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REVIEW

Application of Transgenic Technology in Identification for Gene Function on Grasses

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ABSTRACT

Perennial grasses have developed intricate mechanisms to adapt to diverse environments, enabling their resistance to various biotic and abiotic stressors. These mechanisms arise from strong natural selection that contributes to enhancing the adaptation of forage plants to various stress conditions. Methods such as antisense RNA technology, *CRISPR/Cas9* screening, virus-induced gene silencing, and transgenic technology, are commonly utilized for investigating the stress response functionalities of grass genes in both warm-season and cool-season varieties. This review focuses on the functional identification of stress-resistance genes and regulatory elements in grasses. It synthesizes recent studies on mining functional genes, regulatory genes, and protein kinase-like signaling factors involved in stress responses in grasses. Additionally, the review outlines future research directions, providing theoretical support and references for further exploration of (i) molecular mechanisms underlying grass stress responses, (ii) cultivation and domestication of herbage, (iii) development of high-yield varieties resistant to stress, and (iv) mechanisms and breeding strategies for stress resistance in grasses.

KEYWORDS

Grasses; regulatory genes; protein kinase-like signaling factors; gene function identification; resistance breeding

1 Introduction

Biologists have long relied on traditional genetic methods, utilizing mutants to study positional cloning, identify plant sequences, and confirm gene function through genetic transformation. However, with rapid advancements in structural genomics and high-throughput analysis, the field of functional genomics has emerged [1]. The aim of this study was to analyze the overall functions of genes, including coding genes, small RNA molecules, non-coding RNA, and metabolites, to explain how they interact and influence biological phenotypes. as well as growth, development, and metabolism. The selection and identification of functional genes (including coding and non-coding genes) form the basis for studying how genes regulate biological processes and variations. However, the application of functional genomics relies on the development and utilization of new techniques and methods.

Typically, two approaches are typically used to isolate the functional genes responsible for specific phenotypes or biological processes. Firstly, the traits are studied, and positive genetic screening methods

are used to identify functional genes. Researchers using this approach typically focus on specific phenotypes exhibited by individual organisms or cells and aim to identify genes associated with these phenotypes and reveal their functions; therefore, the method requires the presence of well-defined biological phenotypes. Candidate genes are obtained through molecular biology or genetics methods, followed by evaluation and functional validation.

Alternatively, related traits and gene functions can also be studied using reverse from the perspective of genes (i.e., an identification method based on reverse genetics) [2]. Reverse genetics directly examines the function of a gene or DNA sequence by altering its expression and identifying the resulting mutant phenotype. Numerous methods are used to screen functional genes, such as cDNA library technology [3], RNA interference (RNAi) library technology [4,5], antisense RNA technology, targeted knockout, miRNA-based gene silencing [6], *CRISPR/Cas9* screening technology [7], viral-induced gene silencing (VIGS) technology, insertion mutation technology [7], and other model biological screening technologies [8]. Multiple reverse genetics methods have been used to identify functional genes in various grasses.

Recent advancements in techniques have led to an increased recognition of resistance genes in grasses. Several resistance-related functional genes have been studied in grasses, such as *Festuca elata* [9], *Elymus dahuricus* [10,11], *Agropyron cristatum* [12], *Agrostis stolonifera* [13], *Cynodon dactylon* [14], *Zoysia japonica* [15] and *Paspalum notatum* [16]. These genes can be categorized based on function. The first category comprises functional genes that encode proteins directly protecting grasses under stress, such as *COMT* [9] and other genes affecting biomacromolecules and membrane structures. The second category includes regulatory genes, such as resistance regulatory genes, protein kinase-like signaling factors under stress [14], and transcription factors (TFs) like bZIP [16], MYB/MYC [17] and WRKY [18,19]. Among these, the TF regulatory family is the most studied [20–26]. The proteins encoded by these genes play a role in signal transduction pathways and regulate stress-related gene expression. Studying the genes with key roles in stress responses, understanding the functions of these resistance-related genes in grasses, and analyzing gene function have significant theoretical importance. This research may also lead to applications in cultivating and domesticating herbage and developing new varieties with high yield and resistance to stress.

2 Screening and Identification of Functional Genes in Grasses

2.1 Screening of Functional Genes Based on Forward Genetics Screening Strategy

Functional genes are typically screened using a forward genetic approach, which focuses on specific phenotypes exhibited by individual organisms or cells to identify genes associated with these phenotypes and elucidate their functions [27]. This strategy requires well-defined biological phenotypes, followed by obtaining candidate genes through molecular biology or genetic methods, and subsequently evaluating and validating their functions. Specifically, the screening of functional genes includes five categories: (i) traditional genetic analysis [28–32] (Table 1). (ii) Gene mutation analysis, based on DNA/RNA sequencing [33–37]. (iii) Whole-genome sequencing technology or genome-wide association studies [38–41]. (v) Expression profile analyses [42–45]. (iv) Bioinformatics technology [46–49]. This approach has been extensively used to explore stress-responsive genes in various species, such as *Secale strictum* (C. Presl) [50], *Agrostis stolonifera* [51], *Avena sativa* L. [52], *Paspalum vaginatum*, and *Paspalum fasciculatum* [53,54]. Studies that have used these analytical techniques can serve as valuable references for future investigations of grass gene functions.

2.2 Identification of Functional Genes Based on Reverse Genetics

Recently, the complete genome sequences of model plants (e.g., *Arabidopsis thaliana*) and other plants have allowed for the establishment of large-scale databases of genome sequence information, and the term “reverse genetics” has been coined, with numerous methods available for screening functional genes using

reverse genetics. At present, it mainly includes cDNA library technology [3], RNA interference (RNAi) technology [4], antisense RNA technology, *CRISPR/Cas9* screening technology [7], virus-induced gene silencing (VIGS) technology, *Agrobacterium*-mediated transgenic method [55], and insertion mutation technology [8]. The following is a brief description of the application scope, advantages and disadvantages of three more widely used gene function identification techniques.

Table 1: Commonly screening methods of functional genes method based on forward genetics screening strategy

Method	Application areas	Species example	Reference
Traditional genetic analysis	**Scope:** Refers to classical methods used in genetics to study inheritance patterns, gene mapping, and genetic variation in organisms.	*Polypogon fugax* Nees ex Steud.,	[27–31]
	Technology and Application: Typically include Mendelian crosses, linkage analysis, and genetic mapping using molecular markers like RFLPs (Restriction Fragment Length Polymorphisms) or SSRs (Simple Sequence Repeats).	*Beckmannia syzigachne*	
	Advantages: Traditional genetic analysis has been foundational in understanding the basic principles of genetics and has paved the way for more advanced molecular techniques in contemporary genetic research.		
Gene mutation analysis based on DNA/RNA sequencing	**Scope:** Gene mutation analysis based on DNA or RNA sequencing involves several steps to identify and characterize genetic variations.	*Agrostis stolonifera*,	[32–37]
	Technology and Application: Including ample collection and preparation, library preparation, sequencing, alignment and mapping, variant calling, annotation and prioritization, validation: Interpretation, reporting 9 steps.	*Elymus nutans*,	
	Advantages: Each of these steps requires specialized bioinformatics tools and expertise to accurately analyze sequencing data and interpret genetic variants.	*Poa pratensis* L.	
Whole genome sequencing technology	**Scope:** WGS involves sequencing the entire genome of an organism, which includes all its DNA, including coding and non-coding regions.	*Secale strictum*,	[38–41]
	Technology: WGS utilizes high-throughput sequencing technologies (e.g., Illumina sequencing) to generate millions to billions of short DNA sequences (reads).	*Avena sativa* L.,	

(Continued)

Table 1 (continued)

Method	Application areas	Species example	Reference
	Application: WGS allows comprehensive analysis of an individual's genetic makeup, facilitating studies on population genetics, evolutionary biology, and understanding the genetic basis of complex traits.	*Dactylis glomerata*,	
	Advantages: Provides a comprehensive view of an individual's genetic variation, including rare and novel variants. Enables identification of *de novo* mutations and structural variants.	*Secale strictum (C. Presl)*	
Expression profile analysis	**Scope:** Refers to the systematic study of gene expression patterns across different conditions, tissues, or developmental stages. It aims to quantify and understand how genes are turned on or off in response to various biological factors.	*Agrostis stolonifera*,	[42–45]
	Application and Advantages: It is commonly used for the identification and functional analysis of miRNAs and their target genes related to agronomic traits in some plants, detecting and analyzing the types and expression characteristics of miRNAs and their target genes in different tissues or samples, interpreting the biological functions and metabolic mechanisms of specific miRNAs in some important organs, which can provide important information and basis for the study of the biological functions of miRNAs.	*Dactylis glomerata* L., *Triticum aestivum* L.	
Bioinformatics technology	**Scope and Application:** Bioinformatics technology encompasses a wide range of computational tools, algorithms, databases, and software designed to analyze and interpret biological data, particularly large-scale molecular data generated from genomics, proteomics, metabolomics, and other omics technologies.	*Agrostis stolonifera*, *Avena sativa*, *Zoysiagrasses*, *Elymus nutans*	[46–49]
	Advantages: One of the most widely used techniques, but at the same time needs to be combined with other techniques to discover functional genes.		

2.2.1 CRISPR/Cas Screening and Identification Strategies

Among *CRISPR/Cas* screening and identification strategies, the *CRISPR/Cas9* system stands out as the most efficient method [7,56,57] (Table 2). Numerous studies have shown that transgenic technologies, such as *CRISPR/Cas* and VIGS techniques, are helpful in identifying plant gene function, among which RNAi is a significant reverse genetics tool for analyzing plant gene function and improving genetic quality [56]. Currently, *CRISPR/Cas9* technology finds applications in the treatment of human genetic diseases [58], personalized cell therapy [59], new drug development [60], and improving crop genetics [61]. More than 30 plant species and hundreds of genes have been successfully edited in research aimed at improving crop stress resistance, disease resistance, and insect pests [62,63].

Table 2: Identification method and application of functional genes based on reverse genetics

Method	Application areas	Advantages and disadvantages	Reference
CRISPR/Cas screening	**Technology:** the CRISPR-Cas9 system is the most efficient method. **Application:** CRISPR/Cas9 technology has applications in: treatment of human genetic diseases, personalized cell therapy, new drug development, and improving crop genetics. More than 30 plant species and hundreds of genes have been successfully edited during research related to crop stress resistance, disease resistance, and insect pests.	**Advantages:** high precision; efficiency and scalability, facilitating the study of complex genetic interactions and pathways; permanent modifications; wide applicability; targeting non-coding regions; adaptability, continuous advancements in CRISPR/Cas technology, such as base editing and epigenome editing, enhance its utility and broaden its applications in biological research and biotechnology. **Disadvantages:** off-target effects; efficiency variability, the efficiency of CRISPR/Cas-mediated genome editing can vary depending on the cell type, target gene, delivery method, and guide RNA design; delivery challenges; off-target transcriptome effects; ethical considerations; complexity in analysis; regulatory constraints.	[56–62]
Virus-induced Gene Silencing Techniques (VIGS)	**Scope:** VIGS is a new method for suppressing plant gene expression without requiring a stable transformation regimen or the establishment of large mutant populations. **Technology:** host induction, virus induction, and spray induction.	**Advantages:** rapid and efficient gene knockdown; transient expression; wide host range; no stable integration required, systemic silencing; functional genomics; cost-effective; application to non-model plants; minimal regulatory concerns.	[63–70]

(Continued)

Table 2 (continued)

Method	Application areas	Advantages and disadvantages	Reference
	Application: The diversity of related gene functions has been demonstrated in studies on disease resistance, stress, cell signaling and secondary metabolic biosynthesis pathways. Additionally, VIGS has been widely used to identify of gene functions in vegetable crops and other species, and a series of new correlation models have been constructed.	**Disadvantages:** variable silencing efficiency; partial silencing; off-target effects; viral symptoms; host range limitations; transient nature; limited to specific tissues; size constraints; complex phenotypes; regulatory issues with field applications; technical expertise required.	
Agrobacterium-mediated transgenic method	**Scope:** The procedure of gene function verification, using Agrobacterium-mediated transgenic technology as an example, aims to identify target functional genes for verification through transcriptome sequencing and bioinformatics analysis.	**Advantages:** a model biological screening strategy, widely used owing to its convenient, fast, economical, and easy-to-operate characteristics. Additionally, it helps in identifying the gene family of the target plant in response to abiotic stress at the whole-genome level.	[71–75]
	Technology: Transgenic experiments are conducted to verify the responses and functions of each gene to biological or abiotic stress. **Application:** Agrobacterium-mediated transformation is a powerful and widely used technique in plant biotechnology, and the techniques have been used to study genes in several grass species. Such as crop improvement, biological or abiotic stress tolerance, quality improvement, phytoremediation, products research and development, biofuels and industrial, ornamental plants.	**Disadvantages:** species specificity; integration site variability; regeneration dependency, regeneration protocols can vary significantly between plant species and genotypes, and some plants may be recalcitrant to regeneration; tissue specificity; biosafety concerns; genetic stability.	

2.2.2 Virus-Induced Gene Silencing Techniques (VIGS)

VIGS represents a novel method for suppressing plant gene expression without requiring a stable transformation regimen or the establishment of large mutant populations (Table 2). It can be readily applied to species that are challenging to transform stably or when limited genetic material from a small research population is available. Moreover, it proves beneficial in the study of non-model plant species [64,65]. Currently, VIGS techniques employing host induction [66,67], virus induction [23,26,27], and spray induction [68,69] have been used to elucidate gene function. Research has showcased the diverse roles of related genes in disease resistance, stress responses, cell signaling and secondary metabolic

biosynthesis pathways. Additionally, VIGS has been widely utilized to identify of gene functions in vegetable crops and other species [70,71], leading to the establishment of new correlation models [70,71].

2.2.3 Agrobacterium-Mediated Transgenic Method

The *Agrobacterium*-mediated transgenic method, a prominent model biological screening strategy, is widely favored due to its convenient, fast, economical, and easy-to-operate characteristics (Table 2). Researchers frequently utilize transgenic approaches to overexpress target genes in plants for functional verification purposes. Using *Agrobacterium*-mediated transgenic technology as an example, the process of gene function verification involves identifying target functional genes through transcriptome sequencing and bioinformatics analysis. Additionally, it facilitates comprehensive exploration of the gene families of target plants in response to abiotic stress at the genome-wide level. Transgenic experiments validate the responses and functions of each gene under abiotic stress conditions [72], with several grass species serving as subjects for gene studies [73–76].

3 Application of the Latest Methods of Gene Function Identification in Grass Stress

Stress-inducing factors in grasses include drought, injury, reactive oxygen species (ROS), phosphorus deficiency, salinity, heat, cold, flooding, and heavy metals [77]. Grasses primarily adapt to harsh environmental conditions through dynamic adjustments at morphological, physiological, and biochemical levels, particularly during nutritional and reproductive growth stages. Therefore, exploring, studying, and utilizing the stress-resistance mechanisms of grasses using genomics and molecular biology is crucial. For example, many low-temperature stress response genes have been identified in *A. thaliana*, with most cold-responsive (COR) genes enriched in the starch-sucrose [78]. Studies have demonstrated that proanthocyanidins are important in providing resistance to various stresses (e.g., UV damage, low-temperature damage, drought, flood stress, and bacterial attack).

Numerous studies have highlighted the limitations of single-omics techniques, whereas multi-omics analysis techniques, combining transcriptomics and metabolomics, offer a comprehensive approach to analyzing plant resistance [79]. The multi-technology combination mode of bioinformatics analysis, transcriptomic sequencing analysis, and transgenic technology has been used to identify grass gene function [80]. The combinations of these technologies have been applied to warm-season turf grasses (e.g., *C. dactylon* [81], *Z. japonica* [82–88], and *P. notatum* [89]) and cool-season turfgrasses (e.g., *F. elata* [35–37], *E. dahuricus* [9], *A. cristatum* [13] and *A. stolonifera* [90]). Cold-season turfgrass stress-response genes have been identified and studied [91] Categorizing these studies reveals that researchers usually choose to analyze the resistance mechanism for diverse gene types (Table 3). These categories are summarized below.

3.1 Grass Functional Gene

Functional genes of grasses include three categories (Table 3): The first category is growth regulatory genes, functional identification is primarily performed using transgenic technology or a combination of transgenic technology and gene silencing technology [9,16]. The second group is genes for enzymes that scavenge ROS, these exert detoxification and antioxidant effects [89,92]. The third category is active genes that protect biomolecules, such as osmoregulatory protein-related genes, dehydration protein genes, aquaporin genes, plum genes, and late-embryogenetic-rich proteins [56].

3.2 Grass Regulatory Genes

Grass regulatory genes encompass resistance regulatory genes [10,11], transcription factors (TFs) [93], and protein kinase signaling factors [14] and other protein functional genes involved in stress responses (Table 3). Transgenic technology is commonly employed for identifying these functional genes, often in

combination with gene silencing or knockout techniques for functional validation. The following section provides an overview of the most extensively studied grass regulatory genes in grasses.

3.2.1 Resistance Regulatory Gene

Transgenic technology is the main method for studying resistance-regulatory genes in grasses. *Agrobacterium*-mediated transgenic technology was used to study the H^+- pyrophosphatases (PP) enzyme gene *AtAVP1* from *E. dahuricus*; it was found that H^+-PP enhances low-nitrogen stress tolerance in transgenic *Arabidopsis* and wheat by interacting with a receptor-like protein kinase [10]. This technology was used to transfer genes from wheat and tobacco plants to explore the action mechanism of the type I H^+-PP gene *EdVP1* from *E. dahuricus* under low potassium stress. The results showed that overexpression of the V-type H^+-PP gene *EdVP1* increased the yield and potassium uptake of transgenic wheat [11].

3.2.2 Transcription Factors (TFs)

TFs are central regulators of changes in gene expression [93] and can be categorized based on the characteristics of their structural domains into the following families: WRKY (WRKYGQ), MYB/MYC, NAC (NAM, ATAF, and CUC), Apetala2/Ethylene Responsive Factor (AP2/ERF/EREBP), SQUAMOSA promoter-binding protein-like (SPL), TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP), basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), CBF/dehydration responsive element protein 1 (CBFs/DREB1), HB, Zn-C2H2, MADS, ARF-Aux/IAA, APETALA1/FRUITFULL (AP1/FUL) and others [22]. TFs regulate plant metabolism, development, biosynthesis, other life processes, and response to abiotic stresses; therefore, they represent excellent potential candidate genes that could identify resistant plants [94]. The function of TFs genes is typically determined through transgenic technology. In grasses, the function of TFs genes is usually related to the plant's response to abiotic stress.

WRKY-Like Transcription Factors

TFs in the WRKY family are plant-specific and play crucial roles in responses to abiotic stresses, such as cold, salt, and drought [95,96]. WRKYs are also instrumental in growth and development processes, including seed germination [97], flowering [98,99], anthocyanin biosynthesis [100], and leaf senescence [101]. Most WRKYs positively influence cold tolerance. For instance, in Bermuda grass, more than 100 WRKYs were identified using full-length transcriptome data [99], and 23 displayed cold-induced expression patterns [102]. Using transgenic technology and VIGS, Bermuda grass *CdWRKY2* positively regulated cold tolerance by coordinately activating the *CdSPS1*-involved sucrose synthesis and *CdCBF1*-dependent signaling pathways. Similarly, rice WRKYs, such as *OsWRKY71* and *OsWRKY76*, have been reported to enhance cold resistance [19]. Overexpression of *VaWRKY12* and *VaWRKY33* consistently conferred cold resistance in transgenic *Arabidopsis* and *Vitis vinifera* [103,104]. Additionally, transgenic *Arabidopsis* plants overexpressing *TaWRKY19* showed improved freezing tolerance. These gene identification techniques have also been applied to identify WRKY-like TFs in wheat [105,106].

Researchers have also demonstrated that the Bermuda grass *CdWRKY50* gene negatively regulates plant responses to salt stress using transgenic technology and VIGS [18]. Similar results have been reported in other plants. For instance, in *Arabidopsis*, sodium chloride treatment upregulated the transcripts of two closely related WRKYs, and both *Atwrky33* and *Atwrky25* mutants exhibited salt-sensitive phenotypes, whereas overexpression of *AtWRKY25* and *AtWRKY33* enhanced salt tolerance [107]. Overexpression *GmWRKY54* was reported to improve the salinity and drought resistance of transgenic plants, through the regulation of *STZ/Zat10*, whereas *GmWRKY13* demonstrated different functions [108]. Overexpression *FcWRKY40* enhanced salt tolerance in plants by modulating ion balance and proline accumulation [109]. Additionally, researchers utilized transgenic technology to study the regulatory mechanism of *AmWRKY*-like [94] genes in response to drought stress in *Agropyron mongolicum*, showing that *AmWRKY*-like genes were negatively regulated by natural drought-induced expression. Conversely, *AtWRKY34*, which

may be involved in the C-repeat binding factor (CBF) signaling cascade, negatively modulated the cold response of mature pollen [95].

MYB/MYC-Like Transcription Factors

The MYB family is one of the largest TFs families in plants. Studies have shown that *TaLHY*, a 1R-MYB in *Triticum aestivum*, plays a defensive role against stripe rust infection [110]. Rice *OsMYB48-1*, a MYB-related TFs, is involved in drought and salt stress responses by regulating stress-induced abscisic acid (ABA) synthesis [111]. Recent research highlights the key role of MYB/MYC TFs in various stress responses in grasses, such as cold, salt, and drought [112,113]. For example, using transgenic technology, the TFs gene *LcMYB4* was identified as a positive regulator of cold and frost tolerance in transgenic *A. thaliana*, despite its previously unknown function in Sheepgrass (*Leymus chinensis* (Trin.)) [17]. Another study using *Agrobacterium*-mediated transgenic method and a homologous sequence cloning demonstrate that the *AmrMYB1* and *AmrR2R3MYB* genes of *Agropyron michnoi* Roshev positively regulated drought and salt stress [114]. Studies on the *MwMYB4* gene of *A. mongolicum* Keng using transgenic technology showed that *MwMYB4* overexpression improved drought and low-temperature tolerances [112]. Additionally, a novel MYC-type bHLH TF *ZjICE2* from *Z. japonica* was studied by transgene transfer into *Arabidopsis* [15,82]. Its overexpression enhanced cold, drought, and salt stress tolerances, and was closely related to ICE homologs. In wheat, transgenic technology has been used to study the functions of these TFs [115–117].

bZIP-Like Transcription Factors

bZIP TFs regulate gene activities involved in various biological processes related to plant growth and development; the gene *ABI5* is a member of this family. The abscisic acid (ABA) signaling pathway is crucial during the plant's response to salt stress, with *ABI5* serving as an important TFs in the ABA signal transduction pathway. Transgenic overexpression studies in tobacco were conducted to evaluate the role of *Elymus sibiricus* L. abscisic acid-insensitive 5 (*EsABI5*) in the ABA-dependent regulation of the response of *Siberian wildrye* to salt stress. These studies demonstrated that EsABI5 is involved in anti-salt responses and acts as a negative regulator during salt stress [118]. Jia et al. [119] identified 32 bZIP TFs using a combination of transgenic and gene knockout technologies. Knockout of the homologous gene of the most downregulated auxin-responsive *TabZIP6D_20* in *Arabidopsis* (*AtHY5*) negatively affected the auxin signaling pathway. The auxin-responsive *TabZIP* gene likely has several essential functions in wheat leaf structure in response to auxin. Another study reported that the *Arabidopsis* TF *bZIP29* is involved in leaf and root development by altering cell number in the leaf and root meristem [120].

HEMA-Like Transcription Factors

The HEMA family encodes GluTRs in higher plants. Genetic manipulation of Bermuda grass photosynthetic biosynthesis through *Agrobacterium*-mediated transformation established a highly efficient transformation method by screening crucial embryogenic calli. The approach also improved infection efficiency and photosynthetic capacity assessment by *CdHEMA1* overexpression [80]. The overexpression of *CdHEMA1* enhanced the photosynthetic pigment content, OJIP curve, and reaction center density (RC/CSo), as well as absorption parameters (ABS/CSo, ABS/CSM). Consequently, the performance indices PIABS and PICS were improved compared to the wild type.

bHLH-Like Transcription Factors

The bHLH proteins belong to a superfamily of eukaryotic TFs, which are widely distributed in the animal and plant kingdoms, with various functions in plant development and metabolism [121]. A variety of bHLH TFs involved in plant abiotic stress response pathways have been identified [122]. Zuo et al. [15] identification of *bHLH* genes showed that antisense expression of *ZjbHLH076/ZjICE1* influences tolerance to low temperature and salinity in *Z. japonica*.

AP2/ERF-Like Transcription Factors

The AP2/ERF, a plant-specific TF family, influences various developmental processes and responses to abiotic stresses, such as drought, low temperature, hypoxia, and high salt [123]. Recently, more research has shown that plant hormone-mediate cascade signaling is closely related to stress response, with AP2/ERF TFs playing a key role [124]. The response and adaptation of plants to salt stress require the coordination of many hormones [125], including ABA, which participates in responses to various abiotic stresses. The functions of these TFs in transgenic rice and *A. thaliana* have been identified. Overexpression of *Z. japonica ZjABR1/ERF10* was shown to regulate plant growth and salt tolerance in transgenic *O. sativa* [86]. Jiang et al. [126] reported that a novel transcriptional regulator, *HbERF6*, regulates the *HbCIPK2*-coordinated pathway, enhancing salt tolerance in halophytic *Hordeum brevisubulatum*.

CBFs/DREB1-Like Transcription Factors

A myriad of TFs involved in cold signaling networks has been identified, among which the CBFs/DREB1 playing a central role in cold resistance by directly activating COR gene expression [127]. In *Arabidopsis*, three CBFs (*CBF1*, *CBF2*, and *CBF3*) are essential for cold acclimation, enabling plants to acquire freezing tolerance upon exposure to advanced low non-freezing temperatures [128,129]. The CBFs, along with their transcriptional activators, including inducers of CBF expression 1 (ICE1), constitute the ICE1CBF-COR transcriptional cascade, a well-known cold signaling pathway [127]. Additionally, TFs such as MYB15, CAMTA3, PIF3, EIN3, and BZR1 have been identified as upstream regulators of CBFs [128]. However, most research on the regulatory networks of cold and salt stress responses has focused on model plants, indicating a need for studies on the application of these networks in grass.

TCP-Like Transcription Factors

The TCP TFs are named after the first characterized members, such as Teosinte Branched1 (TB1) of maize [24], CYCLOIDEA (CYC) of *Antirrhinum* [25], and Proliferating Cell Factors one-half (PCF1/2) of rice [26]. TCP proteins (old TFs that appeared approximately 650–800 Mya) have been crucial role in the evolution of plant morphology [130]. Studies have demonstrated the role of TCP proteins in various plant growth and developmental processes [131]. In *A. thaliana* (At), *AtTCP14* and *AtTCP15* regulate embryonic growth and influence seed germination [130]. Meanwhile, *AtTCP5* inhibits the initiation and growth of leaf serrations [132]. Under normal temperatures, *AtTCP17* binds to Cryptochrome 1 (*CRY1*), repressing the formation of Phytochrome-Interacting Factors 4 (PIF4) *AtTCP17* dimer. However, under high temperatures, *CRY1* releases *AtTCP17*, promotes the interaction between the *AtTCP17* and *PIF4*, increases the transcriptional activity of *PIF4*, and regulates thermomorphogenesis [130,133]. Furthermore, *Arabidopsis TCP20* and *TCP22* regulate the circadian rhythm by binding to the CIRCADIAN CLOCK ASSOCIATED1 (CCA1) promoter and activating CCA1 expression [134]. In rice, PCF1/2 modulates the expression of PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) and regulates cell cycle processes [26]. In addition, the CYC-like homolog RETARDED PALEA1 (REP1) regulates palea identity and development in rice [135]. Zhan et al. [20] used transgenic technology to express target genes instantaneously in tobacco leaves, the responses of *ScTCPs* and *sctcp-mir319-3p* in rye (*Secale cereale* L.) to low temperature and drought were studied. The results showed that rye *ScTCP2*, *ScTCP5*, and *ScTCP19* are target genes of *sctcp-mir319-3p*, which affects the stress resistance or tolerance of rye by regulating the expression of these target genes.

Zn-C2H2 Like Transcription Factors

The C2H2-type zinc-finger protein (ZFP) family is one of the largest TFs families in the plant kingdom, with members engage in plant growth, development, and stress responses. Some *DgZFPs* were predicted to function in abiotic stress responses or the regulation of flowering time. The tolerance of the C2H2 ZEP gene family *DgZFPs* of *D. glomerata* (*Dg-ZFP125*) to osmotic and salt stress was studied using transgenic

technology for transplanting yeast. The results indicated that *Dg-ZFP125* enhances the permeability and salt tolerance of *D. glomerata* [136].

APETALA1/FRUITFULL (AP1/FUL)

AP1/FUL genes play crucial roles in vernalization induction, promote plant flowering, determining spikelet primordium, and specifying floral organ identity. In monocotyledonous plants, AP1/FUL genes underwent two significant duplications: the first during monocot formation, giving rise to the *FUL3/OsMADS18* branch, and the second during gramineous plant formation, resulting in the *FUL1/OsMADS14* and *FUL2/OsMADS15* branches [137]. Consequently, studies on these gene have proliferated. For instance, *CRISPR/Cas9* was employed to knockout *BdID1* by targeting different exons of the *B. distachyon* ID transcription factor (*BdID1*) genes in dicotyledonous short-stalked grasses. Subsequent genotyping of transgenic progeny confirmed complete loss of *BdID1* function, highlighting its unique role in the Poaceae family and its association with regulating flowering time pathways [137–139].

3.2.3 Protein Kinase-Like Signaling Factors under Stress of Adversity

MAPKK Genes Mitogen-Activated Protein Kinases (MAPKs)

During long-term adaptive evolution, plants have developed signaling networks to sense and transmit signals. Among stress-activated molecular pathways, MAPK signaling cascades are important signaling modules with critical roles in plant developmental processes, stress responses, and signal transduction [140]. The MAPK cascade consists of three protein kinases: MAPK, MAPK kinase (MAPKK), and MAPK kinase (MAPKKK) [14]. Wang et al. [14] studied the *CdMAPKKKs* gene of Bermuda (*C. dactylon*) grass and showed that *CdMAPKKKs* are commonly associated with regulating numerous biological processes. This technique illustrated how MAPK cascades modulate cold response by modulating *ICE1* protein stability. Other studies based on transgenic technology have shown that the ABA-insensitive five gene of *E. sibiricus* L. engages in the ABA-dependent salt response [140]. The ABA signal transduction and MAPK cascade pathways are important regulators of plant responses to salt stress. The MAPK cascade directly or indirectly participates in the ABA signal transduction pathway, and the ABA signaling pathway regulates the expression of related genes in the MAPK cascade pathway [141,142]. Studies on the responses of other plants (such as the *Vitis vinifera* (*VvMKK2*)) and *Gossypium hirsutum* (*GhMAP3K14-GhMKK11-GhMPK31*) to drought and salt stress, targeting MAPK genes through transgenic and VIGS technologies, have also been reported [143].

CBL/CIPK Protein Kinases

The CBL-CIPK signaling system, comprising calcineurin B proteins (CBLs) and their interacting protein kinases (CIPKs), forms a crucial regulatory network for abiotic stress responses. The system regulates plant responses to abiotic stress by initiating phosphorylation and interpreting calcium (Ca^{2+}) signals [144]. Transgenic technology was used to conduct experimental studies on the regulation of plant salt tolerance by the *CdCBL4-CdCIPk5* complex in *C. dactylon* and rice. Five CBL genes from *C. dactylon* were cloned by homologous cloning using the millet genome as a reference sequence. The interaction between *CdCIPKS* and *CdCBL4* was verified using complementary yeast two-hybrid and bimolecular fluorescence assays. The *CdCBL4-CdCIPKS* complex was found to regulate Na^+ absorption, transport, and excretion under salt stress by modulating the expression of Na^+ transporters and maintaining intracellular sodium and potassium ion balance, thereby enhancing salt tolerance [145].

The Sucrose Non-Fermenting-1 Related Protein Kinase 2 (SnRK2) Family

Members of the *SnRK2* family play crucial roles in abiotic stress responses. Xiang et al. [12] studied the function of the *SnRK2* protein kinase gene (*AcSnRK2.11*) in the protection of *A. cristatum* against stress using transgenic technology to construct expression vectors of yeast and tobacco. *AcSnRK2.11* overexpression increased chlorophyll yield, superoxide dismutase activities, ROS content, peroxidase levels, and soluble

carbohydrate levels. Additionally, overexpression also enhanced freezing tolerance in transgenic yeast and tobacco, suggesting that *AcSnRK2.11* acts as a regulatory factor involved in cold-response pathways.

Chlorophyll Degradation Enzymes (CCEs)

CCEs interact with photosynthetic organs. During leaf senescence, LHCII interacts with five chlorophyll-degrading enzymes (NYC, NOL, PPH, PAO, and RCCR) [146]. *Agrobacterium*-mediated transgenic technology was used to transfer *Agrobacterium* gene into *A. thaliana* or tobacco. Researchers studied the functional characteristics of the chlorophyll-b reductase gene *NYC1* were associated with chlorophyll degradation and photosynthesis in *Z. japonica* (*ZjNYC1*) [84]. Similarly, functional characterization of *ZjPPH*, the pheophytinase gene from *Z. japonica*, was conducted to understand its influence chlorophyll degradation and photosynthesis [83]. Overexpression of *ZjNYC1* and *ZjPPH* decreased the chlorophyll content and promoted senescence. Likewise, Guan et al. [87] used this method to study the mechanism of *Z. japonica* Chlorophyll b Reductase Gene NOL (*ZjNOL*) participation in chlorophyll degradation and photosynthesis. They found that *ZjNOL* simultaneously led to the excessive accumulation of ROS, antioxidant enzyme activation, oxidative stress generation, and consequently accelerating senescence. Similarly, the ectopically expressed chlorophyll-b reductase gene from *Z. japonica* changed the leaf color and chlorophyll morphology in *A. stolonifera* [13]. Functional identification of the H^+-PP enzyme gene family in *E. dahuricus* showed that V-type H^+-PP gene *EdVP1* overexpression from *E. dahuricus* increased the yield, potassium uptake, and low-nitrogen stress tolerance of transgenic plants [10,11].

A study on *A. thaliana* revealed that plants overexpressing *NOL* exhibited significantly lower chlorophyll content compared to wild-type plants These plants also displayed a reduced photosystem II antenna size, indicating that *NOL* was engaged in photosystem II antenna size control [147]. Phylogenetic analysis of the rice (*Oryza sativa*) *OsNOL* gene revealed that the closest protein to *OsNOL* was *OsNYC1*. Rice nol mutants exhibited a phenotype like that of the nyc1 mutant, with significant suppression of chlorophyll b degradation. Additionally, LHCII was selectively preserved throughout senescence, and thylakoid grana were retained even at the late stage of senescence [148]. In perennial ryegrass (*Lolium perenne*), RNA interference of *LpNOL* (NOLi) significantly inhibited chlorophyll breakdown during leaf senescence and altered leaf color, suggesting that *NOL* functions as a chlorophyll b reductase and is associated with the middle color phenotype of this perennial herb [149]. However, many studies have relied on transgenic technology for functional identification, highlighting the need for further research using alternative methods to explore the functional of chlorophyll-degrading enzyme CCE genes.

Hydroxycinnamoyl-CoA Shikimate/Quinate Hydroxycinnamoyl Transferase (HCT)

HCT is an essential enzyme in regulating lignin biosynthesis and composition. Researchers have used transgenic technology to investigate the expression mechanism of the Hydroxycinnamoyl-CoA Shikimate/Quinate Hydroxycinnamoyl Transferase 4 gene from *Z. japonica* (*ZjHCT4*) [88]. The expression of *ZjHCT4* resulted in *A. stolonifera* resulted in excessive elongation and changes in lignin composition by altering the phenylpropionic acid metabolic pathway and hormone response.

Other Protein Functional Genes

In grasses, functional protein genes primarily include those of the calmodulin-like (CML) family [150] and Domain of Unknown Function (DUF) 1644 family [85], with transgenic technology often employed to identify these genes. The CML family serves as an important Ca^{2+} sensor in plants, affecting responses to various stressors. The CML gene *PvCMLs* in *Paspalums vaginatium* has been shown to play a role in the response to salt and cold stress. Additionally, DUF proteins have a specific conserved structural domain, the function of which remains unclear [150,151]. Researcher investigated the function of *ZmDUF1644*, transferred from the halophytic *Zoysia matrella* to the non-halophytic *A. thaliana*, under salt stress

(150 mM NaCl). The results showed that *ZmDUF1644* overexpression enhanced the salt tolerance of *A. thaliana* [85].

In summary, transgenic technology is frequently used to identify gene function in grasses by transferring the target gene to *A. thaliana* [152] or *Nicotiana tabacum* [153,154] for gene function verification. However, a single method often does not suffice for gene function identification in practical applications. The gene function identification techniques described here can be jointly applied or combined with multi-omics analysis techniques (such as transcriptomics and metabolomics) to provide complement insights. This approach aims to rapidly and systematically evaluate gene function and uncover the potential value of functional genes in human health and ecology. Additionally, gene editing using *CRISPR/Cas9* technology has been performed in *Avena sativa* breeding [155]. An efficient screening system for the optimal target sites of *CRISPR/Cas9* gene editing in *A. sativa* was established using the gene gun method, resulting in two mutant plants from *Cas9* editing. These results provide a basis for applying gene editing technology in the molecular breeding of grasses. Furthermore, *CRISPR/Cas9* technology has been used to identify stress-resistance genes (such as *Sh1*, a gene associated with grain shedding) in *A. mongolicum*, and has shown potential in inhibiting the spread of plant DNA viruses [156].

Table 3: Overview of the application of functional identification methods for different functional genes in the identification of grasses genes

Functional type	Gene category	Identification method	Sample species	Identified gene	Reference
Grass functional gene	Growth regulatory genes	Transgenic technology or a combination of transgenic technology and gene silencing technology	*Festuca elata*	Caffeic acid-O-methyltransferase (COMT);	[9,16]
			Paspalum notatum	*GG13*: a candidate gene of Paspalum notatum associated with endosperm development in insensitive *EBN*	
	Functional genes that protect biological macromolecules	Transgenic technology	*Agropyrrhiza mongolica*	Osmoregulatory protein-related genes, dehydration protein genes, aquaporin genes, plum genes, and late-embryogenetic-rich proteins (LEA); *MwLEA3*	[55]
	Genes encoding enzymes that remove ROS	Agrobacterium-mediated transgenic technology combined with knockout technology	*Claviceps paspali*	The *idtF* and *idtP* genes within the indole diterpene biosynthetic gene cluster	[88,91]
Grass regulatory genes	Resistance regulatory gene	Transgenic technology is often used, and combine transgenic technology with gene silencing or gene knockout technology for functional identification	*Elymus dahuricus*	The H^+-pyrophosphatases (PP) enzyme gene (*AtAVP1*); the type I H^+-PP gene (*EdVP1*)	[10,11]
	Transcription TCP factors	Transgenic technology	*Secale cereale* L.	*ScTCPs* and *sctcp-mir319-3p*	[20]

(Continued)

Table 3 (continued)

Functional type	Gene category		Identification method	Sample species	Identified gene	Reference
		HEMA	Transgenic technology	*Cynodon dactylon*	*CdHEMA1*	[79]
		Zn-C2H2	Transgenic technology	*Dactylis glomerata*	*Dg-ZFP125*	[87]
		WRKYs	Transgenic technology or combining transgenic technology and VIGS	*Cynodon dactylon*	*CdWRKY2*, *CdWRKY50*	[101–108]
				Oryza sativa	*OsWRKY71*, *OsWRKY76*	
				Agropyron mongolicum	*FcWRKY40*, *AmWRKY-like*	
		MYB/MYC	Transgenic technology	*Leymus chinensis* (Trin.) Tzvel	*LcMYB4*	[109,111–116]
				Agropyron michnoi Roshev	*AmrMYB1*, *AmrR2R3MYB*	
				Agropyron mongolicum Keng	*MwMYB4*	
				Zoysia japonica	*ZjICE2*	
		bZIP	Transgenic technology or combination of transgenic and knockout technologies	*Elymus sibiricus* L.	Abscisic acid-insensitive 5 (*EsABI5*)	[117,119]
		bHLH	Transgenic technology	*Zoysia japonica*	*ZjbHLH076/ZjICE1*	[120]
		AP2/ERF	Transgenic technology	*Zoysia japonica*	*ZjABR1/ERF10*	[124,125]
				Hordeum brevisubulatum	*HbERF6*	
		CBFs/DREB1	Transgenic technology	Focused on model plants, such as *Arabidopsis thaliana*	*CBF1*, *CBF2*, and *CBF3*	[126–128]
		AP1/FUL	*CRISPR/Cas9* technology	*Brachypodium distachyon*	*BdID1*	[136–138]
	Protein kinase-like signaling factors	CCEs	Transgenic technology	*Zoysia japonica*	Chlorophyll-b reductase gene *NYC1* (*ZjNYC1*) and NOL(*ZjNOL*);	[10,11,83,86,145–148]
				Elymus dahuricus	V-type H^+-PP gene *EdVP1*;	

(Continued)

Table 3 (continued)

Functional type	Gene category	Identification method	Sample species	Identified gene	Reference
			Lolium perenne	*LpNOL* RNA interference gene (*NOLi*)	
	SnRK2	Transgenic technology	*Agropyron cristatum*	*SnRK2*	[12]
	MAPKs	Transgenic technology or a combination of transgenic technology and gene silencing technology	*Cynodon dactylon*	MAPK, MAPK kinase (MAPKK), and MAPK kinase (MAPKKK); CdMAPKKKs;	[14,139–142]
			Elymus sibiricus L.	ABA-insensitive five gene	
	HCT	Transgenic technology	*Agrostis stolonifera*	A Hydroxycinnamoyl-CoA Shikimate/Quinate Hydroxycinnamoyl Transferase 4 gene (*ZjHCT4*)	[87]
	CBL/CIPK	Transgenic technology	*Cynodon dactylon*	*CdCBL4-CdCIPk5* complex	[144]
Other protein functional genes	DUF	Transgenic technology	*Zoysia japonica*	*ZmDUF1644*	[84]
	CML	Transgenic technology	*Paspalums vaginatum*	*PvCMLs*	[149,150]

4 Applications in Genes Functional Identification of Other Plant

Currently, gene silencing, transgenic techniques, and gene knockout techniques are the most widely used methods for identifying gene function. These techniques have been increasingly applied to identify functional genes not only in grasses but also in various economic crops such as cotton (*G. hirsutum*) [157–161], soybean [162], common bean [163], maize [164–166], *Arachis hypogaea* L. [167], sugarcane [168], potato [169], and alfalfa [170], as well as in vegetables like Chinese cabbage (*Brassica rapa* L.) [171,172] and chili peppers (*Capsicum annuum* L.) [173]. They are also used to study flower-forming genes in species such as *Paeonia lactiflora* [174], *Malus halliana* [175], *Phyllostachys edulis* [176], and *Dianthus caryophyllus* [177], resistance genes associated with fruit ripening in grape (*V. vinifera* L.) [178] and sweet cherry [179], and disease-resistant genes against pathogens [180–182] and plant antiviral genes [183].

For example, using *A. thaliana* as a medium, the heterologous overexpression of a gene family in *A. thaliana* identified the function of *PIP5K* family genes related to the development of female inflorescences in *Ricinus communis* Lm [152] and strawberry *SVP* dormant-related genes [153]. Other studies determined the functional identification of homologous genes and the molecular regulatory network of the floral pathway of the *BrcSOC1* gene in *Brassica rapa* ssp. *chinensis* in response to vernalization [171]. Using *N. tabacum* as a carrier, researchers investigated the function of the *SWEET* gene involved in sugar transport in *Hemerocallis fulva* in response to low-temperature stress [154]. Additionally, gene function analysis and upstream transcription factor prediction of the key candidate gene *HaF4D2* (controlling the fatty acid content of sunflowers) showed that seeds of transgenic strains had higher oil content than those of the wild type [72]. In another study, the adenosine triphosphate

(ATP) citrate lyase (*ACL*) gene was cloned by mining the genes related to flower color formation in *P. lactiflora*. The function of *PlACL2* in flower color formation was determined by overexpression in *N. tabacum* and transient silencing in *P. lactiflora* [174]. Simultaneously, *CRISPR/Cas9*, as a new gene editing system, has successfully edited hundreds of genes in more than 30 plant species and has been widely used in researching crop stress [61], disease, and pest resistance [62,63]. For instance, a *CRISPR/Cas9* screening strategy was applied to suppress the gamma response 1 (*TaSOG1*) gene in wheat, which is involved in the DNA damage response (DDR). The mechanism of action of the *TaSOG1* gene in wheat DDR was also investigated [184].

Combining gene function identification techniques is more effective for analyzing plant resistance. For instance, Deng [157] identified the function of Verticillium wilt resistance genes in upland cotton by combining gene silencing technology with transgenic technology and other methods. Furthermore, two resistance response genes from naturally mature tetraploid *Poncirus trifoliata*, specifically the LEA family gene (*PtrLEA7*) and the R2R3-type MYB (*PtrMYB3*), were studied for drought and salt resistance using a tobacco-mediated transgenic technique and VIGS technique, respectively [185]. Studies have shown that when a certain technology is limited in research, other aspects such as innovation, application, and selecting more suitable methods for identifying target gene function need consideration. For example, Zhao [183] first used an easily available vector construction method to study *Papaya* leaf distortion mosaic virus (*PLDMV*) and constructed a variety of *PLDMV* vectors with reporter genes using the *E. coli*-free method. The method was then used to construct viral infectious clones quickly, efficiently, and stably. Compared with traditional methods of *in vitro* transcription or yeast homologous recombination, it had a higher success rate, greater efficiency, and improved cost. The resulting plant virus expression vector construction method will significantly advance subsequent scientific research. Moreover, this method does not require large equipment for nucleic acid extraction or isothermal amplification and provides an effective new approach for constructing unstable plant virus infectious clones in *E. coli* cells. Such methods can be applied to identifying gene functions in grasses.

5 Prospects of Future Research

With the rapid development of high-throughput sequencing technology, an increasing number of whole-genome sequences of plants have been published. However, sequencing efforts for herbage plants remain limited, constraining technical means for gene function research, genetic improvement, and molecular breeding [57]. These factors greatly hinder the development of molecular breeding and functional genomics research in herbage plants. Therefore, it is important to actively explore advanced technologies for identifying gene functions in grasses to facilitate genetic improvement and molecular breeding. Recent studies have highlighted the potential applications of *CRISPR/Cas9* technology, transgenic methods, ethyl methanesulfonate (EMS) technology, and other emerging technologies in this regard.

5.1 Genetic Function Identification of Herbage by Ethyl Methanesulfonate (EMS) Technology

EMS-induced mutagenesis is a potent tool for generating genetic resources to uncover novel genes and characterize their functions, thereby elucidating the molecular basis of important agronomic traits [186]. Moreover, EMS mutagenesis has facilitated multi-omics combined analysis and enabled the identification of related genes through reverse genetics. Gene editing and recombination are innovative breeding methods that significantly enhance plant genetics [187], increasingly becoming primary approaches for genetically improving model plants. Furthermore, EMS mutagenesis is applicable across most plant species [186]. It has been successfully employed in crops, vegetables, and fruits to breed varieties with superior traits [188]. Various plant tissues have been used for mutagenesis, mutagens can cause abundant mutations in different plant organs, such as seeds, bulbs, calluses, and pollen [186]. Specific mutagenesis sites in plants can be selected based on experimental requirements. Recent advances have demonstrated

the effectiveness of mutagenesis breeding technology in enhancing resistance traits in *Arabidopsis*, rice, soybean, and other plants [189], underscoring its potential applicability in grasses.

5.2 Transgenic Technology Is Combined with Other Technologies for the Study of Gene Function in Grasses

In corn, rice, wheat, and other gramineous plants, the transformation rate of plant transgenic techniques, such as *Agrobacterium*-mediated methods, requires improvement [92,190]. Future studies should explore transgenic methods with higher transformation efficiencies, more controllable gene integration, and wider applicability. Combining various genetic transformation methods will enable more in-depth and extensive research on plant genomes and their genetic transformations.

5.3 Virus-Induced Gene Silencing Techniques (VIGS)

VIGS is used to transform plant cells with viral genomes either by direct inoculation or through infection with a modified strain of *Agrobacterium rhizogenes* [157,191]. This technique is extensively studied in functional genomics due to its advantages, such as simple construction, low cost, and short cycles [192]. VIGS can also be used for comparative genomes studies between distinct species. Currently, VIGS techniques have demonstrated the diversity of related gene functions in studies of disease resistance, stress response, cell signaling, and secondary metabolic biosynthesis pathways. This has led to the construction of new correlative models [65], expanding the genetic toolbox for non-model organisms. Additionally, VIGS facilitates virus-induced overexpression (VOX), virus-induced genome editing (VIGE), and host-induced gene silencing (HIGS), and it has been widely used for identifying gene functions in vegetable crops and other species [70,71]. This technique is valuable for identifying gene functions in grasses.

5.4 Deepen the Wide Application of CRISPR/Cas9 Technology in the Identification of Gene Function in Grasses

Gene editing via the *CRISPR/Cas9* system is widely used for targeted genome modifications. As an emerging plant gene editing technology [193,194], it has great research potential in functional genomics. Recently, *CRISPR/Cas9* technology has improved forage varieties of grass plants. Researchers have proposed various effective grassland ecological restoration methods, highlighting the role of *CRISPR/Cas9* in restoring grassland vegetation. This technology can identify genes related to stress resistance, high yield, and quality of native species according to local conditions Additionally, collecting, developing, and utilizing superior grassland plants and restoring grassland ecological functions can significantly shorten the breeding cycle. Therefore, *CRISPR/Cas9* is a promising method for identifying grass gene function in the future.

5.5 Other New Gene Editing Techniques Are Used to Identify the Function of Grasses Genes

In addition to the *Cas9* protein family, studies have shown that the Argonaute (Ago) protein family (e.g., *TtAgo* [160] and *PfAgo* [195]), displays gene editing capabilities. Some studies have confirmed that *NgAgo*-gDNA has potential as a new tool for gene knockouts [196], which can reduce mRNA without producing detectable DNA double-strand breaks to cut RNA instead of DNA. Furthermore, the *CRISPR/Cas13*RNA editor system can be reprogrammed and applied to eukaryotes to combat pathogenic RNA viruses or regulate gene expression, making it useful for identifying grass gene function [197–199]. Recent studies have shown that the *CRISPR/Cas12i* project [200,201] achieves therapeutic genome editing through efficient immune cell editing. While primarily used in medical science, this technology could be applied to plant genome research. Future studies should explore this potential.

6 Conclusion

In summary, identifying and understanding the functional genes that confer stress resistance in grasses is crucial for breeding resilient grass varieties. Advances in functional genomics and transgenic technologies are revolutionizing this field, providing powerful tools for gene function identification and validation. Key functional genes in grasses include disease resistance genes (such as *NBS-LRR* genes and *PRR* genes), biotic and abiotic stress resistance genes (such as DREB/CBF transcription factors, HSP (Heat Shock Proteins), and WRKY transcription factors), and genes related to growth and yield (such as GA signaling genes and photosynthesis-related genes). Common methods for identifying functional genes include Genome-Wide Association Studies (GWAS), Quantitative Trait Loci (QTL) Mapping, CRISPR/Cas9 Gene Editing, RNA Sequencing (RNA-Seq), proteomics and metabolomics, and transgenic technology. Among these, transgenic technology is the most used method in grass gene function identification and is crucial for validating the function of resistance genes in grasses. It involves introducing, modifying, or silencing genes to assess their role in resistance. Future research in grass gene function identification should leverage advanced genomic tools and transgenic technologies. Methods such as GWAS, QTL mapping, *CRISPR/Cas9*, and transcriptomics will be crucial in identifying and characterizing resistance genes. Transgenic approaches will play a vital role in validating these genes' functions and developing grass varieties with enhanced resistance to biotic and abiotic stresses. However, single molecular identification techniques are often limited in studying gene function in grasses. Future advancements in the field will benefit from integrating omics and molecular biology technologies, multiple gene function mining and identification methods, and emerging gene function research tools. This integrated approach will be key to ensuring sustainable agricultural productivity and food security in the face of global challenges. This review of the latest gene function identification methods provides theoretical support for the germplasm development and molecular breeding of resistant grass plants.

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