CRISPR/Cas9 for Functional Analysis in Soybean

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CRISPR/Cas9 is the most powerful reverse genetics approach for fast and precise functional analysis of candidate genes/mutations of interest. Rapid and large-scale analyses of CRISPR/Cas-induced mutagenesis is achievable through Agrobacterium rhizogenes-mediated hairy root transformation. The combination of *A. rhizogenes* hairy root-CRISPR/Cas provides an extraordinary platform for rapid, precise, easy, and cost-effective "in root" functional analysis of genes of interest in legume plants, including soybean.

functional analysis   reverse genetics   CRISPR   gene editing   hairy root

transformation

1. Introduction

In recent years, forward genetic methods have enabled the identification of candidate genes and enzymes involved in a trait or biosynthetic pathway of interest, owing to the fast development of large-scale sequencing techniques, thus leading to a much-increased availability of genome and transcriptome data [1]. These powerful forward genetic techniques have allowed the identification of many quantitative trait loci (QTL) as well as candidate genes and even candidate causal mutations controlling a specific trait [2]; however, the dissection of the exact function of these genetic components remains challenging. In parallel, reverse genetic approaches, such as recombinant DNA technology and mutagenesis, have been applied for functional annotation of genetic components, causal mutations, and candidate genes identified by forward genetic techniques [3]. Reverse genetic methods play a complementary, but necessary role in next-generation sequencing (NGS)-based forward genetic approaches to precisely describe the function of candidate genetic components and subsequently improve agronomic traits [4].

In the past few years, recombinant DNA technology has shown a great advantage over random mutagenesis, where obtaining a desired mutant requires generating and screening large mutant populations. Moreover, reduced viability is another problem associated with induced mutagenesis [2]. On the contrary, site-directed mutagenesis through recombinant DNA technology and the insertion of T-DNA (vector-based mutagenesis) represents a sophisticated method for functional gene analysis and the development of new traits [5]. These approaches require fast and efficient transformation techniques to generate transgenic plants/organs. To date, different transformation methods, including *Agrobacterium*-mediated, imbibition, biolistic, osmotic, liposome, microinjection, pollen tube pathway, shoot apex, infiltration, and silicon carbide-mediated transformation [6][7][8], have been developed to transfer exogenous genetic components to a plant's genome [9]. Currently, *Agrobacterium*-mediated gene transfer

is the most effective and prevalent method [9][10], as it can precisely integrate a DNA sequence into an active section of the host genome with a low copy number and consistent gene expression over generations [11].

Overall, transformation can be conducted in stable or transient manners. Stable transformation refers to the stable integration of exogenous DNA into the host genome, whereas transient transformation is the short-term (transient) expression of gene constructs, resident bacterial plasmid, carrying exogenous DNA in a plant cell. For stable transformation, whole-plant in vitro regeneration is required, which is a time-consuming and costly process. Direct organogenesis, indirect organogenesis, or somatic embryogenesis are the main routes of in vitro regeneration in legumes [12]. Currently, direct organogenesis using cotyledonary nodes or other meristematic explants (e.g., apical/axillary meristems) is the most prevalent in vitro regeneration method for *Agrobacterium*-mediated transformation of legume species [12]. However, some legumes, such as soybean (*Glycine max* L.), are recalcitrant to stable transformation because of limited in vitro regeneration capacity and low DNA transfer rates [1].

In contrast, transient transformation, with no need for complete plant regeneration, provides an efficient alternative for rapid and large-scale functional analysis in soybean plants [13]. This approach has no effect on the stability of the host genome and is independent of the positional effects of the T-DNA integration sites [14]. Therefore, chimeric and composite plants (containing wild-type shoots with transgenic roots) can be generated in an efficient manner, and transgenic roots, with a "hairy root" phenotype, can be analyzed to assess the function of genes of interest, especially genes involved in legume-rhizobium symbiosis and root–microbe interactions [15][16]. Most interestingly, *Agrobacterium rhizogenes*-mediated transformed roots can be used to regenerate whole transformed plants. In general, composite plants can be obtained through in vitro and or in planta (ex vitro) inoculation techniques [17]. Ex vitro hairy root induction is completely independent from laborious in vitro techniques and has great advantages for large-scale gene validation studies. This is especially the case for the functional characterization of genes suspected of controlling root traits. These include genes involved in plant metabolic engineering, plant-pathogen interactions, symbiotic nitrogen fixation (SNF) and nodulation, biotic and abiotic stress tolerance, mycorrhization, phytoremediation, root-shoot interaction, nutrient uptake, and hormone transport [18].

2. CRISPR/Cas9 System for Functional Analysis in Soybean

Transgene-induced overexpression or suppression (RNAi-based knock-down) of a target gene are two important reverse genetic methods to understand a gene's role. Both overexpression and knock-down have been achieved through hairy root transformation in soybean [19][20][21][22][23][24]. There are some pros and cons with both systems. The unpredictable nature of T-DNA insertion sites, and the associated position effects that can impact the level of expression of the introduced transgene, is an important disadvantage of vector-based overexpression studies [25]. Targeted suppression of desired genes in a homology-dependent manner using RNAi is a good way to assess the function of identified candidate genes [26]. RNAi is a quick, easy, and cost-effective sequence-specific method for validating gene function. There are, however, potential pitfalls in this approach, such as limited sequence specificity of siRNAs (off-target activity), transitive silencing, inefficacy and instability, and difficulty in the validation of RNAi knock-down (as the wild-type transgene would be silenced and validation by complementation with a wild-type

gene is impossible) [27]. Targeted genome editing methods can provide more stable and predictable mutant lines to investigate gene function [27].

Simply put, the whole CRISPR/Cas9 genome editing procedure is based on an RNA-guided Cas9 DNA cleavage mechanism [3][28][29][30]. Therefore, a CRISPR/Cas9 construct consists of gene coding for the Cas9 nuclease and an expression cassette for at least one guide RNA (gRNA) embedded in the T-DNA region of a binary vector (**Figure 1**). The subsequent repair of Cas9-induced DSBs by the imprecise non-homologous end-joining (NHEJ) repair process will lead to insertions/deletions (indels), many of which will result in a non-functional or dysfunctional protein. Recent reviews have extensively described CRISPR/Cas9-based genome editing [28][31][32].
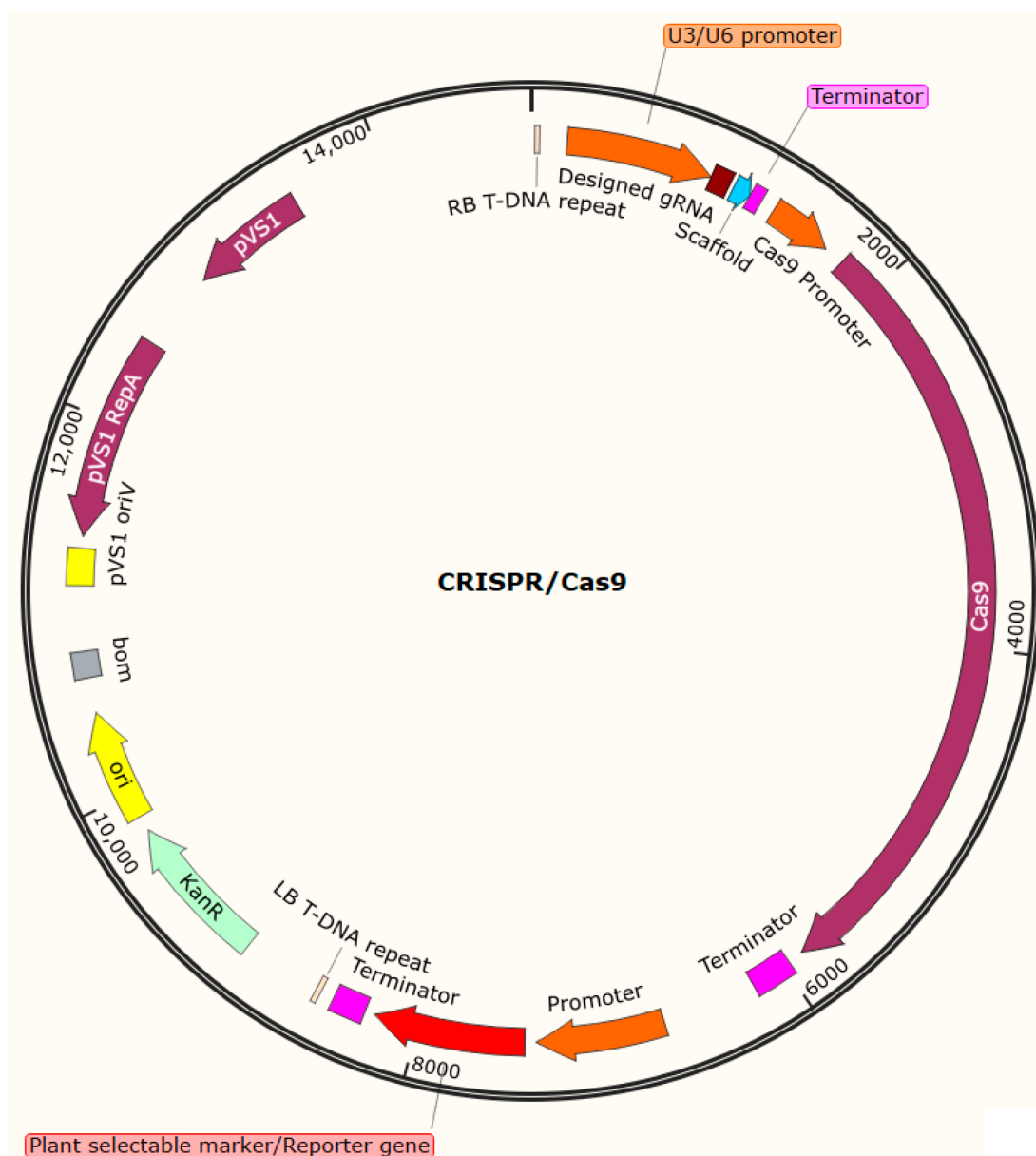

Figure 1. The schematic representation of a binary vector harboring gRNA, Cas9, and selectable marker/reporter gene cassettes in its T-DNA region for targeted genome editing of plants.

2.1. gRNA and Its Components

A CRISPR RNA (crRNA) and a transactivating CRISPR RNA (tracrRNA) are the two components of a single guide RNA (sgRNA) in the natural CRISPR/Cas9 system. In the engineered version of the CRISPR/Cas9 system, the crRNA spacer sequence will be replaced with the designed target sequence at the time of gRNA cassette preparation. The important part of the spacer sequence is the seed sequence that binds to the target DNA, following recognition of the protospacer-adjacent motif (PAM), and therefore it is required for precise target recognition and binding. Depending on the Cas variant, the length of the seed sequence varies from 5–12 nt upstream of the PAM. Mismatches between the seed sequence of a gRNA and the targeted region constitute an important risk for failure in CRISPR activity [33]. The second part of a gRNA—the scaffold sequence—is responsible for generating the required secondary structure to bind to the target sequence and is considered the constant part of a gRNA.

2.2. Critical Criteria in Designing gRNA

2.2.1. Proper Selection of the Target Site(s) within the Locus of Interest

Depending on the desired outcome of editing, different regions within a targeted gene/locus can be selected for mutation induction. In knock-out experiments via a premature termination codon (PTC) strategy, targeting earlier exons is more effective than exons very close to an ATG or intron–exon junction, as PTC-induced loss of function is not common in these regions [33][34].

2.2.2. Determination and Prediction of Off-Target Activity

Success in the induction of desired mutations and preventing off-target editing are two key outcomes of a successful genome editing experiment. These experiments require that great care be taken in designing the gRNA. The cleavage efficiency of candidate gRNAs can be determined in a faster manner through an in vitro screening method rather than through in vivo expression [35]. Predicting off-target activities is another approach to finding the most efficient gRNAs among different candidates. Some of the web-based software for designing gRNAs present information regarding the genome-wide off-target activities of the candidate gRNA [33]. Machine learning algorithms can also predict the mutation induction efficiency of a designed gRNA sequence using repair data obtained from previous studies [36][37]. Although care in designing an optimal site-specific gRNA is the best way to reduce off-target activity, there are some wet-lab procedures that can be used to reduce the frequency of off-target events; these include: (i) reducing gRNA-Cas9 concentration, (ii) using double-nicking mediated by a Cas9 nickase mutant (nCas9), and (iii) using truncated gRNAs (tru-gRNAs) [38].

2.2.3. Nucleotide Features of the Designed gRNA and its Associated Secondary Structure

A GC content of 30–80% and no mismatch to the targeted sequence, especially in the seed region targeting the non-transcribed strand, have been reported as key features of an effective gRNA [33]. Another aspect that should be considered is avoiding multiple "Ts" in the construct [33]. Repeat and anti-repeat (RAR) stem-loop, stem-loop 1, stem-loop 2, and stem-loop 3 are four required stem-loop structures that should be present in an effective gRNA. The formation of a functional Cas9-gRNA-DNA complex does not necessarily depend on the stem-loop 1 structure.

However, stem-loops RAR, 2, and 3 are required and important in plant genome editing experiments [39]. In silico analysis of gRNA secondary structure through web-based software, such as RNA-fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (accessed on 1 February 2022), is an appropriate procedure to predict the efficiency of a designed gRNA [40]. In addition to the predicted secondary structure, information about the free energy (ΔG) of the self-folding potential of the designed gRNA is available in the RNA-fold WebServer. A designed gRNA with self-folding free energy within the range of 0 to 2.0 kcal/mol can lead to the highest cleavage efficiency of Cas9 [40].

2.3. Number of Designed gRNAs

Designing at least two independent gRNAs for each target gene is a solution to minimize the risk of experiment failure in a CRISPR/Cas9 experiment [41]. Designing multiple gRNAs is especially important in species such as soybean, where nearly 75% of the genes are present in multiple copies, so one gRNA is not enough for simultaneous mutation induction in paralogs [42]. Multiple designed gRNAs should be individually inserted between the promotor and gRNA scaffold of a gRNA cassette using several rounds of standard restriction-ligation cloning, which is a tedious and time-consuming procedure [39]. In the following section, researchers highlight different strategies for assembling multiple gRNAs.

2.4. Assembly of Multiple gRNAs and gRNA Processing Strategies

In multiplex gene editing studies, multiple gRNA cassettes designed to target different genes or different regions of a gene at once should be assembled as a single unit and inserted in the destination vector. The first strategy is the insertion of the designed and synthesized double-stranded oligonucleotides in individual gRNA vectors, then isolation of each gRNA cassette (promotor + guide sequence + gRNA scaffold + terminator) from gRNA vectors and their assembly in an intermediate/destination construct using Golden Gate cloning. In Golden Gate assembly, gRNA cassettes should be designed in a way to produce compatible overhanging ends after digestion with type IIS restriction enzymes. After ligation of adjacent units, the resulting gRNA module can then be mobilized into the binary vector of interest using different cloning methods. Zheng et al. [43] provided a Golden Gate-based modular system that can assemble 2, 4, and 6 gRNA units for targeted mutagenesis of soybean [43]. Gibson assembly is another technique for the assembly of various gRNA cassettes and is independent from end-compatibility issues and PCR cleanup steps [44]. The GoldenBraid cloning system has also been used to assemble multiple gRNAs and insert them into destination binary vectors [45]. Modification of existing CRISPR vectors for substitution of other Cas9 proteins, fluorescence proteins, and required resistance genes can be easily performed by using the GoldenBraid cloning system [39][46]. For gene knock-out experiments using gene editing technology, the GoldenBraid technique is well suited as it can carry multiple gRNA constructs leading to an increased possibility of mutagenesis [47]. This technique can also be used for complete disruption of the genes of interest by introducing large deletions [33].

Cloning multiple gRNA cassettes as independent units can present disadvantages such as frequent recombination events and plasmid instability in *E. coli* and *Agrobacterium* because of repeated promotor sequences (promoter

crosstalk effects) [48]. Therefore, the configuration of multiple gRNAs and their simultaneous expression as a single transcript can be more beneficial. In this case, a single polycistronic transcript will be cleaved into individual gRNAs post-transcriptionally using different RNA processing strategies [49]. The CRISPR-associated RNA endoribonuclease Csy4 (from *Pseudomonas aeruginosa*), the tRNA-processing endogenous enzymes, and self-processing ribozymes can be used for post-transcriptional cleavage of a polycistronic transcript [48]. Luo et al. [50] used a polycistronic approach (with Csy4 used to achieve cleavage) and reported a 45.3% mutation induction efficiency when targeting *GW2* paralogs in soybean. A polycistronic tRNA-gRNA (PTG) vector is another processing strategy for multiplexing gRNAs [49].

2.5. Cas9 Nucleases

The Cas protein is responsible for DNA cleavage in a targeted region of the plant genome. Therefore, a high-fidelity Cas enzyme can increase the specificity and efficiency of a targeted genome editing procedure. *Streptococcus pyogenes* Cas9 (SpCas9) is the most robust and widely used Cas enzyme [28]. The presence of a canonical PAM sequence (NGG) in the targeted genomic loci is, however, a prerequisite for cleavage by SpCas9. Therefore, there is a limitation in the number of genomic loci that can be targeted by this protein [51]. Fortunately, different Cas9 variants, with distinct PAM specificities, can be used to expand this range [52]. In a study, three variants of Cas9 (xCas9, SpCas9-NG, and XNG-Cas9) were assessed in soybean [53]. Researchers reported that xCas9 was successful with the NGG and KGA PAMs, SpCas9-NG recognized NGD (NGG, NGA, and NGT), RGC (AGC or GGC), GAA, and GAT PAM sites, whereas XNG-Cas9 cleaved only regions with NGG, GAA, and AGY PAM sequences [53]. These variants of Cas9 can be used to induce mutations in targets devoid of NGG PAMs.

After choosing the most appropriate Cas9 variant, some additional actions are required to increase its efficiency, such as codon optimization, adding nuclear localization signals (NLSs), and the insertion of introns. Grützner et al. [54] compared the efficiency of different Cas9 nucleases in *Arabidopsis*: a human codon-optimized Cas9 with a C-terminal NLS, a maize codon-optimized Cas9 with a C-terminal NLS and with/out an additional N-terminal NLS, and a zCas9 with 13 *Arabidopsis* introns in its sequence that contains a C-terminal NLS with/out an additional N-terminal NLS. They showed that constructs with introns work better than those without introns and two NLSs seem better than one. In the same work, it was found that a Cas9 with introns also proved superior in *Nicotiana benthamiana* and *Catharanthus roseus*.

2.6. Chimeric Deactivated Cas9 Proteins and Their Applications

The Cas9 enzyme can cleave DNA fragments because of its RuvC and HNH nuclease domains. Point mutations in the nuclease domains of SpCas9 can inactivate its RuvC and HNH domains and create a modified Cas9 protein [55]. The D10A mutation (in the RuvC domain) and the H840A mutation (in the HNH domain) both create a nickase enzyme (nCas9). Together, these two mutations result in a nuclease-dead Cas9 (dCas9), which is unable to cleave target DNA while retaining its ability to bind to target DNA with the help of a gRNA [55]. Fusion of nCas9 or dCas9 peptides to other proteins or protein domains can enable other DNA modifications at targeted loci. Such chimeric

proteins can be used for specific purposes such as base editing, transcriptional repression, epigenetic modification, and in vivo labeling (reviewed in [37]).

Multiplex base alterations can be used to create multiple substitutions, thus facilitating the directed evolution of plant genes [48]. In a base editing procedure, gRNAs should be designed such that the targeted base is located between positions 4 and 8 in the gRNA sequences (editing window), counting the end distal to the PAM as position 1 [56]. Two CBEs have been conducted in soybean, and their results showed that the editing window of nCas9-APOBEC1 is located between positions 5–7 in the gRNA sequence, counting the end distal to the PAM as position 1 [57][58].

2.7. Gene Regulatory Elements (GREs) of gRNAs and Cas9 Nuclease Cassettes

Promotors and terminators used in gRNA and Cas9 cassettes are the other critical parameters for achieving an efficient genome edition. Finding the appropriate promoters is crucial as off-target activities can increase markedly when concentrations of the Cas9-gRNA complex are excessive [33]. Two types of RNA polymerase III promoters, including U6/U3, are the most commonly used promoters to express gRNAs in plants [38]. Finding the appropriate variant(s) of U6/U3 promoters is an important step in optimizing gRNA expression as there are various versions of these promoters [33]. Endogenous U6/U3 promoters can lead to better editing outcomes than foreign promoters. In soybean, editing efficiency was significantly increased when the GmU6 promoter (14.7–20.2%) was used instead of the AtU6 promoter (3.2–9.7%) [38]. Similar results were reported when comparing the GmU6-16g-1 promoter (43.4–48.1%) to the AtU6-26 promoter (11.7–18.1%) via soybean hairy-root transformation [59].

Different types of promoters can be used: (i) constitutive (from viruses or plants), (ii) tissue-specific, (iii) inducible, and (iv) developmentally regulated promoters. Constitutive promoters of viral origin (CaMV35S and NOS) derived from plant housekeeping genes (UBIQUITIN or ACTIN) are the most commonly used. Broadly, higher efficiency of plant-derived promoters has been reported [33].

Terminators are other GREs that can affect the stability of Cas9 and gRNA transcripts. RNA Pol II readthrough can interfere with RNA Pol III-mediated transcription of gRNAs can happen when the Cas9 cassette has a weak terminator and both the Cas9 and gRNA expression cassettes are in the same orientation [33]. This can be minimized by using a strong terminator in the Cas9 transcription unit or using an opposite orientation of the gRNA and Cas9 expression cassettes (head-to-head) in the binary vector [34]. A comparative analysis of different terminators revealed that the rbcS-E9 terminator (from *Pisum sativum*) was the best terminator [33].

2.8. Configuration of gRNA and Cas9 Cassettes

Overall, there are three options to achieve co-expression of the gRNA and Cas9 cassettes: (i) a single transcriptional unit (STU), (ii) a two-component transcriptional unit (TCTU), or (iii) a bidirectional promoter system [60][61]. In the STU approach, the expression of both cassettes is jointly driven by a single Pol II promotor. In conventional TCTU, the gRNA is under the control of a pol III promoter (U3 or U6), and the Cas9 cassette is driven by a pol II promoter [61]. When inducible or tissue-specific expression is needed, an STU approach is best [62],

although there can be negative impacts of such a configuration on Cas mRNA maturation and gRNA stability [61]. In a comparison of the STU and TCTU configurations in soybean, TCTU was proven to be a better option than STU [63]. Generally, the editing rate is target-dependent; therefore, finding the best configuration for optimized co-expression of the gRNA and Cas9 requires trial and error [60]. Overall, these various possible components and configurations offer a range of possibilities but also make the optimization of a CRISPR/Cas system complex and multi-factorial (**Figure 2**).
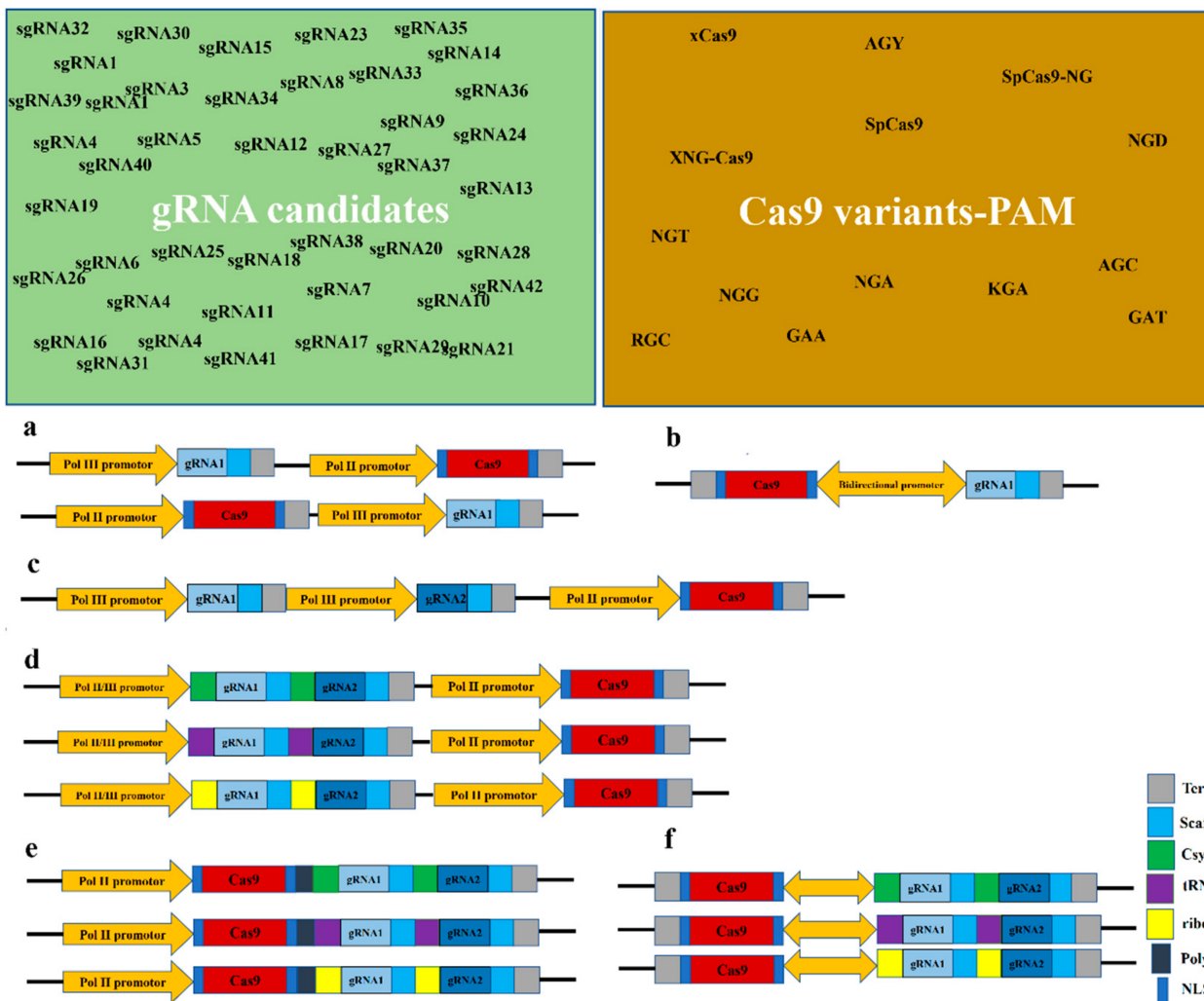

Figure 2. The multi-factorial nature of CRISPR/Cas9-based genome editing procedure considering configuration of gRNA and Cas9 expression cassettes along with different RNA processing strategies in multiplex gRNA experiments. (**a**) Simple TCTU configuration of gRNA and Cas9 cassettes in left and right borders of T-DNA. (**b**) Expression of gRNA and Cas9 cassettes in bidirectional promoter system. (**c**) Simple multiplex gRNA in TCTU system. (**d**) Multiplex gRNA in TCTU system with Csy4, tRNA, and ribozyme processing machines. (**e**) Multiplex gRNA in STU configuration with Csy4, tRNA, and ribozyme processing machines. (**f**) Bidirectional configuration of Cas9 and multiplex gRNA with Csy4, tRNA, and ribozyme processing machines.

References

1. Kuma, K.M.; Lopes-Caitar, V.S.; Romero, C.C.T.; Silva, S.M.H.; Kuwahara, M.K.; Carvalho, M.C.C.G.; Abdelnoor, R.V.; Dias, W.P.; Marcelino-Guimarães, F.C. A High Efficient Protocol for Soybean Root Transformation by Agrobacterium rhizogenes and Most Stable Reference Genes for RT-QPCR Analysis. Plant Cell Rep. 2015, 34, 1987–2000.

2. Sahu, P.K.; Sao, R.; Mondal, S.; Vishwakarma, G.; Gupta, S.K.; Kumar, V.; Singh, S.; Sharma, D.; Das, B.K. Next Generation Sequencing Based Forward Genetic Approaches for Identification and Mapping of Causal Mutations in Crop Plants: A Comprehensive Review. Plants 2020, 9, 1355.

3. Shalem, O.; Sanjana, N.E.; Zhang, F. High-Throughput Functional Genomics Using CRISPR–Cas9. Nat. Rev. Genet. 2015, 16, 299–311.

4. Alonso, J.M.; Ecker, J.R. Moving Forward in Reverse: Genetic Technologies to Enable Genome-Wide Phenomic Screens in Arabidopsis. Nat. Rev. Genet. 2006, 7, 524–536.

5. Bezie, Y.; Tilahun, T.; Atnaf, M.; Taye, M. The Potential Applications of Site-Directed Mutagenesis for Crop Improvement: A Review. J. Crop Sci. Biotechnol. 2021, 24, 229–244.

6. Anjanappa, R.B.; Gruissem, W. Current Progress and Challenges in Crop Genetic Transformation. J. Plant Physiol. 2021, 261, 153411.

7. Joung, Y.H.; Choi, P.-S.; Kwon, S.-Y.; Harn, C.H. Plant Transformation Methods and Applications. In Current Technologies in Plant Molecular Breeding; Springer: Dordrecht, The Netherlands, 2015; pp. 297–343.

8. Rivera, A.L.; Gómez-Lim, M.; Fernández, F.; Loske, A.M. Physical Methods for Genetic Plant Transformation. Phys. Life Rev. 2012, 9, 308–345.

9. Niazian, M.; Sadat Noori, S.A.; Galuszka, P.; Mortazavian, S.M.M. Tissue Culture-Based Agrobacterium-Mediated and in Planta Transformation Methods. Czech J. Genet. Plant Breed. 2017, 53, 133–143.

10. Dalla Costa, L.; Piazza, S.; Pompili, V.; Salvagnin, U.; Cestaro, A.; Moffa, L.; Vittani, L.; Moser, C.; Malnoy, M. Strategies to Produce T-DNA Free CRISPRed Fruit Trees via Agrobacterium Tumefaciens Stable Gene Transfer. Sci. Rep. 2020, 10, 20155.

11. Opabode, J.T. Agrobacterium-Mediated Transformation of Plants: Emerging Factors That Influence Efficiency. Biotechnol. Mol. Biol. Rev. 2006, 1, 12–20.

12. Atif, R.M.; Patat-Ochatt, E.M.; Svabova, L.; Ondrej, V.; Klenoticova, H.; Jacas, L.; Griga, M.; Ochatt, S.J. Gene Transfer in Legumes; Springer: Berlin/Heidelberg, Germany, 2013; pp. 37–100.

13. Collado, R.; Bermúdez-Caraballoso, I.; García, L.R.; Veitía, N.; Torres, D.; Romero, C.; Angenon, G. Epicotyl Sections as Targets for Plant Regeneration and Transient Transformation of Common Bean Using Agrobacterium Tumefaciens. Vitr. Cell. Dev. Biol. Plant 2016, 52, 500–511.

14. Tyurin, A.A.; Suhorukova, A.V.; Kabardaeva, K.V.; Goldenkova-Pavlova, I.V. Transient Gene Expression Is an Effective Experimental Tool for the Research into the Fine Mechanisms of Plant Gene Function: Advantages, Limitations, and Solutions. Plants 2020, 9, 1187.

15. Paolis, A.; Frugis, G.; Giannino, D.; Iannelli, M.; Mele, G.; Rugini, E.; Silvestri, C.; Sparvoli, F.; Testone, G.; Mauro, M.; et al. Plant Cellular and Molecular Biotechnology: Following Mariotti's Steps. Plants 2019, 8, 18.

16. Iantcheva, A.; Mysore, K.S.; Ratet, P. Transformation of Leguminous Plants to Study Symbiotic Interactions. Int. J. Dev. Biol. 2013, 57, 577–586.

17. Zhong, C.; Nambiar-Veetil, M.; Bogusz, D.; Franche, C. Hairy Roots as a Tool for the Functional Analysis of Plant Genes. In Hairy Roots; Springer: Singapore, 2018; pp. 275–292.

18. Alagarsamy, K.; Shamala, L.F.; Wei, S. Protocol: High-Efficiency in-Planta Agrobacterium-Mediated Transgenic Hairy Root Induction of Camellia Sinensis Var. Sinensis. Plant Methods 2018, 14, 17.

19. Subramanian, S.; Stacey, G.; Yu, O. Endogenous Isoflavones Are Essential for the Establishment of Symbiosis between Soybean and Bradyrhizobium Japonicum. Plant J. 2006, 48, 261–273.

20. Hao, Y.-J.; Wei, W.; Song, Q.-X.; Chen, H.-W.; Zhang, Y.-Q.; Wang, F.; Zou, H.-F.; Lei, G.; Tian, A.-G.; Zhang, W.-K.; et al. Soybean NAC Transcription Factors Promote Abiotic Stress Tolerance and Lateral Root Formation in Transgenic Plants. Plant J. 2011, 68, 302–313.

21. Pan, W.-J.; Tao, J.-J.; Cheng, T.; Bian, X.-H.; Wei, W.; Zhang, W.-K.; Ma, B.; Chen, S.-Y.; Zhang, J.-S. Soybean MiR172a Improves Salt Tolerance and Can Function as a Long-Distance Signal. Mol. Plant 2016, 9, 1337–1340.

22. White, L.J.; Ge, X.; Brözel, V.S.; Subramanian, S. Root Isoflavonoids and Hairy Root Transformation Influence Key Bacterial Taxa in the Soybean Rhizosphere. Environ. Microbiol. 2017, 19, 1391–1406.

23. Du, W.; Ning, L.; Liu, Y.; Zhang, S.; Yang, Y.; Wang, Q.; Chao, S.; Yang, H.; Huang, F.; Cheng, H.; et al. Identification of Loci and Candidate Gene GmSPX-RING1 Responsible for Phosphorus Efficiency in Soybean via Genome-Wide Association Analysis. BMC Genom. 2020, 21, 725.

24. Yu, Q.; Liu, Y.-L.; Sun, G.-Z.; Liu, Y.-X.; Chen, J.; Zhou, Y.-B.; Chen, M.; Ma, Y.-Z.; Xu, Z.-S.; Lan, J.-H. Genome-Wide Analysis of the Soybean Calmodulin-Binding Protein 60 Family and Identification of GmCBP60A-1 Responses to Drought and Salt Stresses. Int. J. Mol. Sci. 2021, 22, 13501.

25. Amritha, P.P.; Shah, J.M. Can Genetic Engineering-Based Methods for Gene Function Identification Be Eclipsed by Genome Editing in Plants? A Comparison of Methodologies. Mol. Genet. Genom. 2021, 296, 485–500.

26. Alok, A.; Sharma, S.; Kumar, J.; Verma, S.; Sood, H. Engineering in Plant Genome Using Agrobacterium: Progress and Future. In Metabolic Engineering for Bioactive Compounds; Springer: Singapore, 2017; pp. 91–111.

27. Small, I. RNAi for Revealing and Engineering Plant Gene Functions. Curr. Opin. Biotechnol. 2007, 18, 148–153.

28. Bao, A.; Zhang, C.; Huang, Y.; Chen, H.; Zhou, X.; Cao, D. Genome Editing Technology and Application in Soybean Improvement. Oil Crop Sci. 2020, 5, 31–40.

29. Jaganathan, D.; Ramasamy, K.; Sellamuthu, G.; Jayabalan, S.; Venkataraman, G. CRISPR for Crop Improvement: An Update Review. Front. Plant Sci. 2018, 9, 1–17.

30. Ran, F.A.; Hsu, P.D.; Wright, J.; Agarwala, V.; Scott, D.A.; Zhang, F. Genome Engineering Using the CRISPR-Cas9 System. Nat. Protoc. 2013, 8, 2281–2308.

31. Tyagi, S.; Kumar, R.; Das, A.; Won, S.Y.; Shukla, P. CRISPR-Cas9 System: A Genome-Editing Tool with Endless Possibilities. J. Biotechnol. 2020, 319, 36–53.

32. Rao, M.J.; Wang, L. CRISPR/Cas9 Technology for Improving Agronomic Traits and Future Prospective in Agriculture. Planta 2021, 254, 68.

33. Hassan, M.M.; Zhang, Y.; Yuan, G.; De, K.; Chen, J.-G.; Muchero, W.; Tuskan, G.A.; Qi, Y.; Yang, X. Construct Design for CRISPR/Cas-Based Genome Editing in Plants. Trends Plant Sci. 2021, 26, 1133–1152.

34. Chen, L.; Cai, Y.; Hou, W. Generation of Knockout and Fragment Deletion Mutants in Soybean by CRISPR-Cas9; Humana: New York, NY, USA, 2021; pp. 123–135.

35. Karmakar, S.; Behera, D.; Baig, M.J.; Molla, K.A. In Vitro Cas9 Cleavage Assay to Check Guide RNA Efficiency; Humana: New York, NY, USA, 2021; pp. 23–39.

36. Molla, K.A.; Yang, Y. Predicting CRISPR/Cas9-Induced Mutations for Precise Genome Editing. Trends Biotechnol. 2020, 38, 136–141.

37. Gayen, D.; Karmakar, S. Designing, Performing, and Analyzing CRISPR-Cas9-Mediated Genome Editing Experiments in Leguminous Plants; Humana: New York, NY, USA, 2021; pp. 103–122.

38. Sun, X.; Hu, Z.; Chen, R.; Jiang, Q.; Song, G.; Zhang, H.; Xi, Y. Targeted Mutagenesis in Soybean Using the CRISPR-Cas9 System. Sci. Rep. 2015, 5, 10342.

39. Hussain, B.; Lucas, S.J.; Budak, H. CRISPR/Cas9 in Plants: At Play in the Genome and at Work for Crop Improvement. Brief. Funct. Genom. 2018, 17, 319–328.

40. Hassan, M.M.; Chowdhury, A.K.; Islam, T. In Silico Analysis of GRNA Secondary Structure to Predict Its Efficacy for Plant Genome Editing. Front. Oncol. 2021, 15–22.

41. Miki, D.; Zinta, G.; Zhang, W.; Peng, F.; Feng, Z.; Zhu, J.-K. CRISPR/Cas9-Based Genome Editing Toolbox for Arabidopsis Thaliana. In Arabidopsis Protocols; Humana: New York, NY, USA, 2021; pp. 121–146.

42. Wang, L.; Wang, L.; Zhou, Y.; Duanmu, D. Use of CRISPR/Cas9 for Symbiotic Nitrogen Fixation Research in Legumes. Prog. Mol. Biol. Trans. Sci. 2017, 149, 187–213.

43. Zheng, N.; Li, T.; Dittman, J.D.; Su, J.; Li, R.; Gassmann, W.; Peng, D.; Whitham, S.A.; Liu, S.; Yang, B. CRISPR/Cas9-Based Gene Editing Using Egg Cell-Specific Promoters in Arabidopsis and Soybean. Front. Plant Sci. 2020, 11, 1–15.

44. Gibson, D.G.; Young, L.; Chuang, R.-Y.; Venter, J.C.; Hutchison, C.A.; Smith, H.O. Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases. Nat. Methods 2009, 6, 343–345.

45. Sarrion-Perdigones, A.; Falconi, E.E.; Zandalinas, S.I.; Juárez, P.; Fernández-del-Carmen, A.; Granell, A.; Orzaez, D. GoldenBraid: An Iterative Cloning System for Standardized Assembly of Reusable Genetic Modules. PLoS ONE 2011, 6, e21622.

46. Aliaga-Franco, N.; Zhang, C.; Presa, S.; Srivastava, A.K.; Granell, A.; Alabadí, D.; Sadanandom, A.; Blázquez, M.A.; Minguet, E.G. Identification of Transgene-Free CRISPR-Edited Plants of Rice, Tomato, and Arabidopsis by Monitoring DsRED Fluorescence in Dry Seeds. Front. Plant Sci. 2019, 10, 1–9.

47. Pavese, V.; Moglia, A.; Corredoira, E.; Martínez, M.T.; Torello Marinoni, D.; Botta, R. First Report of CRISPR/Cas9 Gene Editing in Castanea Sativa Mill. Front. Plant Sci. 2021, 12, 1–11.

48. Abdelrahman, M.; Wei, Z.; Rohila, J.S.; Zhao, K. Multiplex Genome-Editing Technologies for Revolutionizing Plant Biology and Crop Improvement. Front. Plant Sci. 2021, 12, 1–15.

49. Raitskin, O.; Patron, N.J. Multi-Gene Engineering in Plants with RNA-Guided Cas9 Nuclease. Curr. Opin. Biotechnol. 2016, 37, 69–75.

50. Luo, Y.; Na, R.; Nowak, J.S.; Qiu, Y.; Lu, Q.S.; Yang, C.; Marsolais, F.; Tian, L. Development of a Csy4-Processed Guide RNA Delivery System with Soybean-Infecting Virus ALSV for Genome Editing. BMC Plant Biol. 2021, 21, 419.

51. Hsu, P.D.; Scott, D.A.; Weinstein, J.A.; Ran, F.A.; Konermann, S.; Agarwala, V.; Li, Y.; Fine, E.J.; Wu, X.; Shalem, O.; et al. DNA Targeting Specificity of RNA-Guided Cas9 Nucleases. Nat. Biotechnol. 2013, 31, 827–832.

52. Ge, Z.; Zheng, L.; Zhao, Y.; Jiang, J.; Zhang, E.J.; Liu, T.; Gu, H.; Qu, L. Engineered XCas9 and SpCas9-NG Variants Broaden PAM Recognition Sites to Generate Mutations in Arabidopsis Plants. Plant Biotechnol. J. 2019, 17, 1865–1867.

53. He, R.; Zhang, P.; Yan, Y.; Yu, C.; Jiang, L.; Zhu, Y.; Wang, D. Expanding the Range of CRISPR/Cas9-Directed Genome Editing in Soybean. aBIOTECH 2021, 1–10.

54. Grützner, R.; Martin, P.; Horn, C.; Mortensen, S.; Cram, E.J.; Lee-Parsons, C.W.T.; Stuttmann, J.; Marillonnet, S. High-Efficiency Genome Editing in Plants Mediated by a Cas9 Gene Containing Multiple Introns. Plant Commun. 2021, 2, 100135.

55. Molla, K.A.; Yang, Y. CRISPR/Cas-Mediated Base Editing: Technical Considerations and Practical Applications. Trends Biotechnol. 2019, 37, 1121–1142.

56. Chen, L.; Cai, Y.; Hou, W. Targeted Base Editing in Soybean Using a CRISPR-Cas9 Cytidine Deaminase Fusion; Humana: New York, NY, USA, 2021; pp. 137–148.

57. Kang, B.-C.; Woo, J.W.; Kim, S.-T.; Bae, S.-J.; Choi, M.; Kim, J.-S.; Kim, S.-G. Guidelines for C to T Base Editing in Plants: Base-Editing Window, Guide RNA Length, and Efficient Promoter. Plant Biotechnol. Rep. 2019, 13, 533–541.

58. Cai, Y.; Chen, L.; Zhang, Y.; Yuan, S.; Su, Q.; Sun, S.; Wu, C.; Yao, W.; Han, T.; Hou, W. Target Base Editing in Soybean Using a Modified CRISPR/Cas9 System. Plant Biotechnol. J. 2020, 18, 1996–1998.

59. Du, H.; Zeng, X.; Zhao, M.; Cui, X.; Wang, Q.; Yang, H.; Cheng, H.; Yu, D. Efficient Targeted Mutagenesis in Soybean by TALENs and CRISPR/Cas9. J. Biotechnol. 2016, 217, 90–97.

60. Hsieh-Feng, V.; Yang, Y. Efficient Expression of Multiple Guide RNAs for CRISPR/Cas Genome Editing. aBIOTECH 2020, 1, 123–134.

61. Zhong, Z.; Liu, S.; Liu, X.; Liu, B.; Tang, X.; Ren, Q.; Zhou, J.; Zheng, X.; Qi, Y.; Zhang, Y. Intron-Based Single Transcript Unit CRISPR Systems for Plant Genome Editing. Rice 2020, 13, 8.

62. Lowder, L.G.; Zhang, D.; Baltes, N.J.; Paul, J.W.; Tang, X.; Zheng, X.; Voytas, D.F.; Hsieh, T.-F.; Zhang, Y.; Qi, Y. A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation. Plant Physiol. 2015, 169, 971–985.

63. Carrijo, J.; Illa-Berenguer, E.; LaFayette, P.; Torres, N.; Aragão, F.J.L.; Parrott, W.; Vianna, G.R. Two Efficient CRISPR/Cas9 Systems for Gene Editing in Soybean. Transgenic Res. 2021, 30, 239–249.