Review

Long Non-Coding RNAs: Rising Regulators of Plant Reproductive Development

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Abstract: Long non-coding RNAs (lncRNAs) exert a multitude of functions in regulating numerous biological processes. Recent studies have uncovered a growing number of lncRNAs within the plant genome. These molecules show striking tissue-specific expression patterns, suggesting that they exert regulatory functions in the growth and development processes of plants. Plant reproductive development is tightly regulated by both environmental and endogenous factors. As plant reproductive development is a crucial aspect of crop breeding, lncRNAs that modulate reproductive development are now particularly worth regarding. Here, we summarize findings that implicate lncRNAs in the control of plant reproductive development, especially in flowering control. Additionally, we elaborate on the regulation mechanisms of lncRNAs, tools for research on their function and mechanism, and potential directions of future research.

Keywords: long non-coding RNAs; reproductive development; flowering time; molecular regulatory mechanisms

1. Introduction

Plant reproduction is essential for flowering plants to pass on genetic traits to the next generations, and it begins with the transition from vegetative to reproductive growth [1–3]. Flowering is a key factor controlling the production of seed and fruit, and both environmental and endogenous factors are involved in regulating flowering time [4,5]. In recent years, studies have shown that many long non-coding RNAs (lncRNAs) can be identified in plants and play regulatory roles in reproductive development [6]. Long non-coding RNAs (lncRNAs) are non-coding RNAs of more than 200 nucleotides, which can play important roles in epigenomic regulation, gene transcription, and expression of protein-coding genes [7]. Initially regarded as “noise” within the genome, lncRNAs have attracted increasing research interest, and several lncRNAs have been functionally characterized at transcriptional and post-transcriptional levels [8–10]. lncRNAs can be regarded as gene regulatory factors, especially in the diagnosis and prognosis of human diseases [11–16]. In plants, the identification of lncRNAs typically receives more attention than research on functions and mechanisms, although continuously emerging studies report specific developmental expression patterns which suggest potentially important biological functions [17–19]. Here, we summarize recent findings on the study of lncRNAs associated with plant flowering and reproductive development, and their regulatory mechanisms. Furthermore, we discuss the technology and potential future research strategies for studying lncRNAs in this field.

2. Identification and Specific Expression Pattern of Plant lncRNAs

Studies on plant lncRNA functions are still at an early stage compared to those on lncRNAs in humans and animals [20,21]. So far, a large number of lncRNAs have been identified from different kinds of plants. (Table 1).

Table 1. List of long non-coding RNAs (lncRNAs) identified in plants.

Plant Species	Numbers of lncRNAs	Approaches	Type(s) of lncRNAs	Reference
<i>Arabidopsis thaliana</i>	1340	In silico-EST	cis-NATs	[22]
	1320	In silico-EST	trans-NATs	[23]
	76	In silico-EST	lncRNAs	[8]
	179	Tiling array	lncRNAs	[24]
	107	RNA-seq	lncRNAs	[25]
	6480	Tiling array	lincRNAs	[26]
	2418	RNA-seq	cis-NATs	[27]
	838	RNA-seq	Intermediate ncRNAs	[28]
	37,238	Tiling array	sense/antisense lncRNAs	[29]
	955	RNA-seq	lncRNAs	[30]
<i>Oryza Sativa Linanaeus</i>	945	In silico-EST and Microarray	sense/antisense lncRNAs	[31]
	7486	In silico-genome	cis-NATs and trans-NATs	[32]
	3819	annotation	cis-NATs	[33]
	2224	RNA-seq	lincRNAs and NATs	[34]
<i>Zea mays L.</i>	1802	In silico-EST	lncRNAs	[35]
	20,163	In silico-EST and RNA-seq	lncRNAs	[36]
	1724	RNA-seq	lncRNAs	[37]
<i>Triticumaestivum L.</i>	125	Microarray and RNA-seq	lncRNAs	[38]
<i>Gossypium</i>	10,820	reproducibility-based RNA-seq	lincRNAs, intronic and antisense lncRNAs.	[39]
	5996	strand-specific RNA sequencing	lincRNAs and NATs	[40]
<i>Populus L.</i>	2542	RNA-seq	lincRNAs	[41]
	17	stress-specific differentially methylated regions sequencing	lncRNAs	[40]
<i>Malus pumila Mill.</i>	54,975	Microarray	sense/antisense lncRNAs	[42]
<i>Setaria italica</i>	584	RNA-seq	lincRNAs and NATs	
<i>Medicago truncatula</i>	503	In silico-genome annotation	lncRNAs	[43]
<i>Pyrus betulifolia</i>	14,478	RNA-seq	lncRNAs	[44]
<i>Prunuspersica</i>	1417	Transcriptome sequencing	lncRNAs	[45]
<i>Brassica rapa L.</i>	1301	RNA-seq	cis-NATs	[46]
<i>Actinidia chinensis Planch</i>	7051	RNA-seq	lncRNAs	[47]
<i>Cucumis sativus L.</i>	3274	RNA-seq	lincRNAs	[48]
<i>Helianthus annuus</i>	6895	RNA-seq	lncRNAs	[49]
<i>Selaginella</i>	230	RNA-seq	lncRNAs	[50]
<i>Sea buckthorn</i>	3428	RNA-seq	lincRNAs anti-sense lncRNAs intronic lncRNAs	[51]
<i>Solanum lycopersicum</i>	3679	RNA-seq	lincRNAs anti-sense lncRNAs intronic lncRNAs	[52]

Current research to identify novel lncRNAs mainly depends on genome-wide transcriptomics, such as DNA microarrays and RNA sequencing [53]. Most plant lncRNAs can be divided into different types, such as intergenic, intronic, sense, and natural antisense lncRNAs (Table 1). The large number of identified lncRNAs shows significant changes in different organs or during stress, suggesting that they are dynamically regulated and might function in development and stress responses [26]. Based on 200 *Arabidopsis thaliana* transcriptomic versions available from databases, a total of 6480 lncRNAs were identified by either organ-specific or stress-induced expression [26]. In maize, 38 lncRNAs were imprinted from full-length cDNA sequences, which were defined by their different relative expression substantially between maternal and paternal alleles [54]. lncRNAs involved in antifungal networks of *Arabidopsis thaliana* were identified using a powerful tool for lncRNA identification, strand-specific RNA sequencing analysis [55]. A total of 245 lncRNAs with poly(A) tails and 58 lncRNAs without poly(A) tails were identified, which were expressed differentially in *Arabidopsis thaliana* seedlings under four stress conditions [56]. RNA sequencing combined with transcriptome analysis was used to identify 2224 tissue-specific lncRNAs in rice, 600 of which were natural antisense transcripts (NATs) [41]. In *Populus trichocarpa*, 2542 drought-responsive long intergenic non-coding RNAs (lincRNAs) were identified by RNA sequencing [41]. In maize and rice, non-directional and strand-specific RNA sequencing experiments were used to profile numerous lncRNAs which were derived from conserved genomic regions. Protein-coding genes flanking or having a sense–antisense relationship to these conserved lncRNAs were mainly involved in development and stress responses, suggesting that the associated lncRNAs might have similar functions [57]. In *Solanum commersonii*, 18,882 lncRNAs were identified by computational modelling predictions and RNA sequencing [58]. In wheat, 125 putative stress-responsive lncRNAs were identified by computational and experimental analyses [59]. In rice, 5515 lncRNAs were also identified during the transition from seed to mature embryo [60]. Comparative transcriptome analysis identified a total of 300 differentially expressed genes (DEGs) and 254 differentially expressed lncRNAs (DELs) between wild-type and vernalized *Brassica rapa* [61]. In *Solanum lycopersicum*, 3679 lncRNAs were discovered by RNA sequencing, and of them, 677 lncRNAs were significantly differentially expressed in ripening mutant fruits, indicating that lncRNAs could be involved in the regulation of fruit ripening [52]. In a recent study, we unraveled 627 lncRNAs as direct targets of the tomato ripening-related transcription factor RIN in a genome-wide range using chromatin immune-precipitation sequencing combined with RNA deep sequencing, and one of these lncRNAs has been shown to be involved in fruit ripening [62].

The most obvious similarities between the identified lncRNAs from different plant species are high tissue-specific expression patterns, suggesting that lncRNAs might play a specific regulatory role in plant development. Although many studies have reported the functions of lncRNAs, there are great necessities to further develop the study on the function and mechanism of lncRNAs [29,41,63–67] (Table 2).

Table 2. The plant tissue-specific lncRNAs and their biological functions.

Name	Species	Tissue Specificity	Biological Function	Refs.
<i>AtR8</i>	<i>Arabidopsis thaliana</i>	Root	Hypoxic stress	[63]
<i>CsM10</i>	<i>Cucumis sativus</i> L.	Apices of seedlings	Sex differentiation	[64]
<i>GmENOD40</i>	<i>Glycine max</i>	Nodule	Nodule formation	[66]
<i>OsENOD40</i>	<i>Oryza sativa</i> L.	Stem	Nodule formation	[67]
<i>Zm401</i>	<i>Zea mays</i>	Pollen	Fertility	[68]
<i>asHSFB2a</i>	<i>Arabidopsis thaliana</i>	Female gametophyte	Vegetative and gametophytic development	[65]

3. Regulation Mechanisms of lncRNAs in Plant Reproductive Development

As a new class of regulator, the mechanisms of lncRNA regulation are shown to be complicated and various, with the growth in functional researches on lncRNAs [69,70]. Research on the interactions between lncRNAs and DNA, microRNAs (miRNAs), or other molecules has become popular, and lncRNA regulatory mechanisms pose substantial difficulties for the study of cancer and inflammatory diseases [71–73]. In these regulatory processes, lncRNAs interact with other biological molecules in several mechanistic properties, such as decoys, scaffolds, guides, and signals [74]. According to studies in plant reproductive development, the regulation mechanisms of lncRNAs can be summarized as follows: First, lncRNAs can compete with miRNAs to target genes and participate in regulating the accumulation of target genes, which is termed endogenous target mimicries (eTMs) [75]. Recently, this mechanism has been previously shown for plant lncRNAs to apparently induce transcription factors, proteins, or microRNAs as decoys or molecular sponges in a target mimicry mechanism, and thus indirectly regulate the transcription and expression of target genes [75–78]. Research on competing endogenous RNAs (ceRNAs) in plants started later, but so far has shown the similarities to animals and human cells. lncRNA *induced by phosphate starvation 1* was the first discovered eTM in plants, which controlled phosphorus balance in plants by sponging miR399 to dissolve its bond with target genes [75,76]. In *Arabidopsis thaliana* and in rice, genome-wide analyses have identified eTMs from intergenic or noncoding genes sponging 20 miRNAs, including miR156, miR159, miR160, miR166, and miR172 [75]. In addition, the lncRNAs *XLOC_0063639* and *XLOC_007072* were identified and confirmed to effectively sponge miR160 and miR166 from their mRNA, thereby controlling floral and seed development in plants [75]. In addition to miRNAs, several lncRNAs have been shown to interact with short interfering RNAs (siRNAs). For example, *Long day specific male fertility associated RNA* (*LDMAR*) has been found to generate an siRNA, *Psi-LDMAR*, which can cause RNA-dependent DNA methylation (RdDM) and lead to repression of *LDMAR* [79]. For example, the siRNA *osa-smR5864w*, derived from lncRNA *P/TMS12-1*, counts 21 nucleotides (nt) and was identified in rice. The siRNA *osa-smR5864w* can trigger photoperiod-sensitive and temperature-sensitive male sterile rice [80]. Over 700 lncRNAs which were specifically enriched in floral organs can generate phased small interfering RNAs (phasiRNAs). The 21 nt phasiRNAs are induced by a cleavage with miR2118, and play important roles in reproductive stages of rice [81].

lncRNAs can also serve as guides that bind to chromatin-modifying enzymes, guide lncRNA–ribonucleoprotein complexes to specific target regions, and elicit changes of chromatin structure in local regions. *Cold induced long antisense intragenic RNA* (*COOLAIR*) regulates the expression of *FLOWERING LOCUS C* (*FLC*), which belongs to a type of transcriptional regulation [82]. So far, the most common model of the lncRNAs regulatory mechanism is as scaffolds to guide the assembly of different types of molecular complexes in the target region, and forming ribonucleoprotein complexes to play a coordinated regulatory role [66,83]. A good example is the plant lncRNA *cold of winter-induced noncoding RNA from the promoter* (*COLD AIR*), which can bind with polycomb repressive complex 2 (PRC2) complex protein CURLY LEAF (CLF) and recruit it to the *FLC* locus, leading to the deposition of histone chromatin marker H3K27 trimethylation (H3K27me3) [6]. *COLD AIR* can both act as a scaffold in recruiting PRC2 complexes and a guide in targeting PRC2 to specific sites, which illustrates the diversity of lncRNAs' biological functions, addressing that some lncRNAs can regulate gene expression in more than one way [82]. Therefore, as more biological mechanisms of lncRNA are revealed, the lncRNA–protein interaction is constantly updated.

4. Function of lncRNAs in Regulation of Plant Reproductive Development

Researchers pay great attention to plant lncRNAs, which is greatly attributed to the discovery of the relationship between lncRNAs and plant flowering [84]. An increasing number of plant lncRNAs from different species have been identified in recent years [38,39,45,85,86]. However, knowledge of their biological functions is relatively limited. At present, research on lncRNA functions in plant reproductive development is mainly limited to plant flowering and pollen development. Here,

we summarize recent studies on lncRNAs that modulate plant reproductive development by a diversity of pathways, as shown in Figure 1.

Figure 1. A simplified schematic of lncRNAs identified in plant reproductive development. Cold induced long antisense intragenic RNA (COOLAIR), cold of winter-induced noncoding RNA from the promoter (COLDAIR), and cold of winter-induced noncoding RNA from the promoter (COLDWRAP) can repress the FLOWERING LOCUS C (FLC) gene to stimulate vernalization; CDF5 LONG NONCODING RNA (FLORE), a natural antisense transcript (NAT) of CYCLING DOF FACTOR 5 (CDF5), promotes CDF5 and increases FLOWERING LOCUS T (FT) transcript level; TCONS_00035129 results in changes of hormone content of gibberellin A₃ (GA₃) in vernalized plants by regulating BraZF-HD21 target genes in *Brassica rapa*; AGLX2 promotes reproductive growth to vernalization; and LDMAR can regulate photoperiodic-sensitive male sterility (PSMS) in rice. The silencing of LDMAR is mediated by Psi-LDMAR through an RNA-dependent DNA methylation (RdDM) pathway; and Zm401 and BcMF11 are involved in regulation of pollen development.

4.1. Vernalization

The timing of flowering greatly affects the production of seeds and fruit [87]. Vernalization, an adaptation to avoid pre-winter flowering, contributes significantly to flowering time in accordance with the seasonal changes for biannual and perennial plants in temperate and boreal climates [5]. *FLC* is key for inhibiting flowering under cold temperatures and is crucial for the vernalization and flowering time control in *Arabidopsis thaliana* [88]. Before exposure to low temperatures during vernalization, the level of *FLC* expression is high. High levels of histone H3K4 trimethylation and H3K36 trimethylation are observed, which are regarded as the “active” marks of gene expression. Conversely, the expression of H3K27me₃ is low [89] (Figure 2). With winter forthcoming, *FLC* expression is repressed by autonomous-pathway genes and constitutive *FLC* repressors [90,91]. Recent studies have shown that non-coding RNAs, such as intronic RNAs [6], antisense lncRNAs [82,92,93],

and small RNAs [7] are involved in modulation of *FLC* expression [94]. The first lncRNA involved in the regulation of *FLC* was called *COOLAIR*, which is located at the 3' end of the *FLC* locus [95,96] and accumulates in the vernalization process [58] (Figure 2). *COOLAIR* comprises the Class I and II polyadenylated antisense *FLC* transcripts, and has been proposed to trigger vernalization-mediated *FLC* silencing [71]. The repression of distal *COOLAIR* transcription has also been shown to be the signal for recruiting *FLOWERING LOCUS D* (*FLD*) to *FLC* chromatin and leads to H3K4 demethylation [75–77] (Figure 2). However, the regulatory role of *COOLAIR* in *FLC* and the vernalization process has yielded controversial results [97,98]. Novel findings show that *FT*, an important gene in the control of flowering time, represses the expression of *FLC* by activating *COOLAIR* [99], which suggests that the feedback regulation of *COOLAIR* expression controls flowering time and seeds dormancy. Another lncRNA, *COLD AIR*, which is 5'-end capped but not polyadenylated and transcribed from the *FLC* intron 1, has been also found to be involved in vernalization [92]. Transcription of *COLD AIR* occurs later than that of *COOLAIR* during vernalization [100]. *COLD AIR* can recruit PRC2 to stimulate H3K9me2 and H3K27me3 accumulation, thereby silencing the expression of *FLC* [6,97] (Figure 2). Recent studies showed that *cold of winter-induced noncoding RNA from the promoter* (*COLDWRAP*) is generated by an *FLC*-inhibiting promoter and necessary for the stable repressive state of *FLC* (Figure 2). Taken together, these results indicate that both *COLD AIR* and *COLDWRAP* are necessary for vernalization [101]. Notably, after *COLD AIR* is silenced by RNA interference, plants will maintain a delay of flowering with the onset of spring [6]. Therefore, interactions between lncRNAs and the maintenance of the *FLC* locus associated with PRC2 warrant further investigation. Subsequently, genome-wide studies using custom-made NAT arrays in *A. thaliana* identified lncRNA *CDF5 LONG NONCODING RNA* (*FLORE*), a NAT of CYCLING DOF FACTOR5 (*CDF5*) [61,68,79,102,103]. *CDF5* delays flowering by directly binding to the promoters of *CONSTANS* (*CO*) and *FT*, whereas *FLORE* promotes the increase of *FT* transcript levels, indicating that the *CDF5/FLORE* NAT pair may be a new regulatory mechanism of flowering [61,68,79,102,103]. The lncRNA *TCONS_00035129* was found to map to the plant hormone signal transduction pathway and to increase the content of plant hormone gibberellin A₃, thereby affecting the process of hormone biosynthesis in vernalization [61] (Figure 1). The first candidate lncRNA in sugar beet, *AGL15X1*, has been shown to be coordinated with the *FT* target genes *BvFT2* and to repress *BvFT1*, thereby promoting vernalization during cold conditions [103].

Figure 2. Schematic illustrations of the regulation of *FLC* regulated by long noncoding RNAs (lncRNAs) and chromatin modifying complexes during the course of vernalization. (a) Before cold, *FLC* is actively transcribed by activation complexes, PAF1, which methylates of H3K4me3 and H3K36me3 at high levels and H3K27me3 at a low level; (b) During cold, lncRNAs *COLDAIR* and *COLDWRAP* recruit the PRC2 complex to repress *FLC*, which also repressed by *COOLAIR*, and the methylation of H3K27me3 increases; (c) After cold, *FLC* is stably silenced by PRC2 complexes, maintaining H3K27me3 through the whole *FLC* chromatin. As the *FLC* locus passes to the next generation, the active chromatin state represented in (a) is re-established.

4.2. Photoperiodic-Sensitive Male Sterility and Pollen Development

The role of pollen development is significant for plant reproductive development [104]. Research showed that lncRNAs also act as a layer in the regulation network of pollen development, which is a complicated physical–chemical process. After the *Nongken 58S* mutant was reported to be associated with photoperiodic-sensitive male sterility (PSMS) in plants [105], further genetic analysis showed that a 1236 nt lncRNA, *LDMAR*, regulated PSMS in rice [106]. *LDMAR* participates in the regulation of rice fertility under long day conditions [106] (Figure 1). Decreased *LDMAR* expression may result in premature programmed cell death in immature anthers, and photosensitive male sterility [106]. Other studies have shown that a siRNA produced by *LDMAR* promoter transcription can induce RNA-directed DNA methylation in the *LDMAR* promoter region, thereby inhibiting *LDMAR* expression [79]. Subsequently, *LDMAR* can be repressed through an RNA-dependent DNA methylation (RdDM) pathway, which is induced by the siRNA *Psi-LDMAR* [79]. In other species, lncRNAs that regulate pollen development were found, including a stamen-specific expression of lncRNA *Zm401* in maize [68]. *Zm401* impaired the expression of pollen developmental genes by regulating the key genes *Zm3-3*, *ZmMADS2*, and *ZmC5*. Reducing *Zm401* expression will cause male sterility in maize (Figure 1). lncRNA *BcMF11* is 828 nt, and was identified in *Brassica campestris* L. spp.

The lowered expression of *BcMF11* will cause pollen grains to be unable to mature [107], suggesting that *BcMF11* was specialized to regulate reproductive development [108]. In addition, when *BcMF11* was silenced, female flower development was normal, whereas tapetum and microspore development were abnormal, which resulted in male pollen abortion and male sterility [109] (Figure 1). Taken together, these data showed lncRNAs are crucial for the regulation of plant pollen development.

5. Perspectives

The functions of lncRNAs in plant reproductive development are investigated continuously and attract increasing research attention. Plant reproductive development is not only one of the most important stages in plant development, but also a crucial process for human life. Therefore, it is important to explore the regulation mechanisms of lncRNAs in plant reproduction [17,103]. Here we summarized several types of methods for studying lncRNA regulation in plant reproductive development (Figure 3).

Figure 3. Summary of the current technologies for studying long noncoding RNAs (lncRNAs) in plant reproductive development. LncRNA expression profiles by RNA-seq; screening lncRNAs with different expression by lncRNA databases; silencing lncRNAs for loss-of-function study with methods like clustered regularly interspaced short palindromic repeats (CRISPR)/-associated protein 9 (Cas9); detecting the interaction between lncRNAs and other molecules by RNA pull down, chromatin Isolation by RNA Purification (ChIRP) and crosslinking-immunoprecipitation (CLIP).

5.1. Technical Challenges

Based on classical methods of lncRNA research, the most important approaches for discovery, prediction, and identification of lncRNAs include microarrays, RNA sequencing, fluorescence in situ hybridization (FISH), and RNA interference (RNAi). The future development of plant lncRNA identification will be largely dependent on the optimization of high-throughput sequencing technology. For example, the paired-end strand-specific RNA sequencing can provide transcript information more accurately in the future, suggesting that the expression of lncRNAs during plant reproductive development will be shown in more detail [110]. In the future, the functions of identified lncRNAs

and data sets of lncRNAs in plant reproductive development need to be updated and made available in databases, such as NCBI-Gene, Ensembl, NONCODE, and LNCipedia [71,111], which at present mainly focus on human diseases and animal cells. Furthermore, new predictive tools which can analyze sequences, chromosome location, and time/tissue specificity of lncRNAs in plants, particularly during reproductive development, are urgently needed. Because of the numerous differences between biological functions of lncRNAs inside and outside the nucleus, more accurate and appropriate cellular localization techniques are needed for the further functional study of lncRNAs identified by high-throughput sequencing. Traditionally, validation of plant lncRNA localization has been performed using FISH, which has been a bottleneck in the study of heterogeneity and subcellular localization of plant RNA molecules in single cells due to the particularity of samples and the limitation of auto fluorescence in vivo.

To address these problems, single-molecule RNA FISH (smFISH) was introduced to visualize individual RNAs using multiple fluorescently labeled oligonucleotide probes specific to the target RNA [112]. SmFISH enables direct quantitation of mRNA, and multiple RNA species can be scored simultaneously in the same cell by multiplexing smFISH probes [113]. It has been applied for subcellular localization of *COOLAIR* at a cellular level [114]. Using the Stellaris smFISH technique, *FLC* and its antisense transcript, *COOLAIR*, did not appear in the same cell in most cases during cold exposure, and their localizations in cells seemed mutually exclusive. The application of smFISH with *COOLAIR* is likely to reveal that the sense transcription and antisense transcription of *FLC* are mutually exclusive at the spatialization level and will provide insights into the regulation function of *COOLAIR* in vernalization of plants. Furthermore, this approach will help to establish a technique to detect the localization and quantification of a single RNA in *Arabidopsis thaliana* [114]. This method can be widely applied to other plants by optimization of the sample processing in order to overcome spontaneous fluorescence in plant green tissues. Considering the multitude of applications in model organisms [115], we believe that smFISH will become a new powerful tool for lncRNA research in plant reproduction.

In order to investigate the functions of lncRNAs, clustered regularly interspaced short palindromic repeats (CRISPR)/-associated protein 9 (Cas9)-induced genome editing technology has been an efficient and powerful tool [116]. Relying on relatively low abundances of off-target sites after knockout of the target gene, the efficiency of DNA disruption, and the inheritance of function fails, CRISPR/Cas9-induced genome editing technology has been considered appropriate to lncRNA loss-of-function analysis [117]. In order to investigate the gene function, the CRISPR/Cas9 system has been successfully applied to create multisite genome knockout mutations. Compared with wild-type fruits, the CRISPR/Cas9-engineered *lncRNA1459* mutations showed significant delays of ripening in *Solanum lycopersicum* [118,119], suggesting that CRISPR/Cas9 technology is a potential avenue for research on lncRNAs involved in the regulation of plant reproductive development [120,121]. Although Cas9 has proven its utility in loss-of-function studies, there is some controversy as a knocked-out DNA molecule may still participate in regulating the transcription of neighboring mRNAs [122]. Generally, the lncRNA functional analysis in plant reproductive development is full of challenges and uncertainties, and therefore the use of newly developed technologies and methods is recommended to benefit from progress made in other fields of research.

5.2. Strategies

In recent years, due to the increasing availability and rapid development of high-throughput transcriptome profiling techniques, an increasing number of lncRNAs of various plant species have been reported. Although the studies on regulation mechanisms of lncRNA were shown to be difficult, we present several avenues for illuminating the regulation mechanism of lncRNAs according to the regulation levels of lncRNA, including post-transcriptional regulation, epigenetic modification, and transcriptional regulation.

In the cytoplasm, ceRNAs, which regulate other RNA transcripts by competing for shared miRNAs, are an emerging model of post-transcriptional regulation [77]. Recent studies have found that lncRNAs can act as “sponges” of miRNAs and bind to miRNAs competitively, preventing the inhibition of target genes by miRNAs [77,123]. If localized in the cytoplasm, a binding miRNA shared by lncRNA and mRNA may occur, which necessitates experiments to test the regulatory relationship between these molecules [124]. However, the ceRNA regulation research is at an early stage in plants, particularly compared with its state in cancer research [3,125–127]. As the first ceRNA was identified in a process of germination in rice, future analysis will need to focus on the regulation mechanisms of ceRNA in plant reproductive development.

If an lncRNA is localized within the nucleus, its potential regulatory role at the chromatin level should be considered. The main research foci to test the hypothesis are: (i) To determine whether lncRNA binds to chromatin; (ii) whether chromatin status is affected by lncRNA; (iii) to identify the locations of specific regulatory regions; (iv) to identify the lncRNA binding proteins; and (v) whether the expression of target genes is affected by lncRNA. lncRNA can also act as a transcriptional coactivator in the nucleus at the transcriptional regulation level. Studies have found that lncRNAs act in *cis* to regulate the expression of nearby mRNAs. In this case, it is necessary to confirm whether there is an expression correlation of the lncRNA and target genes, and whether the expression of mRNA is affected by the intervention of lncRNA. In addition, follow-up studies are needed to examine whether lncRNAs recruit proteins or complexes to later bind to gene promoter regions in order to achieve regulatory functions [128–130]. In contrast, if lncRNAs exert *trans*-regulatory functions, RNA pulldown screening can be used to find lncRNA binding proteins (Figure 3) [6]. For this, it is necessary to first identify the proteins that may bind to lncRNAs, or to find the downstream targets, and second, to analyze the correlation between the expression of the lncRNA and target genes. Finally, it needs to be demonstrated whether the lncRNA and the binding proteins bind to the promoter region of the target gene for expression regulation.

Although many lncRNAs can be identified during flowering and other phases of plant development at the present, our knowledge of the functions and regulation mechanisms of lncRNAs is still limited. Although our knowledge of lncRNA-mediated regulation of vernalization is steadily increasing the understanding of the regulatory mechanisms of lncRNAs, substantial progress in this field is still to be made. The era of determining the function of plant lncRNAs is just beginning. How to map the biological functions and the mechanism of regulation of lncRNAs from animals and diseases to plant reproductive development and how to establish a better prediction and verification of lncRNA in plant reproductive development are extremely difficult. The rapid advancement of technologies will bring new opportunities and breakthroughs in lncRNA research, and the unexplored fields of plant reproductive development will gradually be explored.

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