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Identification, biogenesis, function, and mechanism of action of circular RNAs in plants

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ABSTRACT

Circular RNAs (circRNAs) are a class of single-stranded, closed RNA molecules with unique functions that are ubiquitously expressed in all eukaryotes. The biogenesis of circRNAs is regulated by specific *cis*-acting elements and *trans*-acting factors in humans and animals. circRNAs mainly exert their biological functions by acting as microRNA sponges, forming R-loops, interacting with RNA-binding proteins, or being translated into polypeptides or proteins in human and animal cells. Genome-wide identification of circRNAs has been performed in multiple plant species, and the results suggest that circRNAs are abundant and ubiquitously expressed in plants. There is emerging compelling evidence to suggest that circRNAs play essential roles during plant growth and development as well as in the responses to biotic and abiotic stress. However, compared with recent advances in human and animal systems, the roles of most circRNAs in plants are unclear at present. Here we review the identification, biogenesis, function, and mechanism of action of plant circRNAs, which will provide a fundamental understanding of the characteristics and complexity of circRNAs in plants.

Key words: plant circRNA, identification, biogenesis, function, mechanism

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INTRODUCTION

Circular RNAs (circRNAs) are a distinct class of single-stranded, closed RNA molecules that are ubiquitously expressed in all eukaryotes and prokaryotic archaea (Memczak et al., 2013). Viroids were the first circRNA molecules that were identified in tomato and *Gynura* in the 1970s (Hsu and Coca-Prados, 1979). Although human circRNAs were initially reported around 30 years ago, they were first thought to be by-products of abnormal splicing events. Unlike their homologous messenger RNAs (mRNAs), circRNAs have a closed structure formed by covalent bonding of the 3′ end and the 5′ end after back-splicing and do not have a 5′ cap structure and a 3′ poly(A) tail (Chen, 2020). Therefore, circRNAs are not easily degraded by the exonuclease RNase R and are more stable than linear RNA molecules. Compared with canonical splicing, the efficiency of back-splicing is low, resulting in lower expression levels of the majority of circRNAs than linear RNAs (Misir et al., 2022). Therefore, very few circRNAs are detected in classical RNA sequencing (RNA-seq) datasets, especially poly(A) RNA-seq data. With the development of non-polyadenylated and RNase R-treated RNA-seq technology and circRNA identification algorithms, thousands of circRNAs have been identified in humans, the fruit fly *Drosophila melanogaster* (*D. melanogaster*), the nematode worm *Caenorhabditis elegans*, diverse mammal species, and prokaryotic archaea (Kristensen et al., 2022).

circRNAs are derived from exonic, intronic, and even intergenic regions of the genome, and most circRNAs are derived from one or more exons of protein-coding genes that are referred to as the parental or host genes (Kristensen et al., 2019). The biogenesis of circRNAs is regulated by *cis*-complementary sequences in flanking introns and by specific proteins in humans and animals. There is emerging evidence showing that circRNAs play important roles in various biological processes by forming R-loop structures, acting as microRNA (miRNA) sponges, interacting with RNA polymerase II or other RNA-binding proteins (RBPs), and being translated into polypeptides or proteins in humans and animals (Misir et al., 2022). Therefore, circRNAs are a new class of RNA molecules with multiple regulatory functions.

Over the past several years, circRNAs have been detected in *Arabidopsis*, rice, maize, tomato, cotton, soybean, and other plant species, indicating that circRNAs are also widely present in plants

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and potentially have biological functions (Zhao et al., 2019; Zhang et al., 2020c; Chu et al., 2022; Zhang and Dai, 2022). Aligning circRNAs to plant genome sequences revealed that circRNAs are distributed on each chromosome and even on chloroplast DNA and mitochondrial DNA, and the number of circRNAs derived from each chromosome varies depending on the study (Chu et al., 2017; Liu et al., 2019b; Zhao et al., 2019). Based on the chromosomal location from which they are derived, circRNAs can be classified into exonic circRNAs (EcircRNAs), circular intronic RNAs (ciRNAs), exon–intron circRNAs (ElciRNAs), intergenic circRNAs, intergene-exon circRNAs, and intergene-intron circRNAs (Zhang et al., 2020b). Most circRNAs are usually derived from a single protein-coding gene, and individual circRNAs can be derived from more than one protein-coding gene, such as *agcircRBCS* from the exonic regions of the two genes *RBCS2B* and *RBCS3B* (Zhang et al., 2021a). Alternative back-splicing events are widespread in plant circRNAs. For example, 65%–93% of the host genes generate single circRNA isoforms, and 7%–35% of the host genes generate multiple circRNA isoforms in plants (Chu et al., 2020). The lengths of circRNAs vary from less than 100 bp to thousands of base pairs, but most circRNAs are less than 1000 bp in length (Ye et al., 2015). Compared with the extensive studies of circRNAs in humans and animals, most studies in plants focus on the identification and functional prediction of circRNAs, but the biogenesis, function, and mechanism of action of plant circRNAs are less frequently reported. Although studies on plant circRNAs are still in their preliminary stages, some progress has been made.

IDENTIFICATION OF circRNAs

Bioinformatics tools for identifying circRNAs in plants

circRNAs are a class of covalent RNA molecules with a closed circular structure (Kristensen et al., 2019). The development of RNase R-treated, rRNA-depleted, and high-throughput RNA-seq technology has provided a transformative method for the systematic identification of circRNAs in different species (Lu et al., 2015; Ye et al., 2015). The development of reliable methods to identify circRNAs is very important for studying these molecules. Currently, several tools are available for the prediction of plant circRNAs (Table 1). Usually, these tools prefer RNA-seq datasets as input to identify reads spanning back-splicing junction (BSJ) sites. For BSJ-based circRNA identification tools, two strategies for mapping BSJs are usually used. Most tools, such as MapSplice, find_circ, segemehl, circRNA_finder, CIRCexplorer, and circRNA identifier (CIRI), use a segmented-read-based approach. First, reads that align to the reference genome across their full length are removed. The remaining reads are then analyzed for reads that align with two discontiguous sections of the genome. Among these latter reads, reads that show a change in polarity (i.e., the 3' end of an exon is now located 5' of the same exon; supplemental Figure 1) reflect likely BSJs. The tool known and novel isoform explorer (KNIFE) uses a pseudoreference-based approach. Pseudo-sequences around putative BSJs are first built using a combination of the reference genome and genome annotation. Subsequently, sequencing reads are aligned to the pseudo-sequences to recognize BSJs (supplemental Figure 1).

Because of different alignment strategies (segmented-read-based approach or pseudoreference-based approach), mapper (Mapsplice, Bowtie, segemehl, spliced transcripts alignment to a reference [STAR], Tophat, and burrows-wheeler aligner [BWA]), screening criteria (alignment score, mismatch numbers, splice signals, and number of BSJ reads), and data types (paired-end and/or single-end reads), the circRNA landscape differs depending on the tools of choice, and only a modest overlap is observed between these tools (Hansen et al., 2016). When comparing these tools, it is found that no single tool dominates in all terms of precision, sensitivity, and F1 score (Zeng et al., 2017). For animal and human circRNAs, CIRI identifies the most circRNAs, followed by CIRCexplorer, MapSplice, find_circ, and circRNA_finder (Hansen et al., 2016). Different from animal and human circRNAs, the tools for identifying the most circRNAs differ across species and treatments for plant circRNAs (Chen et al., 2019; Gao et al., 2019; Zhang et al., 2020b; Babaei et al., 2021; Zhou et al., 2021b). Although these circRNA recognition tools are mainly developed based on human and animal datasets, identification of circRNAs in plants still mainly depends on them. Among these tools, CIRI is currently the most used for plant circRNAs, followed by find_circ, CIRCexplorer, circRNA_finder, segemehl, MapSplice, and KNIFE (supplemental Table 1). Because of the differences between these tools, it is suggested that at least two recognition tools are used to identify more circRNAs or more reliable circRNAs in plants (Chen et al., 2021).

Integrated tools, such as CirComPara, PcircRNA_finder, and CircPlant, are available for identifying plant circRNAs. CirComPara is an integrated circRNA prediction algorithm that integrates CIRCexplorer, CIRI, find_circ, and testrealign (Gaffo et al., 2017). PcircRNA_finder is an integrated prediction algorithm that only recognizes EcircRNAs in plants by integrating find_circ, Mapsplice, and segemehl tools (Chen et al., 2016). CircPlant is a tool for the exploration of plant circRNAs by using modified CIRI2 (Zhang et al., 2020d). Pcirc was developed to identify plant circRNAs by using the random forest algorithm of machine learning (Yin et al., 2021). Recently, a plant circRNA identification tool, MeCi, was developed based on common features of plant mitochondrial genomes (Liao et al., 2022). The CirComPara, PcircRNA_finder, CircPlant, PCirc, and MeCi tools for identifying plant circRNAs have not been systematically compared and evaluated with other software.

Bioinformatics tools for the identification of plant circRNAs usually provide a prediction of BSJs and not full-length sequences of circRNAs. Full-length sequences of circRNAs are important for many analyses, such as alternative splicing, protein-coding ability, and miRNA sponges. Computational tools such as CIRCexplorer (Zhang et al., 2014, 2016), circseq_cup (Ye et al., 2017), and CIRI-full (Zheng et al., 2019) can be used to assemble full-length sequences for plant circRNAs using short sequencing reads from RNA-seq (Table 1). The tool circseq_cup has high precision and low sensitivity, and CIRI-full has low precision and high sensitivity. No single assembly algorithm dominates in all cases; therefore, these assembly algorithms should be used together for reliable full-length sequences of circRNAs (Zhang et al., 2022). However, reconstructing the full-length sequences of circRNAs from short reads is challenging and has high false positives given the similarity of circRNAs and their corresponding linear mRNAs. Long-read sequencing technology, such as Nanopore sequencing, has been developed

Tool name	Language	Mapper	Sequencing type	Check splice signals	Platform	Reference
MapSplice	C++	MapSplice	PE		Unix/Linux	Wang et al., 2010
find_circ	Python	Bowtie2	PE, SE	GU-AG	Unix/Linux	Memczak et al., 2013
segemehl	C++	Segemehl	PE, SE		Unix/Linux	Hoffmann et al., 2014
circRNA_finder	Perl	STAR	PE	GU-AG	Unix/Linux	Westholm et al., 2014
CIRCexplorer	Python	TopHat2/TopHat-Fusion, STAR, MapSplice, BWA, segemehl	PE, SE		Unix/Linux	Zhang et al., 2014 and 2016
CIRI	Perl	BWA	PE, SE	mainly GU-AG	Unix/Linux	Gao et al., 2015 and 2018
KNIFE	Python, R, Perl, Shell	Bowtie2, Bowtie	PE		Unix/Linux	Szabo et al., 2015
PcircRNA_finder	Perl, Python	Tophat-Fusion, STAR-Fusion, Bowtie2, find_circ, Mapsplice, segemehl, Tophat	PE	GU-AG GC-AG AU-AC	Unix/Linux	Chen et al., 2016
CirComPara	Python, R, Shell	CIRI, find_circ, CIRCexplorer2_STAR, CIRCexplorer2_BWA, CIRCexplorer2_segemehl, testrealign	PE, SE		Unix/Linux	Gaffo et al., 2017 and 2022
CircPlant	Perl	BWA-MEM, modified CIRI2	PE	GU-AG GC-AG AU-AC	Unix/Linux	Zhang et al., 2020d
PCirc	Python	Bowtie2, Tophat2, Blastn	PE, SE		Unix/Linux	Yin et al., 2021
MeCi	Perl	Blastn	PE		Mac OS X/Linux	Liao et al., 2022
circseq_cup	Perl, Python	TopHat & TopHat-Fusion, STAR, segemehl	PE		Unix/Linux	Ye et al., 2017
CIRI-full	JAVA	BWA, CIRI2, CIRI-AS	PE, SE	Mainly GU-AG	Unix/Linux	Zheng et al., 2019
CIRI-long	Python, C, C++	BWA	Nanopore		Unix/Linux	Zhang et al., 2021b

Table 1. circRNA identification tools used in plants.
PE, paired-end read; SE, single-end read.

to directly sequence full-length circRNA transcripts. CIRI-long was developed to reconstruct the full-length sequence of circRNAs using modified Nanopore long-read sequencing (Zhang et al., 2021b). In plants, modified Nanopore sequencing has been used to identify 3643 full-length circRNAs in rice (Xu et al., 2022b), which may be a good choice in future circRNA studies in plants.

Profiling of circRNAs in plants

Since the first report of circRNAs in *Arabidopsis thaliana* (*A. thaliana*) (Wang et al., 2014), research into plant circRNAs has increased. Recent studies have revealed that circRNAs are also widespread and abundant in plant genomes (Figure 1; supplemental Table 1). Genome-wide identification of circRNAs in plants was first reported in *Oryza sativa* and *A. thaliana*. Subsequently, a growing number of circRNAs have been identified in cereal crops (maize, wheat, and barley), oil crops (soybean, peanut, and *Brassica campestris*), root and tuber crops (potato and taro), sugar crops (sugar beet), beverage crops (tea), fiber crops (cotton and ramie), horticultural crops (tomato, pepper, lettuce, Chinese cabbage, cucumber, watermelon, melon, common bean, alfalfa, grape, longan, citrus, kiwifruit, pear, apple, petunia, moso bamboo, and hazel), medicinal crops (sea buckthorn and *Salvia miltiorrhiza*), and other economic plants, such as tobacco, poplar, and *Eucalyptus grandis* (supplemental Table 1).

As more circRNAs continue to be identified in additional plant genomes, multiple databases have been established to organize these plant circRNAs (Table 2). Examples include PlantCircNet (Zhang et al., 2017), PlantcircBase 7.0 (Chu et al., 2017, 2020; Xu et al., 2022b), LeafcircBase, CircFunBase (Meng et al., 2019), AtCircDB (Ye et al., 2019), CropCircDB (Wang et al., 2019b), and GreenCircRNA (Zhang et al., 2020a). A total of eight plant species are represented in PlantCircNet, 21 in PlantcircBase 7.0, two in CropCircDB, five in LeafcircBase, seven in circFunBase, one in AtCircDB, and 69 in GreenCircRNA. In these databases, circRNA-related information, including their positions in the genome, host genes, and bioinformatics tools, are

Figure 1. Identification of circRNAs in plants (2014 to May 2022).
The numbers represent the total number of circRNAs identified in each study (Bai et al., 2021; Bao et al., 2019; Bian et al., 2021; Chen et al., 2017, 2022; Darbani et al., 2016; Dong et al., 2021; Eisenberg and Levanon, 2018; Fu et al., 2019, 2020; Ghorbani et al., 2018; Han et al., 2021; He et al., 2020; Hong et al., 2020; Jiang et al., 2021; Li et al., 2020a, 2020b, 2021a; Liang et al., 2019; Liu et al., 2017, 2019c, 2020a, 2021a; Luo et al., 2020; Lv et al., 2020; Ma et al., 2021a, 2021b; Medina et al., 2021; Meng et al., 2018; Pan et al., 2018; Qin et al., 2018; Ren et al., 2018; Salih et al., 2021; Shi et al., 2021, 2022; Shu et al., 2021; Sun et al., 2016, 2020, 2021, Tang et al., 2018; Tong et al., 2018; Wang et al., 2017a, 2017b, 2018a, 2018b, 2019c, 2019e, 2020a, 2021a, 2021b, 2021c, 2022a, 2022b, Wu et al., 2020; Xiang et al., 2018; Xu et al., 2019, 2022a; Yang et al., 2020a, 2020b, 2020c, 2022; Yin et al., 2018, 2022; Zeng et al., 2018; Zhan et al., 2021; Zhang et al., 2019a, 2019c, 2020a; Zhao et al., 2017a, 2017c; Zheng et al., 2020; Zhou et al., 2018a, 2018b, 2019, 2020; Zhu et al., 2019; Zuo et al., 2016, 2018, 2019).

provided. The full-length sequences of more than 31 000 circRNAs have been constructed based on CIRCexplorer, circseq_cup, CIRI-full, and CIRI-long in PlantcircBase 7.0 (Xu et al., 2022b). These databases are convenient resources for subsequent studies of plant circRNAs.

Validation of circRNAs in plants

Although numerous circRNAs have been predicted by bioinformatics analyses, few have been experimentally validated (supplemental Table 1), possibly because the expression level of most circRNAs is relatively low. Northern blotting using a probe that spans BSJs is the gold-standard technique for validating circRNAs because it does not include reverse transcription or amplification steps (Figures 2A and 2B). In maize, circRNAs derived from the *Zm26S* rRNA and *Zmcob* and *Zmnad2T2* genes have been validated by northern blotting (Liao et al., 2022). However, northern blotting is a time-consuming and relatively difficult procedure. Therefore, it is rarely used to verify circRNAs. Validation of circRNAs is mainly performed by PCR and Sanger sequencing with sets of divergent and convergent primers in plants and animals (Figures 2A and 2C). BSJs of circRNAs are only present in RNA but not genomic DNA (gDNA); therefore, BSJs can be amplified with divergent primers using cDNA instead of gDNA as templates (Figures 2A and 2C). Whether circRNAs are resistant to RNase R degradation can be determined by PCR or real-time quantitative reverse-transcriptase PCR (qRT-PCR) using divergent primers. Quantification of circRNAs in plants is often performed by qRT-PCR and droplet digital PCR (ddPCR) (Philips et al., 2020a; Zhang et al., 2020b). Because of the low levels of expression of circRNAs, excess divergent primers readily direct the amplification of other cDNAs when the conditions are conducive, resulting in false positives. Thus, DNA gel electrophoresis and Sanger sequencing need to be performed on the qRT-PCR products. ddPCR determines the absolute concentration of a circRNA using the ratio of positive

Names	Number of plant species	Number of circRNAs	Whether to predict circRNA-miRNA-mRNA networks	Latest update	URL
PlantCircNet	8	139 277	yes	2017	http://bis.zju.edu.cn/plantcircnet
PlantcircBase 7.0	21	171 118	yes	2022	http://ibi.zju.edu.cn/plantcircbase/index.php
CropCircDB	2	101 833	yes	2019	http://deepbiology.cn/crop/
LeafcircBase	5	54 049	no	2019	http://bis.zju.edu.cn/LeafcircBase/index.php
CircFunBase	7	1158	yes	2019	http://bis.zju.edu.cn/CircFunBase
AtCircDB	1	84 685	yes	2019	http://www.deepbiology.cn/circRNA
GreenCircRNA	69	213 494	yes	2020	http://greencirc.cn/

Table 2. List of current plant circRNAs databases.

to negative droplets; therefore, ddPCR is likely to be more accurate than qRT-PCR when quantifying circRNAs. However, ddPCR may produce false positives as well.

BIOGENESIS OF circRNAs IN PLANTS

The biogenesis of circRNAs in humans and animals requires cellular spliceosomal machinery. When pre-mRNA processing events are slowed down, nascent RNA can be directed to alternative pathways that facilitate back-splicing, and deletion of splicing factors was found to increase the level of circRNAs in *D. melanogaster* (Liang et al., 2017). In plants, the biogenesis of circRNAs has not been thoroughly investigated (Figure 3). In *Arabidopsis*, the accumulation of circRNAs is significantly increased in the splicing-related mutants *cbp80*, *c2h2*, and *flk* (Philips et al., 2020b), suggesting that the biogenesis of plant circRNAs is also affected by splicing factors (Figure 3A).

In humans and animals, the biogenesis of circRNAs is regulated by specific *cis*-acting elements (Zhang et al., 2014). When the upstream intron sequence flanking the exon is close to the downstream intron sequence flanking the exon, the physical distance between the 3′ acceptor site and 5′ donor site of the exons is reduced, and the loop of the EcircRNA is then formed through back-splicing. This looping can be mediated by base-pairing of reverse complementary sequences (such as inverted repeat *Alu* transposable elements) between the upstream and downstream introns, and short inverted repeats (30–40 nt) are sufficient for circularization (Liang and Wilusz, 2014). Unlike animal circRNAs, most plant circRNAs have fewer repetitive and reverse complementary sequences in intronic sequences flanking EcircRNAs. For example, among the 6074 EcircRNAs in rice and 5152 in *Arabidopsis*, reverse complementary sequences with lengths greater than15 nt between the introns flanking the circRNAs were only found in 46 rice circRNAs and one *Arabidopsis* circRNA (Ye et al., 2015). Similarly, only 20 of the 2354 rice circRNAs contained complementary sequences equal to or greater than 18 nt in the flanking intronic sequences (Lu et al., 2015). Among the 2494 circRNAs in soybean, only 2.7% of the intronic sequences flanking EcircRNAs contained reverse complementary sequences (Zhao et al., 2017b). In moso bamboo, only 10 inverted complementary sequences with lengths of greater than 30 nt were detected in the flanking intronic sequences of 720 EcircRNAs (Wang et al., 2019d; Zhang et al., 2021c). Among the 1432 circRNAs detected in grapevine, only 85 EcircRNAs had flanking intron sequences that contained reverse complementary sequences (Gao et al., 2019). In three species of poplars, only one circRNA in *Populus euphratica*, 11 circRNAs in *Populus pruinosa*, and nine circRNAs in *Populus alba* contained complementary flanking sequences in the flanking introns (Li et al., 2021b). In maize, 17.3% of circRNAs in the flanking regions contained sequences that are related to long interspersed element-1-like elements and their reverse complementary pairs (LLERCPs), and as the number of LLERCPs increases, the accumulation of circRNAs varies, whereas that of linear transcripts decreases (Chen et al., 2018b). These results suggest that the biogenesis of only a few circRNA is regulated by flanking complementary sequences (Figure 3A).

ALKBH5, METTL3, and YTHDC1 affect circRNA biogenesis by modulating the N6-methyladenosine (m6A) levels of RNA, demonstrating that m6A modifications can promote the biogenesis of human circRNAs (Tang et al., 2020; Timoteo et al., 2020). However, the relationship between m6A methylation and circRNA formation in plants has not been reported. The DNA sequences of the flanking introns of EcircRNAs are highly methylated in moso bamboo (Zhang et al., 2021c), suggesting that DNA methylation in flanking introns of circRNAs may contribute to the biogenesis of circRNAs by regulating the transcription and splicing of linear RNA (Figure 3A). This may be due to high levels of DNA methylation in introns that reduce the elongation rate of RNA polymerase II, or affect circRNA biogenesis caused by the competition of some RNA-binding-domain-containing epigenetic factors with splicing factors for binding to flanking intron regions of RNAs (Zhang et al., 2021c). However, whether DNA methylation affects circRNA biogenesis in plants still needs to be further explored.

Looping can also be mediated by dimerization of RBPs, such as quaking (QKI), muscleblind, and fused in sarcoma, that bind to specific motifs in the upstream and downstream introns in humans and animals. Looping can also be disrupted by RBPs in

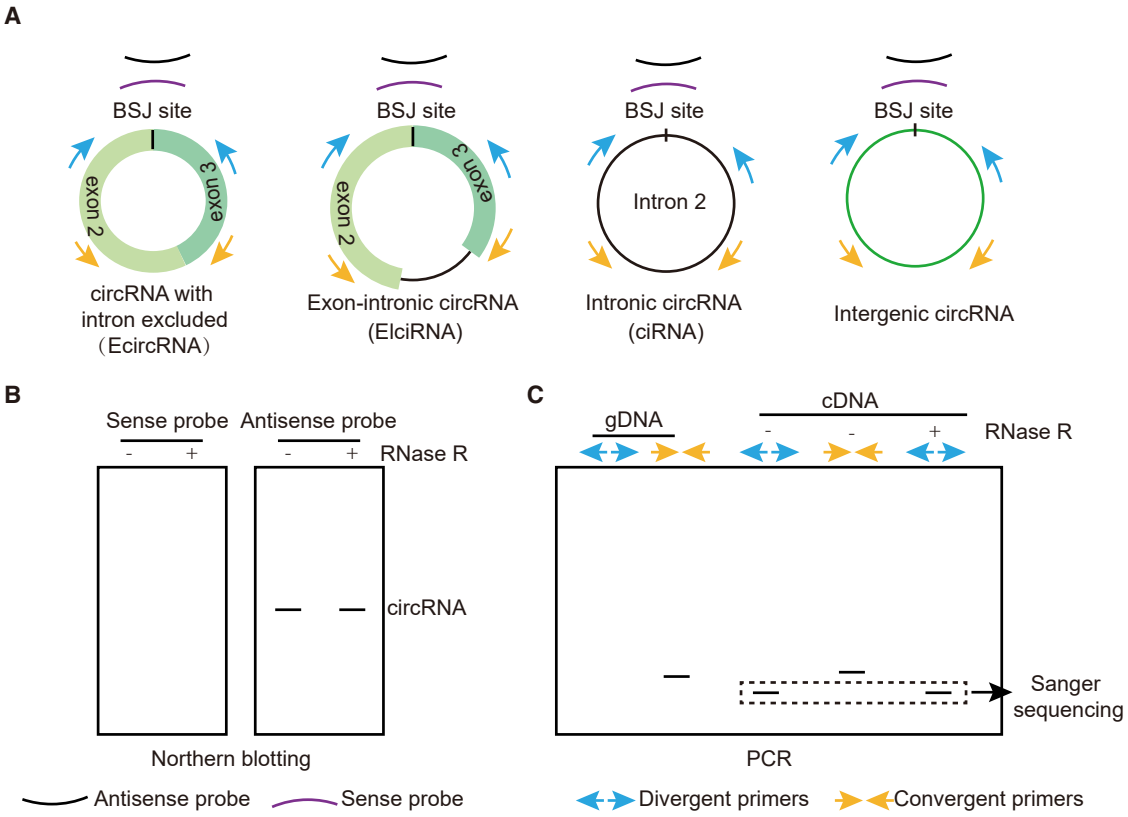

Figure 2. Experimental verification methods for studying plant circRNAs.

(A) Schematic of probes and primers for northern blotting and PCR, respectively.

(B) Identification of circRNAs by northern blotting. For northern blotting, the antisense probe is used as the target probe, and the sense probe serves as the control probe.

(C) Identification of circRNAs by PCR. Divergent primers (blue arrows) and convergent primers (yellow arrows) are designed. For PCR products amplified with divergent primers using cDNA as templates, Sanger sequencing is performed to check whether back-splicing sites (BSJs) are included.

the same systems. For example, adenosine deaminase 1 and ATP-dependent RNA helicase A (DHX9) prevent the formation of looping by editing adenosine to inosine in double-stranded RNA (dsRNA) and by unwinding double-stranded RNA helical structures, respectively (Aktaş et al., 2017; Eisenberg and Levanon, 2018). Immune factors such as NF90/NF110 promote the production of circRNA by stabilizing intronic RNA pairs. However, it is still unclear whether the formation of circRNAs depends on RBPs. Protein homologs of the RBPs QKI, muscleblind, adenosine deaminase 1, fused in sarcoma, and DHX9 have been identified in *Arabidopsis* (Lai et al., 2018). For example, in *Arabidopsis there are* five QKI homologs: AT4G26480, AT5G56140, AT3G08620, AT1G09660, and AT2G38610, all of which are in the K-homology (KH) family (Lai et al., 2018). The roles of these proteins in circRNA biogenesis remain to be investigated (Figure 3A). Circularization of exons correlates with exon skipping in humans (Kelly et al., 2015). In 11 plant species, 38 913 circRNAs were found to overlap with 45 648 alternative splicing (AS) events from their parent isoforms (Wang et al., 2019a), suggesting that the biogenesis of circRNAs may be associated with AS in plants (Figure 3A). However, EcircRNAs can also form R-loop structures with the DNA loci of their parental genes, affecting the formation of exon-skipping AS transcripts (Conn et al., 2017). Therefore, the correlation between EcircRNAs and exon-skipping AS alone may also be because circRNAs affect AS formation.

ciRNAs can be generated from intronic lariat RNA precursors that escape from the debranching step of canonical linear splicing and are produced by the excised introns when the 5′ splice site joins with the branchpoint during splicing and they are degraded by the RNA debranching enzyme 1 (DBR1) for their turnover (Zhang et al., 2013).

Formation and degradation of lariat RNAs during canonical linear splicing are highly conserved in eukaryotes. In *Arabidopsis*, 10 580 lariat RNAs were identified in the wild type and the *dbr1-2* mutant, with higher accumulations of lariat-derived circRNAs in *dbr1-2* mutant plants than in wild-type plants (Zhang et al., 2019d). These results suggest that plant intronic RNAs are also derived from lariat RNAs degraded by DBR1 (Figure 3B).

Unlike circRNAs that are derived from the nuclear genome, mitochondrion-encoded circRNAs (mcircRNAs) may be generated from the degradation of precursor and mature RNAs but not from intron back-splicing (Liao et al., 2022). Mitochondrial RNAs are degraded by coordination of endonucleases and 3′–5′ exonucleases, randomly generating different types of RNA degradation intermediates. These RNA

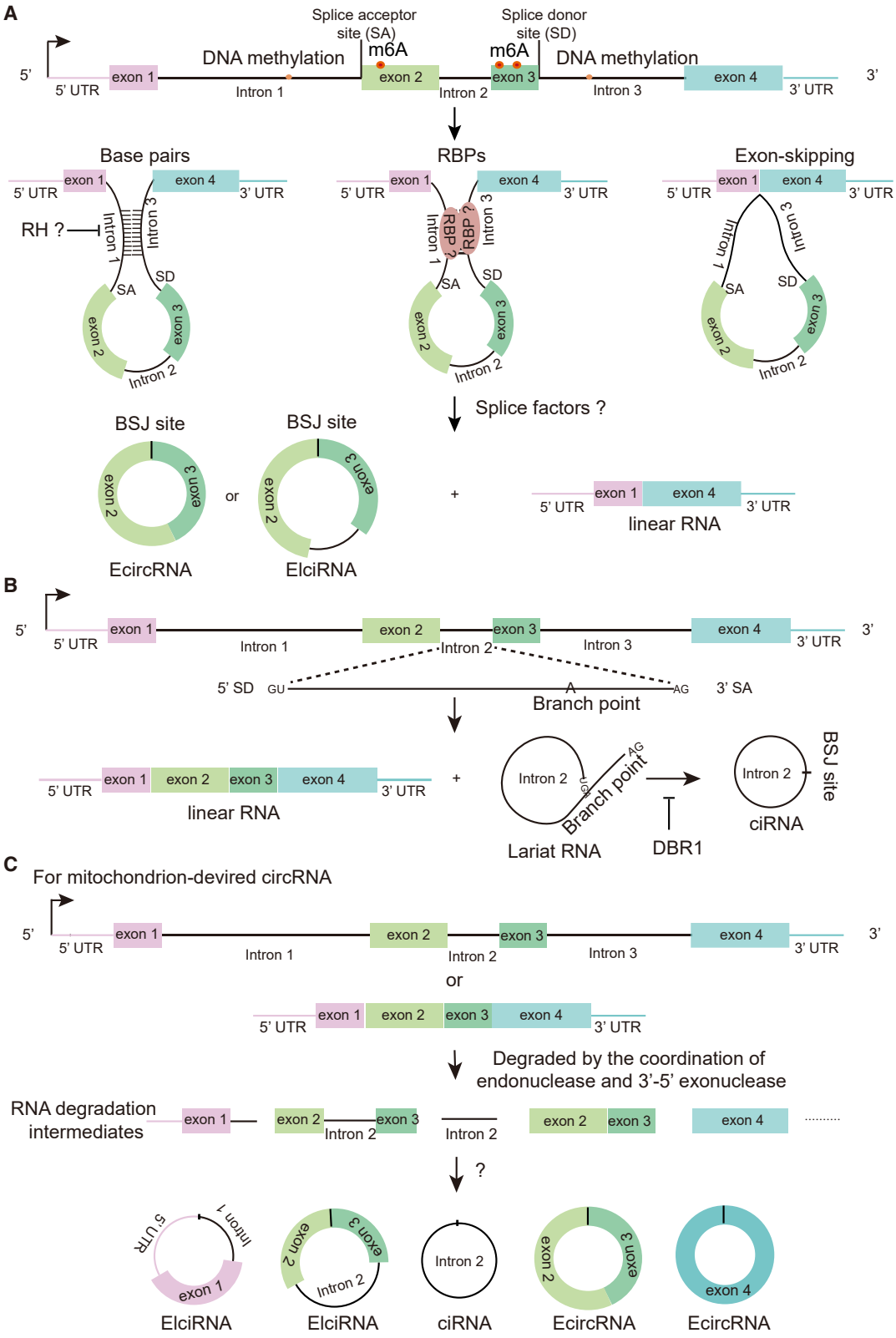

Figure 3. Proposed model of circRNA biogenesis in plants.

(A) The biogenesis of EcircRNAs in plants may be regulated by reverse complementary sequences present in the flanking introns, RNA binding proteins (RBPs), exon-skipping events, N6-methyladenosine (m6A) modifications, or DNA methylation in the flanking introns. RNA helicases may suppress the base complementary pairing to inhibit EcircRNA biogenesis in plants.

(legend continued on next page)

degradation intermediates are circularized by an unknown mechanism to form mcircRNAs (Figure 3C). This process needs further exploration.

FUNCTIONS OF circRNAs IN PLANTS

Putative functions of plant circRNAs

Numerous differentially expressed circRNAs (DEcircRNAs) have been identified during plant growth and development as well in response to biotic (pathogen or virus infection) and abiotic stress (drought, salt, cold, heat, and so on) (supplemental Table 1). Some studies have analyzed the correlation between the expression of circRNAs and their parental genes. For example, only 349 of the 6074 EcircRNAs in rice were significantly correlated to their parental genes based on their expression levels in 18 samples (Ye et al., 2015). In kiwifruit, the expression of 15.3% of EcircRNAs and 19.6% of ciRNAs was positively correlated with parental genes, and 8.3% of exons and 5.2% of introns were negatively correlated with parental genes (Wang et al., 2017c). These studies suggest that differences in most circRNA expression might not be a result of differences in parent gene expression and somehow be regulated in a tissue- or stress-specific fashion. However, the function of these DEcircRNAs needs to be determined experimentally.

Confirmed functions of plant circRNAs

To further study the roles of circRNAs in plants, the functions of a few circRNAs have been determined through overexpression in transgenic plants (Figure 4; supplemental Table 2; supplemental Figure 2). Overexpression of ciRNAs is achieved by overexpressing gDNA containing introns and overexpressing the open reading frame (ORF) sequence as a control (supplemental Figure 2). Overexpression of the ciRNA *lariat41* in *A. thaliana* causes curly leaves, late flowering, altered phyllotaxy, and reduced fertility (Li et al., 2016; Cheng et al., 2018). Although the biogenesis of only a few circRNAs relies on reverse complementary sequences, overexpression of EcircRNAs in plants requires the assistance of reverse complementary sequences and flanking introns (supplemental Figure 2). The reverse complementary sequences promote the circularization of exons, and flanking introns promote the precision of their circularization (Tan et al., 2017; Gao et al., 2019). Six transgenic lines overexpressing *PSY1-circ1* from the key carotenoid biosynthesis gene *Phytoene Synthase 1* (*PSY1*) in the tomato cultivars 'Ailsa Craig' and 'Micro Tom' show significant decreases in lycopene and β-carotene accumulation and produce yellow fruits (Tan et al., 2017). A transgenic line (OE25) overexpressing *PSD-circ1* from *Phytoene Desaturase* (*PDS*) in 'Micro Tom' produces yellow fruits with photo-bleached leaves, petals, and sepals and has severely reduced lycopene and β-carotene contents (Tan et al., 2017). Overexpression of a circRNA from exon 6 of the *SEPALLATA3* (*SEP3*) gene in *A. thaliana* results in plants with reduced stamen numbers and additional petals (Conn et al., 2017). Overexpression of grapevine *Vv-circATS1*, a circRNA derived from *glycerol-3-P acyltransferase*, enhances cold tolerance in *A. thaliana* (Gao et al., 2019). Transgenic *Arabidopsis* plants overexpressing *circGORK*, which is derived from exons 2 and 3 of the *Guard cell outward-rectifying K+ channel* (*GORK*) gene, are hypersensitive to abscisic acid and show greater drought tolerance (Zhang et al., 2019b). Overexpressing *Circ_0003418* from *Potri.014G020900*, a gene that encodes the really interesting new gene (RING)-type E3 ligase XBAT32, in poplar callus under normal conditions or high temperature significantly increases the accumulation of ethylene and malondialdehyde, suggesting that *Circ_0003418* reduces the tolerance of poplar callus to high temperature (Song et al., 2021). Rice lines that overexpress *circR5g05160*, derived from *LOC_Os05g05160*, which encodes a putative plant immunity regulator, MPK14, have much smaller disease lesions and less fungal biomass when infected by *Magnaporthe oryzae*, suggesting that *circR5g05160* enhances tolerance to rice blast disease (Fan et al., 2020).

At present, the functions of a few intronic and intergenic circRNAs have been determined by deletions using CRISPR-Cas9 (Figure 4 and supplemental Figure 2), which edits the DNA sequences of introns and intergenic regions without affecting the protein-coding capacity of ORFs. In rice, at least two independent T1 deletion lines for the ElciRNA *Os06circ02797* and the intergenic circRNA *Os05circ02465* were generated using a multiplexed CRISPR-Cas9 strategy, and their expression of parental or flanking genes was not affected (Zhou et al., 2021a). Two deletion lines of *Os05circ02465* showed high salt tolerance with significantly lower germination rates. Seedlings of the *Os06circ02797* mutants *Os06circ02797Δ1* and *Os06circ02797Δ2* showed a rapid growth phenotype with higher chlorophyll *a/b* content after seed germination (Zhou et al., 2021a). The results of these studies indicate that circRNAs are essential to control plant growth and development and responses to environmental stimuli.

CRISPR-Cas9 has also been used to knock down EcircRNAs by editing the complementary sequences in the flanking introns in humans and animals (Zheng et al., 2016). However, editing strategies are difficult to design, and the knockdown efficiency is unstable. The flanking introns of only a few EcircRNAs in plants contain reverse complement sequences; therefore, application of this method to knock down EcircRNAs in plants is limited. Currently, the knockdown of circRNAs, including all types of circRNAs, by RNAi (small interfering RNA [siRNA]/short hairpin RNA [shRNA]) is a common method used in humans and animals (Li et al., 2018). In plants, only one case of EcircRNA knockdown using siRNA has been reported to date (Song et al., 2021). To knock down circRNAs using RNAi, siRNAs are designed from the junction sites of candidate circRNAs to construct knockdown vectors (supplemental Figure 2). To avoid complementation of the siRNAs with homologous linear RNA, which could affect the expression of linear RNA, the length of the overlapping sequences is strictly limited to ~10 nt (Song et al., 2021). The knockdown vector for *Circ_0003418* was

(B) ciRNAs are derived from lariat RNAs produced from canonical splicing events. RNA debranching enzyme 1 (DBR1) promotes the degradation of lariat RNAs and inhibits the production of ciRNAs.

(C) Mitochondrion-encoded circRNAs (mcircRNAs) may be generated from the degradation of precursor and mature RNAs. Mitochondrial RNAs are degraded by coordination of the endonuclease and 3′–5′ exonuclease, which randomly generate different types of RNA degradation intermediates. These RNA degradation intermediates are circularized by an unknown mechanism to form mcircRNAs.

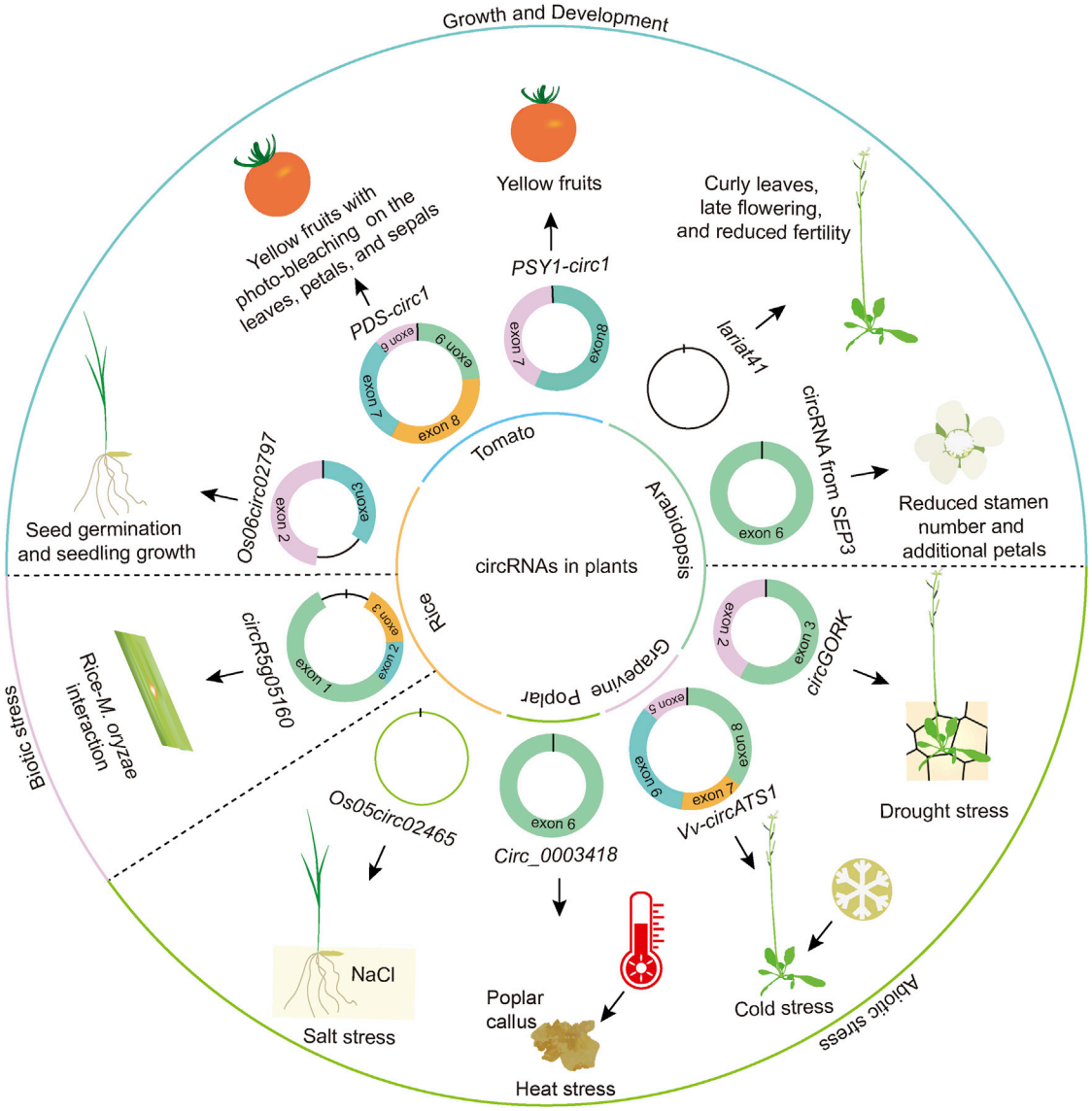

Figure 4. Confirmed functions of circRNAs in plants.
circRNAs from *A. thaliana*, rice (*O. sativa*), tomato (*Solanum lycopersicum*), grapevine (*Vitis vinifera*), and poplar (*Populus tomentosa*) play important roles during plant growth and development (*Os06circ02797*, *PDS-circ1*, *PSY1-circ1*, *lariat41*, and circRNA from *SEP3*) and the responses to abiotic (*circGORK*, *Vv-circATS1*, *Circ_0003418*, and *Os05circ02465*) and biotic stress (*circR5g05160*).

transformed into poplar callus, and the relative expression level of *Circ_0003418* was found to be significantly lower than in the control. However, the knockdown of *Circ_0003418* significantly decreased the abundance of mRNA transcribed from its parental gene, and the authors did not mention the phenotype of the poplar callus after knocking down *Circ_0003418* (Song et al. (2021). Therefore, it remains a challenge to knock down the levels of circRNAs without affecting the expression of their parental genes.

Because overlapping sequences between a circRNA and its cognate linear RNA are hard to distinguish, the CRISPR-Cas13 RNA editing system has been used in humans and animals because the knockdown of various types of circRNAs is more precise than RNAi technology, and there is no effect on the cognate linear RNAs (Li et al., 2021c; Koch, 2021). In the future, an attempt could be made to use CRISPR-Cas13 to knock down plant circRNAs to study their functions. Recently, by targeting splicing signals (GU/AG) involved in back-splicing and canonical splicing, base editors have been shown to efficiently suppress the production of circular but not linear RNAs (Gao et al., 2022). Hence, EcircRNAs with GU/AG splicing signals in plants could be knocked down by editing GU/AG elements in the future.

MECHANISMS OF ACTION OF circRNAs IN PLANTS

Inhibiting the expression of parental genes through formation of R-loops

circRNAs negatively regulate the expression of their parental genes by base-pairing with the genomic sequence of the parental

genes to form R-loop structures, accompanied by increased expression of the corresponding AS transcripts (Xu et al., 2020). In *A. thaliana*, a circRNA derived from exon 6 of the *SEPALLATA3* (*SEP3*) gene regulated exon skipping by forming a circRNA:DNA R-loop, which resulted in down-regulation of *SEP3* transcription and up-regulation of the AS transcript *SEP3.3* missing exon 6 (Conn et al., 2017). In maize, three types of circRNAs from the centromeric retrotransposon *CRM1* bound to the centromere through R-loops (Liu et al., 2020b). However, the effects of their presence on the transcription of *CRM1* and their corresponding alternative spliceosomes have not been reported. DNA:RNA hybrid immunoprecipitation and sequencing in *Populus trichocarpa* have shown that overexpression of *circ-IRX7* in protoplasts of stem-differentiating xylem increased the levels of R-loop structures, resulting in decreased expression of its parental gene *PtrIRX7* and increased expression of its corresponding AS transcript *PtrIRX7-S* (Liu et al., 2021b). Some circRNAs reduce the expression level of their parental genes, but it is unknown whether they affect the expression of their corresponding AS transcripts. For example, overexpression of *Os08circ16564* in rice reduces the expression level of its parental gene (*AK064900*) in transgenic plants (Lu et al., 2015). Delivery of *ag-circRBCS* synthesized *in vitro* into *Arabidopsis* seedlings using the delaminated layered double hydroxide lactate nanosheet method significantly depresses the expression of *RBCS* (Zhang et al., 2021a). Overexpression of *PSY1-circ1* derived from the *PSY1* inhibits the accumulation of mRNA from its parental gene *PSY1* in tomato (Tan et al., 2017). In bamboo, protoplast transformation experiments indicated that overexpression of *circ-bHLH93* decreases the expression of its linear transcript *bHLH93* (Wang et al., 2019d). Moreover, 38 913 circRNAs were found to overlap with 45 648 AS events of the parental isoforms in 11 plant species (Wang et al., 2019a). In *P. trichocarpa*, 8932 R-loop peaks overlapped with 181 circRNAs and 672 AS events (Liu et al., 2021b). The results of these studies suggest that the mechanism by which circRNAs regulate the expression of parental genes and the cognate AS transcripts is through the formation of R-loop structures with the genomic locus, which may be ubiquitous in plants (Figure 5A). In future studies, the mechanism by which these circRNAs form R-loop structures needs to be examined by DNA:RNA hybrid immunoprecipitation and sequencing and R-loop dot-blotting experiments, and phenotypic analyses of stable transformants harboring distinct splicing isoforms or circRNAs also need to be performed.

Acting as miRNA sponges

CircRNAs can regulate the expression of mRNAs by acting as miRNA "sponges" (Hansen et al., 2013; Zhong et al., 2018). That is, circRNAs interact with miRNAs and compete with mRNAs to reduce the binding of miRNAs to mRNAs, thereby inhibiting the degradation and increasing the relative expression of mRNAs (Figure 5B). The circular structures of circRNAs make them more stable than mRNAs, allowing them to compete with mRNAs for binding to miRNAs. In plants, potential circRNA-miRNA-mRNA networks have been predicted in different species under various experimental treatments (supplemental Table 1), suggesting that plant circRNAs may also function by acting as miRNA sponges. For example, Huang et al. (2021) identified 21 DEcircRNAs as potential miRNAs sponges in rice. Also, characterization of co-expression networks and qRT-PCR analysis showed that circRNAs may function as miRNA sponges. For example, the levels of circRNA 3:39 797 208|39 848 986 and its putatively regulated mRNAs decreased during flag leaf development and senescence, suggesting that circRNA 3:39 797 208|39 848 986 might regulate the expression of these mRNAs by binding to osa-miR2925 (Huang et al., 2021). In addition, the *QS-circQS-miR6024* and *AO2-circAO2-miRX282* networks were identified in tobacco, suggesting that circRNAs might regulate the expression of their parental genes by functioning as miRNA sponges (Chen et al., 2019). *Os06circ02797* was predicted to have many putative *OsMIR408* binding sites by bioinformatics analysis, and seven of nine putative *OsMIR408* target genes were downregulated in the *os06circ02797Δ1* mutant, indicating that *Os06circ02797* may function as a sponge for *OsMIR408* (Zhou et al. 2021a). However, direct evidence and systematic research demonstrating the interaction between circRNAs and miRNAs have yet to be reported.

Interacting with RBPs

circRNAs can bind RBPs to function as protein sponges, decoys, scaffolds, and recruiters in humans and animals (Huang et al., 2020) (Figure 5C). Because the specific RNA sequences bound by plant RBPs are not known, there are only a few studies that have focused on predicting the interactions between plant circRNAs and RBPs. RNA immunoprecipitation sequencing and colocalization experiments in *A. thaliana* have shown that lariat RNAs are associated with the DCL1/HYL1 dicing complex *in vivo* and competitively inhibit the binding of HYL1 with pre-miRNA, resulting in reduced miRNA accumulation (Li et al., 2016). In *Arabidopsis*, circRNAs were identified by Argonaute (AGO) immunoprecipitation libraries, showing that these circRNAs may affect the function of miRNAs by interacting with the AGO protein (Capelari et al., 2019). Extracellular RNAs in apoplastic fluid contained circRNAs that formed circRNA-protein complexes with proteins, possibly including GRP7 and AGO, and were protected from degradation when treated with RNase A (Karimi et al., 2022). Whether these circRNAs interact with the GRP7 or AGO proteins remains to be determined by RNA pull-down, RNA immunoprecipitation, and RNA electrophoretic mobility shift assay experiments.

ElciRNAs can promote the transcription of their parental genes in *cis* via interaction with host U1 small nuclear ribonucleoprotein particles and RNA polymerase II (Li et al., 2015), and ciRNAs can interact with the RNA polymerase II complex to enhance the transcription of their parental genes in *cis* (Zhang et al., 2013; Figure 5D). Interaction between circRNAs and RNA polymerase II has not been reported in plants.

Being translated into peptides or proteins

While generally, circRNAs are not thought to be translated into proteins or peptides because of their lack of a 5′ cap structure, there is increasing evidence to suggest that circRNAs can be translated into proteins or peptides via a cap-independent mechanism that recruits translation initiation factors through internal ribosome entry sites (IRESs) or m6A modifications (Pamudurti et al., 2017; Yang et al., 2017; Figure 5E). In plants, circRNAs are also predicted to have translational potential. A total of 165

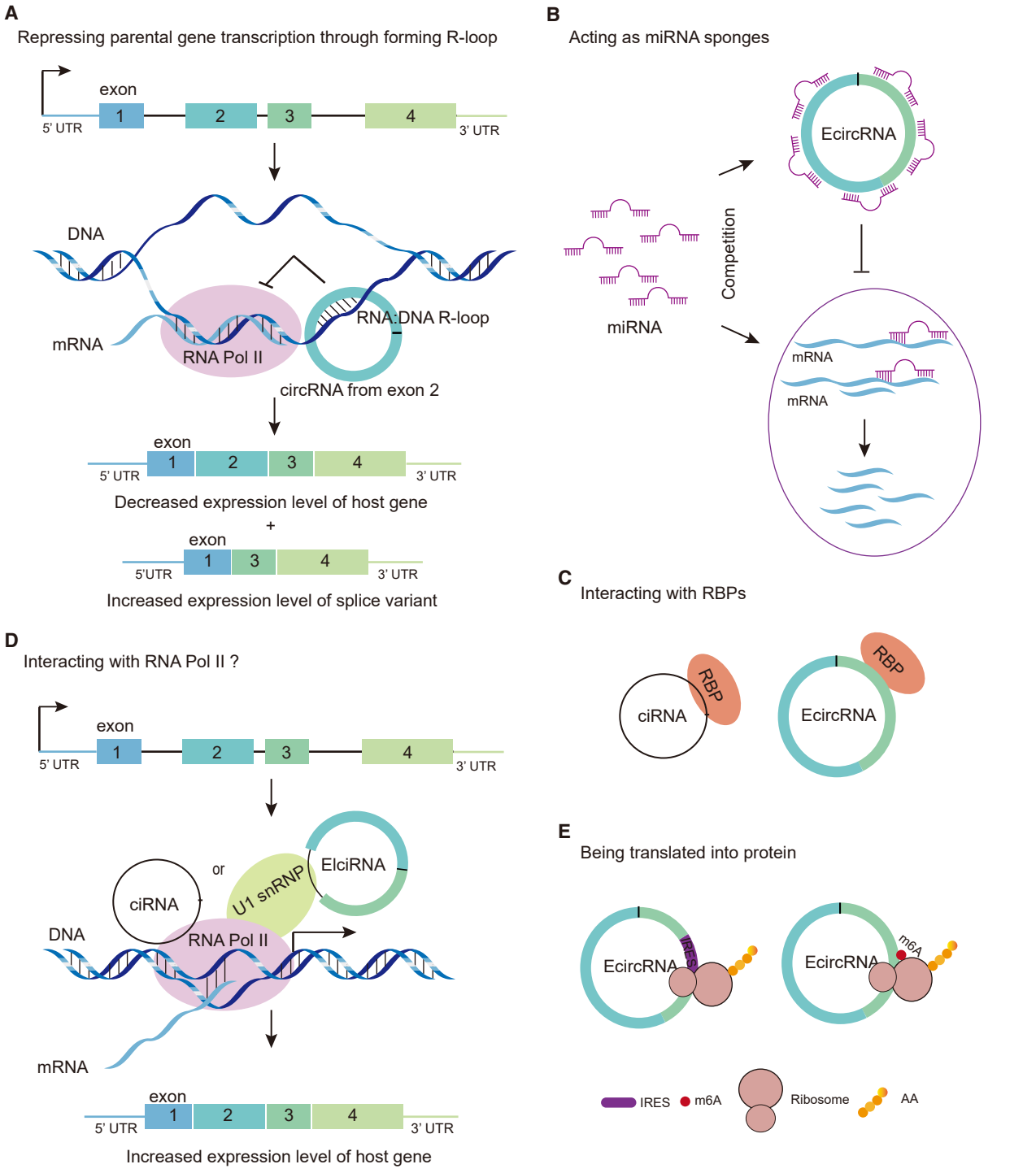

Figure 5. Proposed model for the mechanism of action of circRNAs in plants.

(A) EcircRNAs inhibit the expression of host genes or parental genes and promote the production and expression of corresponding exon-skipping AS transcripts by forming R-loop structures with the genomic DNA locus of host genes.

(B) circRNAs act as miRNA sponges. circRNAs interact with miRNAs and compete with mRNA to bind to miRNAs, inhibiting the degradation of miRNAs on mRNA and increasing the expression of mRNAs.

(C) EcircRNAs or ciRNAs interact with RBPs.

(D) ElciRNAs and ciRNAs promote the transcription of their parental genes via interaction with host U1 small nuclear ribonucleoprotein particles and RNA polymerase II or the RNA polymerase II complex, respectively.

(E) CircRNAs with open reading frames (ORFs) can be translated into peptides or proteins via internal ribosome entry sites (IRESs) or m6A modifications near the ORFs.

soybean circRNAs were predicted to contain at least one IRES element and an ORF, indicating their potential to encode polypeptides or proteins (Chen et al., 2018a). A CircCode tool has been developed to evaluate the coding ability of circRNAs. Based on the ribosome profiling (Ribo-Seq) datasets downloaded from NCBI, 1569 circRNAs in *A. thaliana* may have translation potential (Sun and Li, 2019). In maize, 69 circRNAs were found to be capable of being translated into proteins, and 27 of these were predicted to be translated with high confidence (with at least two Ribo-Seq reads spanning the junction sites through Ribo-Seq sequence data) (Luo et al., 2019). In the giant bamboo species *Phyllostachys edulis*, Wang et al. (2020b) identified 46 circRNAs that contained the m6A modification by Nanopore-based direct RNA-seq, and 11 of these could potentially be translated into long, continuous polypeptides. Liao et al. (2022) identified 1463 mcircRNAs that bound to ribosomes and 358 mcircRNA-derived polypeptides in maize and *Arabidopsis* by analyzing mitopolysome and mitoribosome profiling data and mass spectrometry-based proteomics data, respectively. These studies suggest that plant circRNAs have translational potential, although this needs to be confirmed experimentally.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Recent advances in plant RNA-seq have enabled the identification of many circRNAs that accumulate during normal growth and development and in the responses to abiotic and biotic stress across multiple plant species. These findings not only uncovered a previously unexpected complexity of plant transcriptomes but have also revealed unique characteristics and functions that distinguish these circRNAs from linear RNAs. Identification of circRNAs mainly relies on next-generation sequencing data from rRNA removal and RNase R-treated RNA-seq libraries to identify BSJs. Currently, several tools have been used to predict plant circRNAs. However, these tools yield different results, and only a modest overlap was observed between them (Hansen et al., 2016). Therefore, it is recommended that at least two recognition tools are used to identify more or more reliable circRNAs (Chen et al., 2021). It is difficult to capture full-length circRNAs and to accurately quantify them. The emergence of third-generation nucleotide sequencing technologies (PacBio and Nanopore) makes it possible to detect and accurately quantify full-length circRNAs, and Oxford Nanopore sequencing can detect modifications in circRNAs, which is helpful to study the functions and mechanisms of action of circRNAs (Wang et al., 2020b). However, the library preparation process for long-read sequencing of circRNAs is challenging given their lack of free 3′ ends. In addition, Nanopore sequencing tends to have higher error rates and costs compared with short-read sequencing. Therefore, circRNAs may be better identified using a combination of next-generation and third-generation sequencing. New detection methods with more comprehensive abilities and higher precision for predicting circRNAs will contribute to revealing the complexity of the circRNA world in plants.

Formation of only a few EcircRNAs relies on the presence of reverse complementary sequences in their flanking introns in plants. Whether the biogenesis of EcircRNAs is dependent on RBPs or exon-skipping events or other potential mechanisms remains unclear and needs further exploration. For circRNAs derived from organellar (mitochondrial and chloroplast) genomes, it is presently unknown whether their formation depends on the coordination of endonucleases and 3′–5′ exonucleases, so further experimentation is needed. Compared with studies on the biogenesis of plant circRNAs, studies showing how plant circRNAs are degraded are rare. circRNAs in humans and animals have been reported to be degraded by the miRNA-mediated AGO2 protein, the endonuclease RNase L, or the gene silencing key factor GW182 protein (Hansen et al., 2011; Liu et al., 2019a; Jia et al., 2019), whereas the participation of proteins or enzymes involved in circRNA degradation in plants needs to be investigated. Research is needed to determine whether circRNAs are produced post-transcriptionally or during co-transcriptional processes.

Although the functions of only a few circRNAs have been reported, it is becoming apparent that they play important roles during plant growth and development and the responses to biotic and abiotic stress. The function of circRNAs in plants still needs to be explored further, which may provide new insights for improving agronomic traits in crops. The functions of circRNAs are closely related to their cellular localization, which can be determined by fluorescence *in situ* hybridization and nucleocytoplasmic separation (Gao et al., 2019; Song et al., 2021). Currently, except for *Vv-circATS1*, which is localized in the nucleus and cytoplasm, and *Circ_0003418*, which is localized in the nucleus, the localization of most circRNAs in plants is unknown, and our understanding of how the biogenesis of circRNAs contributes to their unique regulatory patterns, particularly in different biological contexts, remains obscure. Nuclear export of circRNAs in human and animal cells utilizes two separate pathways based on length, in which UAP56 regulates the nuclear export of long circRNAs, and URH49 is required for the nuclear export of short circRNAs. However, it is not clear whether the trafficking of plant circRNAs also depends on these two proteins.

The potential for plant circRNAs to form R-loop structures, act as miRNA sponges, interact with RBPs, and be translated into proteins is gradually being discovered and needs to be investigated further using genetic, biochemical, and molecular biological methods. It is also possible that circRNAs in plants could function through other unknown mechanisms. Although research on plant circRNAs has progressed rapidly in the past few years, we still do not know how many circRNAs have explicit physiological roles because of the low abundance of most circRNAs. With the increased research focus on circRNAs, it is highly likely that there will be a comprehensive understanding of the function of plant circRNAs in the near future.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

AUTHOR CONTRIBUTIONS

Conceptualization, R.L. and G.L.; writing – original draft, R.L. and Y. M.; writing – review & editing, G.L. and G.T; supervision, G.L. and G.T.

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