 2021; Chen, Kibayashi, et al. 2025). scCRISPR directly resolves the interplay between genetic variation and phenotypic plasticity at single-cell resolution. This approach systematically integrates cellular heterogeneity with perturbation outcomes, establishing causal genotype-to-phenotype relationships within complex biological systems, and has already assisted in resolving some practical issues (Figure 1E).

scCRISPR auxiliary analysis diverse biological processes in animal models with high-resolution, including immune response (Chen et al. 2021), developmental programming (Umhoefer et al. 2025), and genetic variation (Rubin et al. 2019). The inherent inaccessibility and molecular sparsity of single cells introduce inevitable complexities in scCRISPR experimental design, particularly across three critical dimensions: (1) CRISPR perturbation strategy targeting CREs; (2) Delivery and disturbance

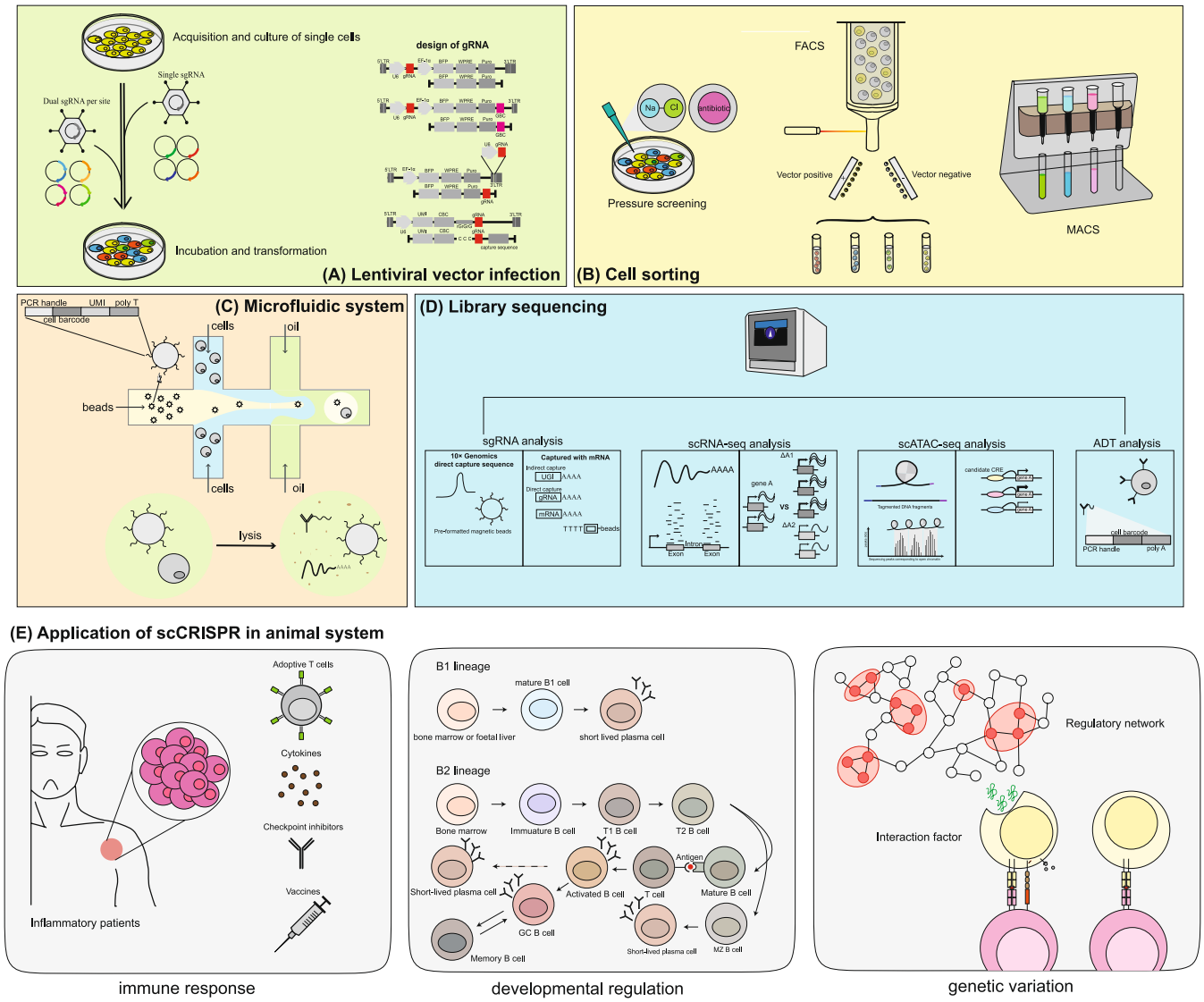

FIGURE 1 | scCRISPR process overview and practical applications. (A) The process of viral infection. Cells in culture are infiltrated using vectors such as lentiviruses (Chen, Kibayashi, et al. 2025; Shifrut et al. 2018) to deliver gRNA components into the cells. Vectors can be single-target or multi-target, and different types of vectors have different infiltration efficiencies (Choo et al. 2021). Three different gRNA design options are used in order to subsequently read the gRNA counterpart information from the sequencing data. (B) Cell sorting. Sorting of already infiltrated cells to screen out infiltration failures to reduce experimental error. Common sorting protocols include the following: Pressure screening (e.g., osmotic pressure or antibiotics, Shifrut et al. 2018), Fluorescence-Activated Cell Sorting (Chen, Kibayashi, et al. 2025) and Magnetic-Activated Cell Sorting. (C) Microfluidic system. Microfluidic systems use magnetic beads and cells that enter from different channels and are encapsulated by oil to form oil droplets, with individual cells and individual magnetic beads encapsulated in a single oil droplet (Dixit et al. 2016). (D) Libraries sequencing. Four different sequencing libraries are available, gRNA libraries, scRNA libraries, scATAC libraries and antibody-specific libraries. (E) Application of scCRISPR in animal systems. Aids in resolving immune responses and refining immune agents (Chen, Kibayashi, et al. 2025); mines key nodes in cell development and differentiation (Loo et al. 2020); maps gene regulatory networks and interplay factors.

in plant systems; (3) Co-capture of gRNA and the cellular transcriptome; (4) Noise assessment of single-cell data. Here we provide a brief introduction, including the reasons why these problems arise and the solutions available.

2.1 | CRISPR Perturbation Strategy Targeting CREs

In knockout screening experiments, editing tools play a critical role in shaping the interpretation of knockout outcomes. The conventional CRISPR-Cas9 system generates double-strand breaks in DNA by directing SpCas9, resulting primarily from small insertions or deletions (indels) introduced via the error-prone non-homologous end-joining (NHEJ) repair pathway. This approach remains the benchmark for gene knockout studies. By introducing indels into the upstream non-coding region of *CLV3*, expression levels were fine-tuned, enabling quantitative modulation of fruit locule number (Rodríguez-Leal et al. 2017). However, for most CREs, random, small-scale sequence mutations are often insufficient to completely disrupt their function due to the presence of

multiple, dispersed transcription factor binding sites, thereby leading to confounding results.

Recent studies have highlighted the CRISPR-Cas12a promoter editing system for crop improvement, a system that generates mismatched double-strand breaks (DSBs) thereby resulting in larger deletions (Zhou et al. 2023; Tang et al. 2016). Cas12a creates double-stranded DNA breaks resulting in sticky ends. By designing a pair of gRNAs targeting both sides of a CRE, Cas12a can efficiently mediate the deletion of large DNA fragments, leading to the complete removal of the entire CRE module. For instance, in rice, a continuum of grain size traits was achieved through promoter editing of *OsGBSS1* (Zhou et al. 2023). Recently, dCas9 has achieved widespread application by targeting promoter or enhancer regions to reversibly, controllably, and without altering DNA sequences, repress or enhance transcription initiation and elongation.

Notably, early CRISPR interference/activation (CRISPRi/a) systems in plants exhibited suboptimal efficacy when directly adapted from animal models. CRISPR activation (CRISPRa) and interference (CRISPRi) systems enable the regulation of endogenous gene expression by fusing catalytically inactive Cas proteins (dCas9) with transcriptional activation or repression domains. However, directly applying CRISPRa/i to analyse CRE function remains challenging, as the efficacy of activation or inhibition is critically dependent on the precise positioning of gRNAs within the CRE. For example, when targeting promoter regions, it is essential to avoid occupying repressive elements to ensure successful activation (Li et al. 2017). Additionally, the design of highly efficient CRISPRi systems for plant systems remains suboptimal (Pan et al. 2021).

Therefore, the current and more clearly defined role of CRISPRa/i in CRE research is as an indirect perturbation tool, whereby targeting TFs disrupts upstream regulators within the transcriptional regulatory network and subsequently enables observation of changes in downstream CRE activity and target gene expression (Oikawa et al. 2015). This strategy places CREs within their native regulatory context, thereby allowing inference of regulatory relationships between specific TFs and their target CREs through the systematic activation or inhibition of different TFs (Piatek et al. 2015), ultimately enabling the construction of a transcriptional regulatory network. This indirect strategy of perturbing TFs to read out CRE activity circumvents the technical uncertainties associated with directly targeting CREs, offering a viable approach for deciphering the dynamic regulatory logic of CREs.

Furthermore, most naturally occurring CRE variants occur as single nucleotide polymorphisms (SNPs). For detailed functional dissection of key SNPs within CREs, base editors have been utilised in the context of knockout experiments. Base editors combine the targeting specificity of CRISPR systems with the catalytic activity of deaminases, enabling precise nucleotide conversion without inducing double-strand breaks in DNA (Lapinaite et al. 2020). By introducing single-base changes within transcription factor binding motifs, the individual contribution of each SNP to transcription factor recruitment and transcriptional output can be evaluated. In recent years, base editors have been successfully implemented in several crops (Li et al. 2024), for example, the recently developed Cas12a-based base editor has achieved highly efficient multiplex gene editing in rice (Cheng et al. 2023). Their potential for the functional analysis of CREs is increasingly being explored, thereby offering new avenues for establishing refined genotype–phenotype associations.

2.2 | Delivery and Disturbance in Plant Systems

However, the extended application of single-cell technology in plant systems is limited by unique structural constraints, particularly the cell wall, which is a flexible but highly elastic matrix composed of complex polysaccharides (Pu et al. 2025). The plant cell wall impedes direct gRNA vector delivery, necessitating enzymatic digestion to achieve cellular access. Here, we briefly outline experimental approaches that may prove effective.

The protoplast system serves as a core platform for transient expression in plants and single-cell omics research by modulating cell membrane permeability to facilitate the entry of exogenous carriers (Figure 2A). In scCRISPR screening, the protoplast-based approach offers distinct advantages: it enables the batch preparation of large quantities of protoplasts and is relatively simple to perform. However, enzymatic digestion substantially compromises cellular viability. Treated plant cells exhibit rapid metabolic decline within 24 h, which precludes extended transformation protocols (Yan et al. 2023). Further consideration is that when mixing gRNA libraries with protoplast suspensions, individual cells may take up multiple distinct gRNA plasmids, resulting in multiple perturbations within a single cell. Under these circumstances, the observed transcriptomic changes cannot be unambiguously assigned to the perturbation of specific CREs, thereby confounding signal interpretation and complicating downstream data analysis. Although optimising plasmid concentration and transfection conditions can partially reduce the probability of multiple transfections, achieving true 'one-perturbation-per-cell' remains challenging when library complexity is high.

An alternative approach involves the use of viral vector delivery systems. Plant viruses, functioning as natural nucleic acid carriers, are capable of infecting plant cells with high efficiency and subsequently replicating within them (Shen et al. 2024). Among these, *Agrobacterium tumefaciens*, a gram-negative bacterium, is extensively utilised in plant genetic engineering for the delivery of expression plasmids in plant tissue culture experiments. For instance, in maize, the transformation and genome editing efficiency of the inbred line B104 was enhanced by optimising the *Agrobacterium*-mediated transformation method using a ternary vector system (Kang et al. 2022). Notably, recent studies have highlighted avenues for high-throughput gene function screening using viral vectors (Zhu et al. 2025), and viral delivery systems have been refined for plants, thus facilitating efficient genome editing across multiple species (Lowensohn et al. 2026). Collectively, these advances suggest that viral vector delivery systems could provide the technical foundation for scCRISPR screens.

Comparing the two delivery strategies, the protoplast system offers higher throughput and shorter experimental cycles but

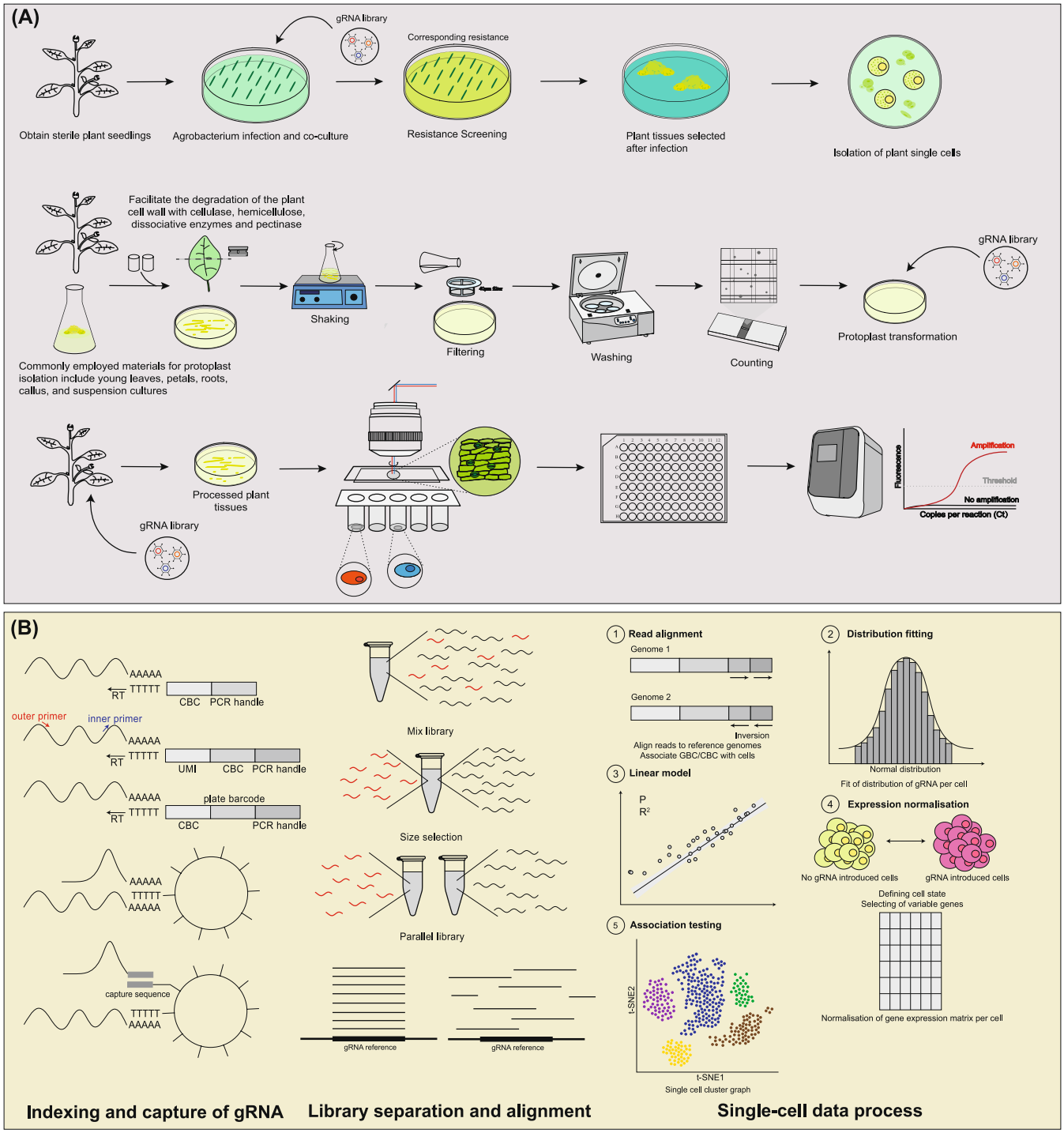

FIGURE 2 | Possible approaches of scCRISPR in plants. (A) Isolation and separation of single plant cells following infection. Several single-cell infection and isolation protocols are commonly used in plant cells. *Agrobacterium* dissociates plant tissue after infection; extraction and isolation of protoplasts (Chen et al. 2023); laser microdissection (Woronik et al. 2022); laser microdissection and pressure catapulting and laser capture microdissection (Hildebrandt et al. 2023). (B) Capture and analysis of gRNA and single-cell data. gRNA capture includes: indexing and capture; library separation and alignment. Brief data analysis steps: read alignment, distribution fitting, linear model, expression normalisation, association testing.

requires careful evaluation and correction of noise introduced by multiple perturbations. The viral vector system, in contrast, holds a theoretical advantage in achieving 'one-to-one' perturbations and may be more suitable for studies involving highly complex libraries. Other alternatives, such as plant suspension cell lines, callus tissue, or embryonic cells, require selection based on the specific characteristics of different plant species (Figure 2A). Suspension cell lines, established by agitating callus tissue in liquid medium, exhibit rapid proliferation and a high degree of homogeneity (Ge et al. 2023). Embryogenic cells, derived from embryogenic callus or suspension cultures, possess high division and regeneration potential, making them an optimal material for obtaining totipotent protoplasts in grass crops.

Other plant single-cell acquisition methods, including laser microdissection (LMD), laser microdissection and pressure catapulting (LMPC), laser capture microdissection (LCM), and tissue culture-derived suspension cell lines, exhibit inherent throughput limitations such as slow cell acquisition (Figure 2A). Methodological efficiency for single-cell acquisition exhibits substantial interspecies variation across plant systems, necessitating protocol optimisation and workflow adaptation to reduce experimental duration while ensuring enhanced viability-preserving cell recovery.

2.3 | Co-Capture of gRNA and the Cellular Transcriptome

Accurate cell identity annotation is foundational to single-cell experimental design, which is the basis of biologically meaningful interpretation of heterogeneity and perturbation responses. For the design of scCRISPR, identification of gRNA determines the specific perturbation effect introduced. Consequently, establishing a precise and comprehensive mapping between cells and their associated gRNAs is essential. However, the absence of polyA-tail in gRNA prevents their synchronous capture with cellular transcripts using standard scRNA-seq protocols. This limitation complicates the precise assignment of specific gRNAs (and relevant perturbations) to different cells.

gRNA-specific capture technologies offer novel strategies to address this limitation. Here describes three predominant gRNA design strategies for gRNA-phenotype co-localisation (Figure 1A): (1) Dual-promoter co-expression: This system employs separate U6 and EF1α promoters driving independent expression cassettes: one for the gRNA transcript and another for reporter genes fused to a gRNA barcode (GBC). This architecture enables detection of the gRNA identity within single cell. GBCs exhibit strict one-to-one correspondence with their cognate gRNAs (Dixit et al. 2016). Co-captured with the single-cell transcriptome, they enable perturbation traceability at single-cell resolution without requiring additional sequencing. (2) LTR recombinant engineering: This approach integrates a U6-gRNA expression cassette into the functionally inert region of the viral 3′ long terminal repeat (3′LTR) (Pierce et al. 2021; Liang et al. 2023; Liscovitch-Brauer et al. 2021). This enables co-encapsulation of polyadenylated gRNA transcripts within viral particles, permitting synchronous capture alongside the cellular transcriptome in standard scRNA-seq workflows. (3) Direct gRNA capture: This strategy employs engineered gRNA scaffolds containing custom affinity tags or structural motifs within their backbone architecture (Replogle et al. 2020). By obviating the need for engineered barcode sequence, this direct capture approach mitigates risks of cellular toxicity, off-target effects, or transcriptional interference inherent to alternative systems. Furthermore, recently developed single-cell multi-omics platforms (e.g., CITE-seq, Frangieh et al. 2021; TEA-seq, Swanson et al. 2021) have demonstrated utility in concurrent genotype–phenotype profiling through the introduction of antibody-labelled gRNA capture probes (Frangieh et al. 2021).

Despite their utility, the inherent divergence between animal and plant systems often results in suboptimal efficiency when such tools are directly applied in plants. Consequently, adapting and optimising key components of these methodologies for plant-specific contexts represents a critical pathway toward their effective implementation. First is intracellular PCR labelling (Figure 2B): delivering a plasmid library carrying identical gRNA with distinct random barcodes into cells, then adding barcode to the gRNA via intracellular PCR before sequencing. This may be one of the most feasible approaches for implementation in protoplasts (Satpathy et al. 2019). Second, refer to the capture protocols used in animal (Radtke et al. 2022), concatenating gRNA with a barcode sequence transcribable by Pol II, enabling direct capture using single-cell library preparation reagents. Finally, modifying specific plant viral vectors such as tobacco mosaic virus to impart functionality comparable to animal systems like lentivirus holds immense potential. Such engineered plant viral vectors could potentially overcome cell wall barriers to achieve efficient delivery and diffusion.

2.4 | Noise Assessment of Single-Cell Data

Screening analyses for scCRISPR can be unified as a two-stage framework comprising data standardisation and association inference (Wang 2021) (Figure 2B). Data standardisation primarily processes raw sequencing data to mitigate or eliminate technical artefacts originating from experimental or sequencing procedures, thereby preserving true biological variation and ensuring subsequent analytical accuracy; association inference is used to analyse the relationship between different variables, which is highly superior when dealing with large-scale experimental data. However, critical analytical limitations continue to impede robust interpretation of single-cell genomics data. For instance, in comparative assessments of phenotypically distinct cell populations post-clustering, CREs may exhibit differential activity across subpopulations. Such heterogeneous activity patterns can drive divergent cellular responses to identical regulatory stimuli, thereby introducing additional biological variation (Buenrostro, Wu, Litzenburger, et al. 2015; Zamanighomi et al. 2019).

The intrinsic biological features of single-cell experimental designs (e.g., replicate observations absence and cellular heterogeneity) combined with technical platform limitations (e.g., capture efficiency and library preparation protocols) generate diverse and complex noise sources (Datlinger et al. 2021). This multidimensional constellation of confounding factors necessitates implementing multiscale quality control frameworks to achieve high-fidelity signal extraction. Development of noise assessment frameworks critically depends on computational biology advances. The MIMOSCA framework, introduced in Perturb-seq, enables technical noise and biological signal disentanglement through latent variable modelling (Dixit et al. 2016). The MAGeCK series developed for scCRISPR incorporates three algorithmic sets to successively optimise gRNA significance definition, gene prioritisation, and intracellular gene perturbation effect identification (Yang et al. 2020; Li et al. 2014, 2015). These algorithms further enable the unbiased reconstruction of hierarchical regulatory sequence–functional gene–phenotypic networks. For challenges including unquantified raw cellular expression profiles and heightened sample heterogeneity, iterative Expectation–Maximisation (EM) algorithms may likewise be employed to estimate the true cluster proportions

of perturbed cells alongside their cellular responses (Meng et al. 2024). Interactive computational platforms housing extensive datasets are additionally accessible for reference (Yang et al. 2025).

2.5 | scCRISPR Deciphers Plant *Cis*-Regulatory Networks

The advancement of single-cell histology, coupled with the CRISPR/Cas system, presents novel possibilities for refining the causal regulatory network of CREs. Single-cell transcriptomics, the first modality integrated into CRISPR/Cas platforms, has played a pivotal role in elucidating key genes and regulatory pathways in animal systems (Figure 1E). Recent advances enable the simultaneous sequencing of gRNAs, transcriptomes, and epigenomes, providing comprehensive intracellular genomic data (Ma et al. 2020). This significantly enhances the functional characterisation of CREs, particularly in human disease research. It enables the characterisation of immune responses, the development of immunotherapeutics, and provides critical insights into the regulatory mechanisms governing immune cell differentiation (Figure 1E), highlighting its significant applied value.

scCRISPR technology was initially pioneered in animal model systems and has undergone substantial methodological innovation since its inception. Nevertheless, for this technology still in its nascent stage within plant systems, a significant translational gap persists between methodological development and the derivation of substantively meaningful biological insights. The dynamic regulatory network of CREs is an integral part of the gene regulatory pathway, precisely regulating plant growth, development, environmental response and adaptive evolution. CREs are involved in plant sensing and responding to environmental changes, such as light, water and temperature, to enhance stress tolerance. In addition, they play an important role in the evolutionary process, revealing the evolutionary history and potential adaptive mechanisms. The capacity to profile the epigenome at single-cell resolution offers a unique perspective in plant science for deciphering cell fate determination in complex tissues. It facilitates temporal tracking of chromatin accessibility reprogramming and its coupling to transcriptional regulatory networks in defined cellular contexts. As an illustration, single-cell resolution profiling of the maize regulatory architecture reveals both evolutionarily conserved and lineage-specific chromatin remodelling dynamics at CREs (Marand et al. 2021). Further integration with multimodal omics analyses (e.g., scATAC-seq + RNA-seq co-profiling) enables comprehensive delineation of the spatiotemporal dynamics governing the chromatin accessibility–transcription factor binding–gene expression regulatory cascade. This provides mechanistic insights into the molecular basis of plant developmental plasticity and environmental adaptation. The following outlines several prospective avenues through which this technology could contribute to elucidating plant *cis*-regulatory networks.

Revealing cell type-specific functions of CRE. A given CRE can mediate distinct functions across cell types by regulating different target genes, depending on cell-specific chromatin contexts and transcription factor combinations. For example, by mapping high-resolution root single-cell atlases in Arabidopsis, we directly identified numerous cell type-specific enhancer candidate sequences (Shulse et al. 2019). Perform scCRISPR screening within specific cell populations to project transcriptional responses onto cell clusters, observing whether CRE perturbation effects are specific to a particular cell type.

Analysing CRE hierarchical regulation and synergistic effects. The same CRE can execute distinct functions across cell types by regulating different target genes, depending on variations in chromatin accessibility and TF occupancy. Previous studies have demonstrated that CRISPRi can be used to screen for multiple enhancers (Xie et al. 2017), which found that most enhancers exert independent and additive effects on gene expression, but specific combinations exhibit synergistic effects. scCRISPR enables the simultaneous delivery of multiple gRNAs targeting distinct candidate CREs within the same locus. By comparing transcriptional outcomes following single versus combinatorial perturbations, the functional relationships among these elements, such as synergy, additivity, or redundancy, can be systematically determined.

Constructing cell state-specific gene regulatory networks. Unlike traditional correlation-inferred gene regulatory networks, scCRISPR establishes direct causal links through perturbation–response pairing. This enables the reconstruction of higher-resolution regulatory networks with inherent hierarchical information. For example, the MAESTRO algorithm (Jin et al. 2020) integrates scCRISPR screening data and scRNA-seq data to construct cell type-specific regulatory networks and identify key regulatory factors.

3 | Application of scCRISPR in Deciphering Plant *Cis*-Regulatory Network

scCRISPR integrates CRISPR gene editing strategies and single-cell sequencing technologies, offering unique advantages for biological research and translational applications through its unique high-resolution profiling and specific targeting capabilities, which can reveal cell type-specific functions obscured by population averaging effects, but may entail higher costs and more complex data integration. Over the past decade, scCRISPR has evolved into a key method for large-scale perturbation screening to study genomic function. To date, more than six thousand relevant studies have been published, which have been conducted mainly in animal systems.

Technological breakthroughs in plants will directly impact the resolution of core biological questions unique to plants. As sessile organisms, plants cannot move freely, which endows them with complex pathways for responding to environmental signals. scCRISPR facilitates the direct resolution of which CREs become activated and which target genes are reprogrammed in specific cell types under defined environmental or developmental conditions. This capability enables the precise mapping of cell type–specific regulatory networks in response to external signals. In meristematic tissues, which retain developmental plasticity throughout the life cycle, scCRISPR enables the identification of key CREs governing cell fate decisions at single-cell resolution. This approach provides a novel mechanistic

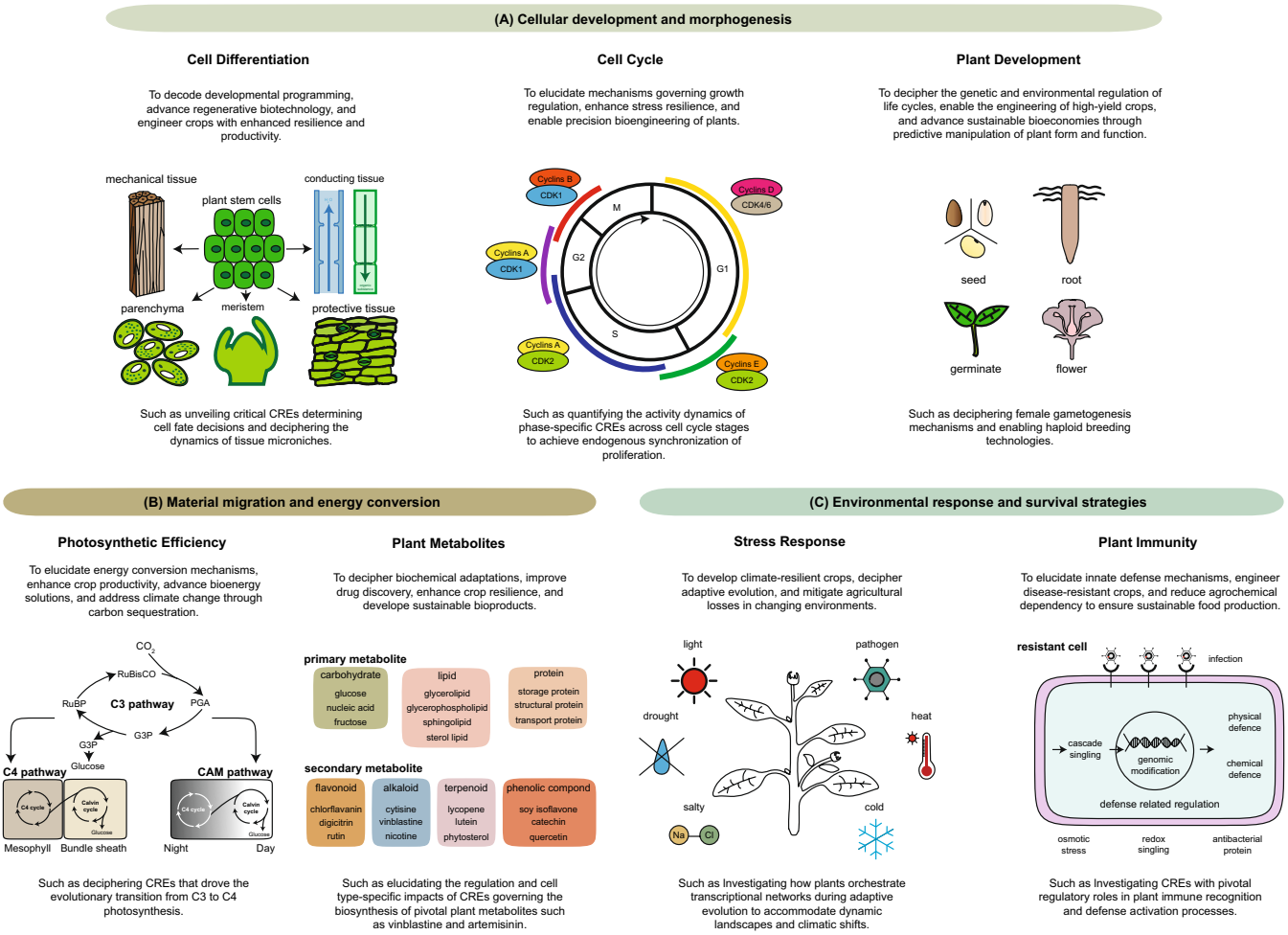

FIGURE 3 | Future applications of CRE regulation network. This figure synthesises the objectives and exemplary applications of scCRISPR for interrogating CRE-functions across three distinct plant research domains: (A) Cellular development and morphogenesis, (B) Material migration and energy conversion, and (C) Environmental response and survival strategies. Specifically including photosynthetic efficiency, plant metabolites, stress response, plant immunity, cell differentiation, cell cycle, and plant development.

perspective for dissecting plant developmental physiology. The precise targeting capacity of scCRISPR further enables specific disruption of homologous CREs derived from particular ancestral subgenomes in polyploid crops such as wheat and cotton. This approach permits the analysis of subgenome-preferential CRE activity and allele-interaction logic at single-cell resolution, yielding mechanistic insights that can inform precision editing strategies in polyploid crop improvement.

scCRISPR holds significant potential for plant research by enabling mechanistic dissection of cell cycle regulation and differentiation determinants through cell type-resolved interrogation of *cis*-regulatory landscapes across developmental trajectories. This platform empowers targeted genomic perturbations to optimise biosynthetic pathways, amplify agronomic traits, and fortify stress tolerance while enabling comprehensive functional genomics interrogation. We provide a concise overview of scCRISPR applications targeted, including plant growth and development regulation, photosynthetic efficiency, plant metabolism, biotic and abiotic stress response, and plant rhythms. Collectively, these approaches enable dissection of evolutionary divergences across species and mechanistic validation of phenotype-CRE functional relationships.

Plant developmental ontogeny and growth dynamics (Figure 3A). CREs undergo dynamic regulation throughout plant ontogeny, including vegetative growth, floral development, and fruit ripening, thereby orchestrating stage-specific expression of developmental genes at critical transitions. For instance, conserved noncoding sequence (CNS) motifs within tomato promoter regions modulate flowering regulation by altering transcriptional activity of *UFO*, a conserved floral regulator (Lanctot et al. 2025); similarly, targeted modification of the tomato *KLUH* promoter modulates fruit-size phenotypes through *cis*-regulatory engineering (Li et al. 2022). *Cis*-regulatory regions in plant genomes harbour valuable reservoirs of regulatory variation. Yet a single type of plant tissue inherently comprises phenotypically and functionally diverse cell populations. This cellular heterogeneity poses distinct challenges for elucidating the dynamic mechanisms governing complex physiological activities in plants. scCRISPR enables single-cell resolution perspectives, which unlock new possibilities for exhaustively analysing the spatiotemporal dynamics of plant physiology.

Photosynthetic performance enhancement (Figure 3B). Photosynthesis constitutes a fundamental biochemical process in plants and an integral component of Earth's terrestrial

carbon cycle. The RuBisCO-catalysed C3 pathway generates photorespiratory byproducts under elevated temperatures, reducing photosynthetic efficiency, and thus the decline in photosynthesis capacity due to global warming is one of the major challenges facing plants. Throughout the long evolutionary process, plants have developed divergent photosynthetic pathways (Tu et al. 2022). The C4 pathway is evolutionarily derived from C3 photosynthesis, with key enzymatic and genetic components originating from C3 ancestors (Matsuoka et al. 1994, 1993). As part of the plant genome, CREs potentially mediate this evolutionary transition (Mendieta et al. 2024; Kajala et al. 2012). For instance, the C4 photosynthetic enzyme genes in *E. baldwinii* deploy environmentally responsive and cell type-specific CREs to achieve adaptive integration of environmental sensing, developmental programming, and metabolic optimisation—exemplifying tripartite co-evolutionary dynamics (Chen, Jia, et al. 2025). In C4 plants, CO_2 storage and utilisation take place in separate cells, so cell type has an equally crucial effect on photosynthetic efficiency. scCRISPR helps identify CREs that have been renewed during evolution and determines the cellular-level compartments in which they mediate the spatiotemporal regulation of photosynthetic genes and enzyme activities, with important implications for existing C3-to-C4 engineering to achieve the crossing of key technological barriers.

Metabolic network engineering (Figure 3B). Plant metabolism constitutes a crucial bridge between genotype and phenotype. These metabolites not only underpin fundamental physiological processes but also serve as essential precursors for pharmaceutical and industrial applications (Venegas-Molina et al. 2021), exemplified by vinblastine, a potent cytokinesis inhibitor widely utilised in antineoplastic therapy (Caputi et al. 2018). *Cis*-regulatory motifs serve as binding sites for transcription factors, orchestrating the biosynthesis and spatiotemporal accumulation of specialised plant metabolites (Huffaker et al. 2011). For instance, transcriptional activation of isopentenylflavane and picric acid biosynthetic genes in hops requires combinatorial control by multiple *cis*-regulatory elements (Duraisamy et al. 2016). Biosynthetic pathways for plant metabolites are precisely regulated through both cell-type specificity and spatial–temporal dynamics. scCRISPR enables single-cell CRE perturbation profiling across environmental contexts, providing transformative insights into cell-type-specific metabolite biosynthesis patterns and advancing human utilisation through metabolic engineering.

Stress response (Figure 3C). The fixed nature of plants precludes escape from environmental stress, necessitating adaptive adjustments in morphology, physiology, biochemistry, and metabolism to adapt to changing conditions (Sharma et al. 2022). For example, under drought conditions, the phytohormone ABA coordinates stomatal closure to reduce transpiration (Brenner and Schmülling 2012). The binding of CREs to transcription factors is an important component of the plant stress pathway, which makes them an important target for the engineering of stress-tolerant plants. The interaction between CREs and transcription factors constitutes an integral component of plant stress signalling pathways, positioning CREs as prime targets for engineering stress-resilient plants. For instance, a 108 bp insertion within the promoter of wheat drought-responsive gene *NAC 071A* significantly altered its transcriptional output (Mao et al. 2022). Cellular stress responses include disruption and re-establishment of homeostasis. Precise regulation of cell-type-specific chromatin states is therefore essential to resolve signalling dynamics, spatiotemporal organisation, and turnover kinetics of stress-responsive CREs, thereby enabling rapid, high-fidelity adaptation to environmental perturbations.

Circadian rhythm. Environmental systems exhibit cyclical variations, exemplified by seasonal oscillations and circadian rhythms (Wang et al. 2020). The endogenous circadian oscillator orchestrates physiological processes in plants, including bud formation and floral initiation (Miwa et al. 2006). Genetic regulatory mechanisms mediate the entrainment of endogenous circadian rhythms to external *Zeitgebers*, enabling plants to achieve adaptive synchronisation with cyclical environmental changes. The circadian oscillator is regulated by interconnected feedback loops, with CREs constituting integral components. For example, the evening element (EE) motif is enriched in promoters of evening-phase circadian genes (Harmer and Kay 2005). This enables environmental regularity of physiological processes through changes in transcript abundance (Greenham et al. 2020). Notably, CREs coordinating co-expressed circadian genes exhibit high evolutionary conservation (Movahedi et al. 2011; Noda et al. 2025). Reprogramming CREs to modulate circadian gene networks enables precise tuning of core physiological pathways, thereby enhancing phenotypic plasticity for adaptation to diverse *edapho-climatic* conditions and mitigating region-specific cultivation failures.

4 | Future Challenges, Opportunities, and Plant-Specific Development Pathways of scCRISPR

The transition of plant scCRISPR screening from a conceptual framework to practical implementation confronts considerable hurdles. The foregoing comprehensive discussion of CRISPR tools, delivery systems, single-cell capture techniques, and the generation and analysis of data noise suggests that, despite significant progress in each of these areas, future breakthroughs will increasingly hinge upon the judicious selection of modules specifically tailored to the unique characteristics of plant systems. This approach is directed toward achieving core breakthroughs and pioneering novel integrated solutions.

The primary challenge at present is establishing efficient and reliable standardised processes, with the core focus on the coordinated optimisation of three key stages: delivery, capture, and parsing. (1) Enhancing conversion efficiency and authenticity: Existing plant protoplast systems exhibit significant issues with high cellular wounding stress and difficulties in preservation and regeneration, directly impacting the relevance of screening results. Developing milder cell wall enzymatic hydrolysis protocols, utilising cell nuclei instead of whole cells, or creating delivery systems capable of penetrating cell walls all hold promising potential. Meanwhile, the CRISPR system still requires refinement to provide stable and controllable gene regulation at the single-cell level. (2) Developing a plant cell identification solution: This is the key bottleneck for achieving large-scale, high-complexity screening. As mentioned earlier, systems that demonstrate excellent efficacy in animals prove extremely inefficient in plants, necessitating the exploration of

new approaches. (3) Preservation of cellular spatial positioning information: Conducting CRE research under conditions closer to the natural state remains one of the ultimate goals, which necessitates overcoming the limitations of the protoplast system. The combination of tissue dissociation with snRNA-seq may serve as an alternative strategy to achieve in situ perturbation and nuclear readout within specific tissues. The integration of scCRISPR with spatial transcriptomics may hold greater exploratory value, enabling precise localisation of CRE perturbation effects to specific cellular layers and spatial microenvironments.

Technological advances deepen scientific understanding. scCRISPR screening will propel plant functional genomics toward predictive design paradigms, catalysing the generation of large-scale, high-resolution datasets on genomic regulation. This will forge links between CREs, TFs, target gene outputs, and cellular phenotypes. Further integration with machine learning will enable models to predict CRE activity in specific cell types and environments, as well as their response patterns to external perturbations, based on DNA sequence features and chromatin states. In the future, tracking the dynamic regulatory changes of CREs during plant development and under various environmental stresses, establishing quantitative relationships between sequence variations and expression outputs, and reverse-engineering artificial CRE elements to achieve specific expression patterns may all become achievable.

In the future, tracking the dynamic regulatory changes of CREs during plant development and under various environmental stresses, establishing quantitative relationships between sequence variations and expression outputs, and reverse-engineering artificial CRE elements to achieve specific expression patterns may all become attainable. Ultimately, these capabilities will not only help elucidate the fundamental principles governing plant development and adaptation, such as the regulatory codes underlying core biological processes like cell fate determination, organogenesis, and environmental responses, but also provide transformative technological solutions to pressing global challenges, including food security and sustainable agriculture. scCRISPR will truly become the pivotal bridge connecting genotypes to phenotypes and linking fundamental research to breeding applications, propelling plant science into an era of dynamic, quantitative, and causal analysis at single-cell resolution.

Acknowledgements

This study was supported by the finance science and technology project of Xinjiang Uyghur Autonomous Region (2023A01) and the Tianchi Talent Introduction Plan of Xinjiang Uyghur Autonomous Region. This study was also supported by the National Key Research and Development Program of China (grant no. 2021YFF1000900).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

References

Adrian, J., S. Farrona, J. J. Reimer, M. C. Albani, G. Coupland, and F. Turck. 2010. "Cis-Regulatory Elements and Chromatin State Coordinately Control Temporal and Spatial Expression of FLOWERING LOCUS T in Arabidopsis." *Plant Cell* 22: 1425–1440.

Alvarado, V. Y., A. Tag, and T. L. Thomas. 2011. "A Cis Regulatory Element in the TAPNAC Promoter Directs Tapetal Gene Expression." *Plant Molecular Biology* 75: 129–139.

Angeloni, A., and O. Bogdanovic. 2019. "Enhancer DNA Methylation: Implications for Gene Regulation." *Essays in Biochemistry* 63: 707–715.

Azodi, C. B., J. P. Lloyd, and S. H. Shiu. 2020. "The Cis-Regulatory Codes of Response to Combined Heat and Drought Stress in *Arabidopsis thaliana*." *NAR Genomics and Bioinformatics* 2: lqaa049.

Bai, M., J. Yuan, H. Kuang, et al. 2020. "Generation of a Multiplex Mutagenesis Population via Pooled CRISPR-Cas9 in Soya Bean." *Plant Biotechnology Journal* 18: 721–731.

Bai, X., Y. Huang, Y. Hu, et al. 2017. "Duplication of an Upstream Silencer of FZP Increases Grain Yield in Rice." *Nature Plants* 3: 885–893.

Batut, P. J., X. Y. Bing, Z. Sisco, J. Raimundo, M. Levo, and M. S. Levine. 2022. "Genome Organization Controls Transcriptional Dynamics During Development." *Science* 375: 566–570.

Bogdanovic, O., A. Fernandez-Miñán, J. J. Tena, et al. 2012. "Dynamics of Enhancer Chromatin Signatures Mark the Transition From Pluripotency to Cell Specification During Embryogenesis." *Genome Research* 22: 2043–2053.

Brenner, W. G., and T. Schmülling. 2012. "Transcript Profiling of Cytokinin Action in Arabidopsis Roots and Shoots Discovers Largely Similar but Also Organ-Specific Responses." *BMC Plant Biology* 12: 112.

Buenrostro, J. D., B. Wu, H. Y. Chang, and W. J. Greenleaf. 2015. "ATAC-Seq: A Method for Assaying Chromatin Accessibility Genome-Wide." *Current Protocols in Molecular Biology* 109: 21–29.

Buenrostro, J. D., B. Wu, U. M. Litzenburger, et al. 2015. "Single-Cell Chromatin Accessibility Reveals Principles of Regulatory Variation." *Nature* 523: 486–490.

Caputi, L., J. Franke, S. C. Farrow, et al. 2018. "Missing Enzymes in the Biosynthesis of the Anticancer Drug Vinblastine in Madagascar Periwinkle." *Science* 360: 1235–1239.

Chen, K., J. Chen, X. Pi, L. J. Huang, and N. Li. 2023. "Isolation, Purification, and Application of Protoplasts and Transient Expression Systems in Plants." *International Journal of Molecular Sciences* 24: 16892.

Chen, K. Y., T. Kibayashi, A. Giguelay, et al. 2025. "Genome-Wide CRISPR Screen in Human T Cells Reveals Regulators of FOXP3." *Nature* 642: 191–200.

Chen, L., Y. Jia, Z. Zhou, et al. 2025. "Genomic and Cis-Regulatory Basis of a Plastic C(3)-C(4) Photosynthesis in *Eleocharis baldwinii*." *Advanced Science* 12: e15681.

Chen, S., N. E. Sanjana, K. Zheng, et al. 2015. "Genome-Wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis." *Cell* 160: 1246–1260.

Chen, Z., E. Arai, O. Khan, et al. 2021. "In Vivo CD8(+) T Cell CRISPR Screening Reveals Control by Fli1 in Infection and Cancer." *Cell* 184: 1262–1280.

Cheng, Y., Y. Zhang, G. Li, et al. 2023. "CRISPR–Cas12a Base Editors Confer Efficient Multiplexed Genome Editing in Rice." *Plant Communications* 4: 100601.

Chiou, J., R. J. Geusz, M. L. Okino, et al. 2021. "Interpreting Type 1 Diabetes Risk With Genetics and Single-Cell Epigenomics." *Nature* 594: 398–402.

Choo, X. Y., Y. M. Lim, K. Katwadi, et al. 2021. "Evaluating Capture Sequence Performance for Single-Cell CRISPR Activation Experiments." *ACS Synthetic Biology* 10: 640–645.

Datlinger, P., A. F. Rendeiro, T. Boenke, et al. 2021. "Ultra-High-Throughput Single-Cell RNA Sequencing and Perturbation Screening With Combinatorial Fluidic Indexing." *Nature Methods* 18: 635–642.

Deb, A., and S. Kundu. 2015. "Deciphering Cis-Regulatory Element Mediated Combinatorial Regulation in Rice Under Blast Infected Condition." *PLoS One* 10: e0137295.

Díaz-Valenzuela, E., R. H. Sawers, and A. Cibrián-Jaramillo. 2020. "Cis- and Trans-Regulatory Variations in the Domestication of the Chili Pepper Fruit." *Molecular Biology and Evolution* 37: 1593–1603.

Dixit, A., O. Parnas, B. Li, et al. 2016. "Perturb-Seq: Dissecting Molecular Circuits With Scalable Single-Cell RNA Profiling of Pooled Genetic Screens." *Cell* 167: 1853–1866.

Duraisamy, G. S., A. K. Mishra, T. Kocabek, and J. Matoušek. 2016. "Identification and Characterization of Promoters and Cis-Regulatory Elements of Genes Involved in Secondary Metabolites Production in Hop (*Humulus lupulus*. L)." *Computational Biology and Chemistry* 64: 346–352.

Ernst, J., P. Kheradpour, T. S. Mikkelsen, et al. 2011. "Mapping and Analysis of Chromatin State Dynamics in Nine Human Cell Types." *Nature* 473: 43–49.

Feng, D., Z. Liang, Y. Wang, et al. 2022. "Chromatin Accessibility Illuminates Single-Cell Regulatory Dynamics of Rice Root Tips." *BMC Biology* 20: 274.

Frangieh, C. J., J. C. Melms, P. I. Thakore, et al. 2021. "Multimodal Pooled Perturb-CITE-Seq Screens in Patient Models Define Mechanisms of Cancer Immune Evasion." *Nature Genetics* 53: 332–341.

Franssen, H. J., O. Kulikova, V. Willemsen, and R. Heidstra. 2017. "Cis-Regulatory PLETHORA Promoter Elements Directing Root and Nodule Expression Are Conserved Between *Arabidopsis thaliana* and *Medicago truncatula*." *Plant Signaling & Behavior* 12: e1278102.

Ge, X., J. Xu, Z. Yang, et al. 2023. "Efficient Genotype-Independent Cotton Genetic Transformation and Genome Editing." *Journal of Integrative Plant Biology* 65: 907–917.

Gilbert, L. A., M. H. Larson, L. Morsut, et al. 2013. "CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes." *Cell* 154: 442–451.

Greenham, K., R. C. Sartor, S. Zorich, P. Lou, T. C. Mockler, and C. R. McClung. 2020. "Expansion of the Circadian Transcriptome in *Brassica rapa* and Genome-Wide Diversification of Paralog Expression Patterns." *eLife* 9: e58993.

Guo, M., F. Yang, L. Zhu, et al. 2024. "Loss of Cold Tolerance Is Conferred by Absence of the WRKY34 Promoter Fragment During Tomato Evolution." *Nature Communications* 15: 6667.

Han, X., Y. Zhang, Z. Lou, et al. 2023. "Time Series Single-Cell Transcriptional Atlases Reveal Cell Fate Differentiation Driven by Light in Arabidopsis Seedlings." *Nature Plants* 9: 2095–2109.

Harmer, S. L., and S. A. Kay. 2005. "Positive and Negative Factors Confer Phase-Specific Circadian Regulation of Transcription in Arabidopsis." *Plant Cell* 17: 1926–1940.

Hildebrandt, L., T. Zimmermann, and D. Pröfrock. 2023. "Laser Microdissection Pressure Catapulting (LMPC): A New Technique to Handle Single Microplastic Particles for Number-Based Validation Strategies." *Analytical and Bioanalytical Chemistry* 415: 3041–3049.

Hsu, P. D., E. S. Lander, and F. Zhang. 2014. "Development and Applications of CRISPR-Cas9 for Genome Engineering." *Cell* 157: 1262–1278.

Huffaker, A., F. Kaplan, M. M. Vaughan, et al. 2011. "Novel Acidic Sesquiterpenoids Constitute a Dominant Class of Pathogen-Induced Phytoalexins in Maize." *Plant Physiology* 156: 2082–2097.

Jaitin, D. A., E. Kenigsberg, H. Keren-Shaul, et al. 2014. "Massively Parallel Single-Cell RNA-Seq for Marker-Free Decomposition of Tissues Into Cell Types." *Science* 343: 776–779.

Jin, X., S. K. Simmons, A. Guo, et al. 2020. "In Vivo Perturb-Seq Reveals Neuronal and Glial Abnormalities Associated With Autism Risk Genes." *Science* 370: eaaz6063.

Jung, Y. L., W. Zhao, I. Li, et al. 2024. "Epigenetic Profiling Reveals Key Genes and Cis-Regulatory Networks Specific to Human Parathyroids." *Nature Communications* 15: 2106.

Kajala, K., N. J. Brown, B. P. Williams, P. Borrill, L. E. Taylor, and J. M. Hibberd. 2012. "Multiple Arabidopsis Genes Primed for Recruitment Into C_4 Photosynthesis." *Plant Journal* 69: 47–56.

Kang, M., K. Lee, T. Finley, H. Chappell, V. Veena, and K. Wang. 2022. "An Improved Agrobacterium-Mediated Transformation and Genome-Editing Method for Maize Inbred B104 Using a Ternary Vector System and Immature Embryos." *Frontiers in Plant Science* 13: 860971.

Kaya-Okur, H. S., S. J. Wu, C. A. Codomo, et al. 2019. "CUT&Tag for Efficient Epigenomic Profiling of Small Samples and Single Cells." *Nature Communications* 10: 1930.

Lanctot, A., A. Hendelman, P. Udilovich, G. M. Robitaille, and Z. B. Lippman. 2025. "Antagonizing Cis-Regulatory Elements of a Conserved Flowering Gene Mediate Developmental Robustness." *Proceedings of the National Academy of Sciences of the United States of America* 122: e2421990122.

Lapinaite, A., G. J. Knott, C. M. Palumbo, et al. 2020. "DNA Capture by a CRISPR-Cas9-Guided Adenine Base Editor." *Science* 369: 566–571.

Li, J. F., J. E. Norville, J. Aach, et al. 2013. "Multiplex and Homologous Recombination-Mediated Genome Editing in Arabidopsis and Nicotiana Benthamiana Using Guide RNA and Cas9." *Nature Biotechnology* 31: 688–691.

Li, Q., Q. Feng, A. Snouffer, B. Zhang, G. R. Rodríguez, and E. van der Knaap. 2022. "Increasing Fruit Weight by Editing a Cis-Regulatory Element in Tomato KLUH Promoter Using CRISPR/Cas9." *Frontiers in Plant Science* 13: 879642.

Li, W., J. Köster, H. Xu, et al. 2015. "Quality Control, Modeling, and Visualization of CRISPR Screens With MAGeCK-VISPR." *Genome Biology* 16: 281.

Li, W., H. Xu, T. Xiao, et al. 2014. "MAGeCK Enables Robust Identification of Essential Genes From Genome-Scale CRISPR/Cas9 Knockout Screens." *Genome Biology* 15: 554.

Li, X., J. Xie, C. Dong, et al. 2024. "Efficient and Heritable A-to-K Base Editing in Rice and Tomato." *Horticulture Research* 11: uhad250.

Li, Z., D. Zhang, X. Xiong, et al. 2017. "A Potent Cas9-Derived Gene Activator for Plant and Mammalian Cells." *Nature Plants* 3: 930–936.

Liang, J., J. Wei, J. Cao, et al. 2023. "In-Organoid Single-Cell CRISPR Screening Reveals Determinants of Hepatocyte Differentiation and Maturation." *Genome Biology* 24: 251.

Liscovitch-Brauer, N., A. Montalbano, J. Deng, et al. 2021. "Profiling the Genetic Determinants of Chromatin Accessibility With Scalable Single-Cell CRISPR Screens." *Nature Biotechnology* 39: 1270–1277.

Liu, B., Y. He, X. Wu, et al. 2024. "Mapping Putative Enhancers in Mouse Oocytes and Early Embryos Reveals TCF3/12 as Key Folliculogenesis Regulators." *Nature Cell Biology* 26: 962–974.

Loo, C. S., J. Gatchalian, Y. Liang, et al. 2020. "A Genome-Wide CRISPR Screen Reveals a Role for the Non-Canonical Nucleosome-Remodeling BAF Complex in Foxp3 Expression and Regulatory T Cell Function." *Immunity* 53: 143–157.

Lowensohn, T. N., W. B. Cody, C. Tsai, et al. 2026. "An In Planta Single-Cell Screen to Accelerate Functional Genetics." *bioRxiv*. https://doi.org/10.1101/2025.08.06.668818.

Luo, L., M. Gribskov, and S. Wang. 2022. "Bibliometric Review of ATAC-Seq and Its Application in Gene Expression." *Briefings in Bioinformatics* 23: bbac061.

Ma, S., B. Zhang, L. F. LM, et al. 2020. "Chromatin Potential Identified by Shared Single-Cell Profiling of RNA and Chromatin." *Cell* 183: 1103–1116.e1120.

Mao, H., S. Li, B. Chen, et al. 2022. "Variation in Cis-Regulation of a NAC Transcription Factor Contributes to Drought Tolerance in Wheat." *Molecular Plant* 15: 276–292.

Marand, A. P., Z. Chen, A. Gallavotti, and R. J. Schmitz. 2021. "A Cis-Regulatory Atlas in Maize at Single-Cell Resolution." *Cell* 184: 3041–3055.

Marinov, G. K., and Z. Shipony. 2021. "Interrogating the Accessible Chromatin Landscape of Eukaryote Genomes Using ATAC-Seq." *Deep Sequencing Data Analysis* 2243: 183–226.

Matsuoka, M., J. Kyozuka, K. Shimamoto, and Y. Kano-Murakami. 1994. "The Promoters of Two Carboxylases in a C4 Plant (Maize) Direct Cell-Specific, Light-Regulated Expression in a C3 Plant (Rice)." *Plant Journal* 6: 311–319.

Matsuoka, M., Y. Tada, T. Fujimura, and Y. Kano-Murakami. 1993. "Tissue-Specific Light-Regulated Expression Directed by the Promoter of a C4 Gene, Maize Pyruvate, Orthophosphate Dikinase, in a C3 Plant, Rice." *Proceedings of the National Academy of Sciences of the United States of America* 90: 9586–9590.

Mendieta, J. P., X. Tu, D. Jiang, et al. 2024. "Investigating the Cis-Regulatory Basis of C(3) and C(4) Photosynthesis in Grasses at Single-Cell Resolution." *Proceedings of the National Academy of Sciences of the United States of America* 121: e2402781121.

Meng, Q., L. Wei, K. Ma, et al. 2024. "scDecouple: Decoupling Cellular Response From Infected Proportion Bias in scCRISPR-Seq." *Briefings in Bioinformatics* 25: bbae011.

Miwa, K., M. Serikawa, S. Suzuki, T. Kondo, and T. Oyama. 2006. "Conserved Expression Profiles of Circadian Clock-Related Genes in Two Lemna Species Showing Long-Day and Short-Day Photoperiodic Flowering Responses." *Plant & Cell Physiology* 47: 601–612.

Movahedi, S., Y. van de Peer, and K. Vandepoele. 2011. "Comparative Network Analysis Reveals That Tissue Specificity and Gene Function Are Important Factors Influencing the Mode of Expression Evolution in Arabidopsis and Rice." *Plant Physiology* 156: 1316–1330.

Neelakandan, A. K., D. A. Wright, S. M. Traore, X. Chen, M. H. Spalding, and G. He. 2022. "CRISPR/Cas9 Based Site-Specific Modification of FAD2 Cis-Regulatory Motifs in Peanut (*Arachis hypogaea* L)." *Frontiers in Genetics* 13: 849961.

Nekrasov, V., B. Staskawicz, D. Weigel, J. D. G. Jones, and S. Kamoun. 2013. "Targeted Mutagenesis in the Model Plant Nicotiana Benthamiana Using Cas9 RNA-Guided Endonuclease." *Nature Biotechnology* 31: 691–693.

Noda, Y., F. Wang, S. Chankaew, et al. 2025. "Diurnal Regulation of SOS Pathway and Sodium Excretion Underlying Salinity Tolerance of *Vigna marina*." *Plant, Cell & Environment* 48: 3925–3938.

Oikawa, T., H. Maeda, T. Oguchi, et al. 2015. "The Birth of a Black Rice Gene and Its Local Spread by Introgression." *Plant Cell* 27: 2401–2414.

Pan, C., X. Wu, K. Markel, et al. 2021. "CRISPR–Act3.0 for Highly Efficient Multiplexed Gene Activation in Plants." *Nature Plants* 7: 942–953.

Park, P. J. 2009. "ChIP-Seq: Advantages and Challenges of a Maturing Technology." *Nature Reviews. Genetics* 10: 669–680.

Piatek, A., Z. Ali, H. Baazim, et al. 2015. "RNA-Guided Transcriptional Regulation In Planta via Synthetic dCas9-Based Transcription Factors." *Plant Biotechnology Journal* 13: 578–589.

Pierce, S. E., J. M. Granja, and W. J. Greenleaf. 2021. "High-Throughput Single-Cell Chromatin Accessibility CRISPR Screens Enable Unbiased Identification of Regulatory Networks in Cancer." *Nature Communications* 12: 2969.

Pu, J., J. Ma, H. Zhai, et al. 2025. "Atomic Force Microscopy Imaging of Plant Cell Walls." *Plant Physiology* 197: kiae655.

Puchta, H., B. Dujon, and B. Hohn. 1996. "Two Different but Related Mechanisms Are Used in Plants for the Repair of Genomic Double-Strand Breaks by Homologous Recombination." *Proceedings of the National Academy of Sciences of the United States of America* 93: 5055–5060.

Radtke, A. J., C. J. Chu, Z. Yaniv, et al. 2022. "IBEX: An Iterative Immunolabeling and Chemical Bleaching Method for High-Content Imaging of Diverse Tissues." *Nature Protocols* 17: 378–401.

Replogle, J. M., T. M. Norman, A. Xu, et al. 2020. "Combinatorial Single-Cell CRISPR Screens by Direct Guide RNA Capture and Targeted Sequencing." *Nature Biotechnology* 38: 954–961.

Rodríguez-Leal, D., Z. H. Lemmon, J. Man, M. E. Bartlett, and Z. B. Lippman. 2017. "Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing." *Cell* 171: 470–480.

Rubin, A. J., K. R. Parker, A. T. Satpathy, et al. 2019. "Coupled Single-Cell CRISPR Screening and Epigenomic Profiling Reveals Causal Gene Regulatory Networks." *Cell* 176: 361–376.

Salomon, S., and H. Puchta. 1998. "Capture of Genomic and T-DNA Sequences During Double-Strand Break Repair in Somatic Plant Cells." *EMBO Journal* 17: 6086–6095.

Satpathy, A. T., J. M. Granja, K. E. Yost, et al. 2019. "Massively Parallel Single-Cell Chromatin Landscapes of Human Immune Cell Development and Intratumoral T Cell Exhaustion." *Nature Biotechnology* 37: 925–936.

Shalem, O., N. E. Sanjana, and F. Zhang. 2015. "High-Throughput Functional Genomics Using CRISPR-Cas9." *Nature Reviews Genetics* 16: 299–311.

Shan, Q., Y. Wang, J. Li, et al. 2013. "Targeted Genome Modification of Crop Plants Using a CRISPR-Cas System." *Nature Biotechnology* 31: 686–688.

Sharma, M., P. Kumar, V. Verma, R. Sharma, B. Bhargava, and M. Irfan. 2022. "Understanding Plant Stress Memory Response for Abiotic Stress Resilience: Molecular Insights and Prospects." *Plant Physiology and Biochemistry* 179: 10–24.

Shen, Y., T. Ye, Z. Li, et al. 2024. "Exploiting Viral Vectors to Deliver Genome Editing Reagents in Plants." *aBIOTECH* 5: 247–261.

Shifrut, E., J. Carnevale, V. Tobin, et al. 2018. "Genome-Wide CRISPR Screens in Primary Human T Cells Reveal Key Regulators of Immune Function." *Cell* 175: 1958–1971.

Shulse, C. N., B. J. Cole, D. Ciobanu, et al. 2019. "High-Throughput Single-Cell Transcriptome Profiling of Plant Cell Types." *Cell Reports* 27: 2241–2247.

Skene, P. J., and S. Henikoff. 2017. "An Efficient Targeted Nuclease Strategy for High-Resolution Mapping of DNA Binding Sites." *eLife* 6: e21856.

Song, X., X. Meng, H. Guo, et al. 2022. "Targeting a Gene Regulatory Element Enhances Rice Grain Yield by Decoupling Panicle Number and Size." *Nature Biotechnology* 40: 1403–1411.

Sun, Y., L. Shang, Q. H. Zhu, L. Fan, and L. Guo. 2022. "Twenty Years of Plant Genome Sequencing: Achievements and Challenges." *Trends in Plant Science* 27: 391–401.

Swanson, E., C. Lord, J. Reading, et al. 2021. "Simultaneous Trimodal Single-Cell Measurement of Transcripts, Epitopes, and Chromatin Accessibility Using TEA-Seq." *eLife* 10: e63632.

Tang, X., X. Zheng, Y. Qi, et al. 2016. "A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants." *Molecular Plant* 9: 1088–1091.

Tu, X., S. Ren, W. Shen, et al. 2022. "Limited Conservation in Cross-Species Comparison of GLK Transcription Factor Binding Suggested Wide-Spread Cistrome Divergence." *Nature Communications* 13: 7632.

Umhoefer, J. M., M. M. Arce, S. Whalen, et al. 2025. "Cis-Regulatory Element and Transcription Factor Circuitry Required for Cell-Type Specific Expression of FOXP3." *bioRxiv*. https://doi.org/10.1101/2024.08.30.610436.

Vatov, E., U. Ludewig, and U. Zentgraf. 2021. "Disparate Dynamics of Gene Body and Cis-Regulatory Element Evolution Illustrated for the Senescence-Associated Cysteine Protease Gene SAG12 of Plants." *Plants* 10: 24.

Venegas-Molina, J., F. J. Molina-Hidalgo, E. Clicque, and A. Goossens. 2021. "Why and How to Dig Into Plant Metabolite-Protein Interactions." *Trends in Plant Science* 26: 472–483.

Wang, F., T. Han, Q. Song, et al. 2020. "The Rice Circadian Clock Regulates Tiller Growth and Panicle Development Through Strigolactone Signaling and Sugar Sensing." *Plant Cell* 32: 3124–3138.

Wang, H., J. Ren, S. Zhou, et al. 2024. "Molecular Regulation of Oil Gland Development and Biosynthesis of Essential Oils in Citrus spp." *Science* 383: 659–666.

Wang, L. 2021. "Single-Cell Normalization and Association Testing Unifying CRISPR Screen and Gene Co-Expression Analyses With Normalisr." *Nature Communications* 12: 6395.

Wang, M., Z. Li, Y. Zhang, et al. 2021. "An Atlas of Wheat Epigenetic Regulatory Elements Reveals Subgenome Divergence in the Regulation of Development and Stress Responses." *Plant Cell* 33: 865–881.

Wolter, F., and H. Puchta. 2018. "Application of CRISPR/Cas to Understand Cis- and Trans-Regulatory Elements in Plants." *Methods in Molecular Biology* 1830: 23–40.

Woronik, A., K. Kiontke, R. S. Jallad, R. A. Herrera, and D. H. Fitch. 2022. "Laser Microdissection for Species-Agnostic Single-Tissue Applications." *Journal of Visualized Experiments* 181: 10-3791.

Wu, J., F. Gao, T. Li, et al. 2021. "Genome-Wide Cis-Regulatory Element Based Discovery of Auxin-Responsive Genes in Higher Plant." *Genes* 13: 24.

Xie, L., X. Gong, K. Yang, et al. 2024. "Technology-Enabled Great Leap in Deciphering Plant Genomes." *Nature Plants* 10: 551–566.

Xie, S., J. Duan, B. Li, P. Zhou, and G. C. Hon. 2017. "Multiplexed Engineering and Analysis of Combinatorial Enhancer Activity in Single Cells." *Molecular Cell* 66: 285–299.

Xin, H., X. Liu, S. Chai, et al. 2024. "Identification and Functional Characterization of Conserved Cis-Regulatory Elements Responsible for Early Fruit Development in Cucurbit Crops." *Plant Cell* 36: 2272–2288.

Xu, Q., S. Huang, G. Guo, et al. 2022. "Inferring Regulatory Element Landscapes and Gene Regulatory Networks From Integrated Analysis in Eight Hulless Barley Varieties Under Abiotic Stress." *BMC Genomics* 23: 843.

Yan, T., Q. Hou, X. Wei, et al. 2023. "Promoting Genotype-Independent Plant Transformation by Manipulating Developmental Regulatory Genes and/or Using Nanoparticles." *Plant Cell Reports* 42: 1395–1417.

Yang, B., M. Zhang, Y. Shi, et al. 2025. "PerturbDB for Unraveling Gene Functions and Regulatory Networks." *Nucleic Acids Research* 53: D1120–D1131.

Yang, L., Y. Zhu, H. Yu, et al. 2020. "scMAGeCK Links Genotypes With Multiple Phenotypes in Single-Cell CRISPR Screens." *Genome Biology* 21: 19.

Yin, X., A. K. Biswal, J. Dionora, et al. 2017. "CRISPR-Cas9 and CRISPR-Cpf1 Mediated Targeting of a Stomatal Developmental Gene EPFL9 in Rice." *Plant Cell Reports* 36: 745–757.

Zamanighomi, M., S. S. Jain, T. Ito, D. Pal, T. P. Daley, and W. R. Sellers. 2019. "GEMINI: A Variational Bayesian Approach to Identify Genetic Interactions From Combinatorial CRISPR Screens." *Genome Biology* 20: 137.

Zhou, J., G. Liu, Y. Zhao, et al. 2023. "An Efficient CRISPR–Cas12a Promoter Editing System for Crop Improvement." *Nature Plants* 9: 588–604.

Zhu, H., X. Qin, L. Wei, et al. 2025. "Engineered Geminivirus Replicons Enable Rapid In Planta Directed Evolution." *Science* 390: eady2167.