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# Functions of long intergenic non-coding (linc) RNAs in plants

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**Abstract** Whole transcriptome analyses in many organisms have revealed that most transcribed RNAs do not encode proteins. These non-coding RNAs likely contribute to the regulation of gene expression during the development of multicellular organisms. In eukaryotes, the roles of small RNAs, one class of non-coding RNAs, in transcriptional and post-transcriptional regulation have been well characterized. However, the functions of a second class of non-coding RNAs, long intergenic noncoding (linc) RNAs, are relatively unknown, especially in plants. Recent advances in RNA-seq and tiling microarray technologies have revealed the presence of many lincRNAs across plant species. This review focuses on the functions of lincRNAs that have been recently reported in plants. One of the most well characterized functions of lincRNAs is to epigenetically regulate gene expression by recruiting proteins for chromosome modification to specific loci. Second, lincRNAs are known to inhibit the physical interaction between microRNAs (miRNAs) and their target mRNAs thus controling protein levels of the target mRNAs. Lastly, lincRNAs control alternative splicing by binding and sequestering the proteins required for alternative splicing.

**Keywords** Long intergenic non-coding RNA (lincRNA) · Polycomb repressive complex2 (PRC2) · MicroRNAs (miRNAs) · Endogenous target mimic (eTM) · Alternative splicing

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## Introduction

Deep transcriptome analyses have revealed that a substantial portion of the human genome is transcribed (Consortium 2012; Djebali et al. 2012). However, only a small portion of the transcriptome is translated into proteins (Ponting et al. 2009). The transcripts that are not translated are termed non-coding RNAs (ncRNAs). The abundance of ncRNAs suggests that they could play important regulatory roles in the development of complex organisms.

These ncRNAs are composed of divergent sub-groups according to RNA length. Small RNAs (less than 200nt), which include microRNAs (miRNAs) and small interfering RNAs (siRNAs), have been well characterized in regard to the regulation of transcription and post-translation in eukaryotes, including plants (Chitwood and Timmermans 2010). Long intergenic non-coding RNAs (lincRNAs), (longer than 200nt), are defined as transcripts that lack an open reading frame of more than 100 amino acids, are located at least 500 bp away from annotated protein coding genes on the chromosome, and encode no transposable elements (Liu et al. 2012). Thousands of lincRNAs in the human genome have been identified, but their function remains unclear. While the expression level of lincRNAs in humans is over ten times lower than the average expression level of the protein coding genes, they are expressed in a tissue-specific manner and induced by stress, suggesting an important functional role (Cabili et al. 2011). Indeed, many lincRNAs are expressed in the mouse embryonic stem (ES) cells (Guttman et al. 2011). Knockdown of a group of these lincRNAs was shown to disrupt pluripotency and change the expression levels of differentiation markers (Guttman et al. 2011).

One well characterized lincRNA function in animals is the recruitment of the protein complexes that mediate

epigenetic changes at specific chromosomal loci. Many lincRNAs are associated with polycomb repressive complex 2 (PRC2), which tri-methylates histone H3 at lysine 27 to silence transcription of local genes (Hekimoglu and Ringrose 2009). For example, *Xist* lincRNA is expressed from the inactive X chromosome and recruits PRC2 in a cis-regulated manner (Chaumeil et al. 2006). The *HOX* transcript antisense RNA (*HOTAIR*) is transcribed from the *HOXC* homeotic locus and represses the expression of the *HOXD* locus in a trans-regulated manner through recruitment of the PRC2 complex (Li et al. 2013). This lincRNA is vital for body patterning during mammalian development. More than 800 *HOTAIR* occupancy sites have been identified within the gonome using Chromatin Isolation by RNA purification followed by sequencing (ChiRP-Seq) (Chu et al. 2011). Furthermore, a significant pattern of co-occupancy between *HOTAIR* and the PRC2 complex has been discovered, suggesting that *HOTAIR* guides PRC2 to many target sites on the chromosome (Chu et al. 2011).

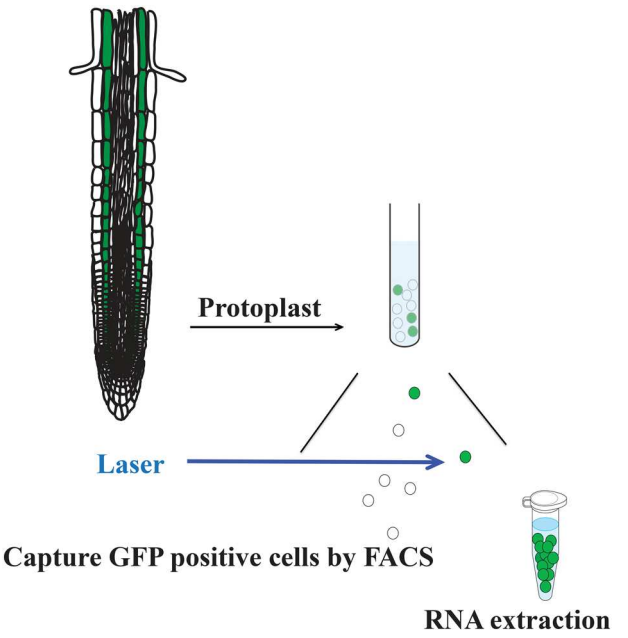


**Fig. 1** The whole transcriptome analysis at the specific cell type. GFP is expressing in the specific cell types of *Arabidopsis* roots (*upper right*). The tissues are collected from these GFP marker lines and rapidly converted to protoplasts by enzymatic digestion (*upper left*). GFP-positive cells are isolated using a fluorescence activated cell sorter. Total or poly-A selected RNAs are extracted from enriched cells for transcriptome analysis (*lower right*)

## Discoveries of LincRNAs in plants

Recent advances in sequence technology and computational analysis have revealed that lincRNAs are also present in diverse plant species. In *Arabidopsis*, thousands of ncRNAs have been identified by expressed sequence tags (EST) and tiling microarrays (Jouannet and Crespi 2011; MacIntosh et al. 2001; Marker et al. 2002; Rymarquis et al. 2008; Song et al. 2009). However, it was reported that a large portions of plant ncRNAs are natural antisense RNAs (Matsui et al. 2008; Okamoto et al. 2010). Thus far, over 6,000 lincRNAs have been identified by a strict systematic search for only lincRNAs (Liu et al. 2012). As with mammals, in plants it has been shown that lincRNAs are more lowly expressed (about 30–60 times lower than mRNAs in *Arabidopsis*) than mRNA transcripts and that they are most highly expressed at the level of specific tissues (Liu et al. 2012). Cell type specific transcriptome analyses in plants are very likely to uncover even more lincRNAs. These analyses are challenging, because plant cells are tightly connected to each other by the cell walls. One method to isolate specific cells is to digest the cell wall enzymatically and then use fluorescence-activated cell sorting (FACS) to enrich for specific cell types (Fig. 1) (Birnbaum et al. 2005, 2003; Brady et al. 2007; Iyer-Pascuzzi and Benfey 2010; Petricka et al. 2012). This approach combined with RNA-seq was used to generate whole transcriptome profiles for all cell types and developmental zones in the *Arabidopsis* root (Li et al. 2016). This analysis was used to identify 1,200 lincRNAs from a previous database (Liu et al. 2012) and an additional 430 novel lincRNAs, the majority which were found to be expressed in specific cell types (Li et al. 2016). The identification of additional lincRNAs was likely enabled by the enhanced sensitivity acquired by measuring cell type specific expression, unlike previous transcriptome analyses which used whole organs or tissues. Indeed, only 25% of these lincRNAs are detected in more than half of the cell types, which strongly suggests that lincRNAs play a cell type specific role in the root. Furthermore, cell type specific transcriptome analyses have provided 26 co-expression networks of protein coding genes and lincRNAs that are differentially expressed across all cell types of the *Arabidopsis* root suggesting that lincRNAs regulate gene expression in specific cell types (Li et al. 2016).

Transcriptome data across plant species suggests that lincRNAs are important for plant development. In wheat, over 100 putative stress responsive lincRNAs have been identified (Xin et al. 2011). These lincRNAs are again expressed in a tissue-specific manner and induced by powdery mildew infection and/or heat stress. In maize, over 20,000 putative lincRNAs have been identified using the EST and RNA-seq databases, which contain the results of 30 different experiments (Li et al. 2014). Substantial portions of these lincRNAs were predicted to be the precursors of small RNAs and only 1,704 of these were considered to be high-confidence lincRNAs. In rice, over 1,000 lincRNAs have been identified from tissue-specific whole

transcriptome analyses at two different developmental stages and from 40 public RNA-seq datasets (Zhang et al. 2014). LincRNAs have also been identified from other plant species such as sorghum (Qi et al. 2013), peach (Wang et al. 2013), and Populus (Chen et al. 2015; Shuai et al. 2014). Huge amounts of expression data in many plants suggest that lincRNAs are important for plant development. However, the functions of only a few lincRNAs have been experimentally tested and characterized in plants.

## Transcriptional regulation by lincRNA

Plant development is regulated by both environmental signals and innate genetic programs. For example, certain plants flower in the spring after sensing prolonged exposure to winter cold. This vernalization response is an environmentally induced epigenetic switch that silences the expression of the floral repressor, *FLOWERING LOCUS C* (*FLC*), in *Arabidopsis*. Winter cold triggers the enrichment of histone tri-methylation at the *FLC* locus through PRC2. This epigenetic change results in silencing of *FLC*.

*COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) recruits PRC2 to the *FLC* locus and silences expression of *FLC* by vernalization-mediated epigenetic control (Heo and Sung 2011). *COLDAIR* is a long intronic ncRNA (1.1 kb) that is transcribed from the first intron of the *FLC*. The expression of *COLDAIR* is induced by cold treatment, reaches maximum levels at 20 days of cold, and then returns to basal levels. In the opposite pattern, the expression of *FLC* is repressed by chromosomal change after 20 days of cold treatment. *In vitro* assays suggest that COLDAIR physically associats with several components of the PRC2 complex *in vitro* assay. An RNA-immunoprecipitation assay showed direct interaction between *COLDAIR* and one component of PRC2 *in vivo* during cold treatments. Their maximum interaction occurred after 20 days of cold treatments. When *COLDAIR* was knocked down in plants using RNA interference, there was a late flowering phenotype even after vernalization. Interestingly, *FLC* expression was still repressed in the *COLDAIR* knock down lines, but repression of *FLC* was not maintained after cold treatment in the *COLDAIR* knock down lines. The enrichment of one component of PRC2 in relation to *FLC* is strongly reduced and not induced by cold in the *CLODAIR* knock down lines. Also, the levels of Histone H3 lysine 27 tri-methylation at the *FLC* locus were largely impaired in the *COLDAIR* knock down lines. These findings showed that *COLDAIR* recruits PRC2 to the target site of chromatin to repress gene expression (Heo and Sung 2011). To date, this is the only example in plants of regulation of gene expression by the histone H3 lysine 27 tri-methylation induced by a non-coding RNA.

As an example for transcriptional regulation by lincRNA, it has been shown that *HIDDEN TREASURE 1* (*HID1*) negatively controls the gene expression of *PHYTOCHROME INTERACTING FACTOR 3* (*PIF3*) (Wang et al. 2014). Light signal is one of the important environmental signals to regulate plant development. The *PIF* genes encode basic helix-loop-helix transcription factors that are repressors of photomorphogenesis, resulting in a short hypocotyl, opened cotyledons, and chlorophyll synthesis in response to light exposure. The *pif3* mutant shows a hyper-photomorphogenic phenotype. The *hid1* mutant has abnormal photomorphogenesis resulting in a longer hypocotyl under continuous red light (Wang et al. 2014). In the *hid1* T-DNA mutant, the T-DNA is inserted into a polycistoronic cluster of four lincRNAs at the intergenic site resulting in decreased accumulation of all four lincRNAs. However, only one lincRNA could rescue the *hid1* phenotype. This lincRNA is 236nt RNA and contains a potential open reading frame (ORF) encoding a 44-amino acid peptide. Plants with a *HID1* mutation that disrupts a potential ORF but retain RNA structure could rescue the *hid1* mutant phenotype. *HID1* is predicted to form four stem-loop structures based upon computater simulations. Two stem loops of *HID1* were essential for rescue of the *hid1* phenotype. These experiments showed that *HID1* functions as a lincRNA to control photomorphogenesis. Over 600 genes were significantly changed in the *hid1* mutant compared to wild type plants grown under red light. Of these genes, a small set of red light responsive genes was upregulated. One of these, *PIF3*, was upregulated and PIF3 protein accumulated in the *hid1* mutant under red light. These results suggest that *HID1* negatively regulates *PIF3* expression. *HID1* was found mostly associated with chromosomes, suggesting that *HID1* may directly regulate *PIF3* expression. ChiRP followed by qPCR showed that *HID1* physically associates with the proximal region of the *PIF3* promoter (Wang et al. 2014). These results showed that *HID1* transcriptionally regulates *PIF3* under red light (Fig. 2a).

## Target mimics of miRNAs

MicroRNAs (miRNAs) are small RNAs of about 21nt that post-transcriptionally regulate target mRNAs via complementary sequences by site-specific cleavage and the translational repression of the target mRNAs. LincRNAs have been shown to bind to miRNAs via complementary sequences as decoy RNAs to block the interaction between miRNA and their authentic target mRNAs. In one example, a lincRNA functions as a decoy RNA, when plants are grown under a phosphorus (Pi) limited condition.

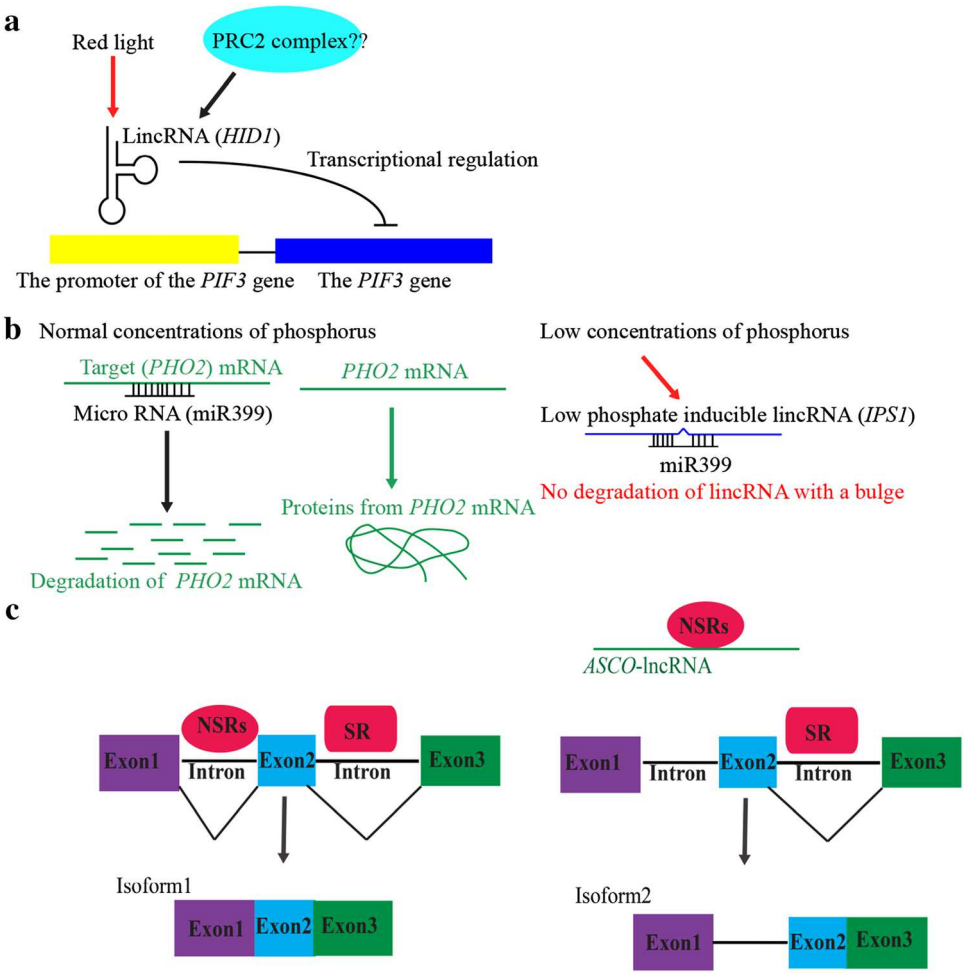


**Fig. 2** Functions of lincRNAs in plants. **a** Transcriptional regulation of the *PIF3* gene by *HID1* under *red light*. *Red light* induces accumulation of *HID1*. *HID1* physically associates with the proximal region of the *PIF3* promoter. That binding negatively regulates transcription of the *PIF3* gene. *HID1* may recruit PRC2 complex for transcriptional regulation of *PIF3*. **b** Function of a lincRNA (*IPS1*) as endogenous target mimicry (eTM). In normal concentrations of phosphorus (Pi), *PHO2* mRNA is degraded by miR399 guided cleavage via sequence complementary (*left*). *IPS1* is induced under low Pi concentrations and binds to miR399 (*right*). However, the pairing is interrupted by a mismatch loop at the expected miR399 cleavage site, because degradation of *IPS1* is impaired. That results in sequestering miR399 from *PHO2* mRNAs. Finally, *PHO2* mRNA is more intact by eTM effect of *IPS1*. **c** Alternative splicing regulation by *ASCO*-lncRNA. NSRs are required for alternative splicing to produce isoform1. For example, the intron between exon1 and exon2 is spliced out by NSRs (*left*). However, *ASCO*-lncRNA binds to NSRs and inhibits physical interaction between NSRs and the intron of pre-mRNA (*right*). Isoform2 is produced more efficiently by *ASCO*-lncRNA. (Color figure online)

Pi is an essential nutrient for plant growth. Under Pi-limited conditions, plants adapt by changing root architecture and morphology to enhance Pi acquisition. Moreover, remobilization of internal Pi is also essential for plant development (Franco-Zorrilla et al. 2007). Mutants with altered internal Pi concentrations show developmental defects. The *phosphate2* (*pho2*) mutant, which has a mutation in the *UBIQUITIN-CONJUGATING ENZYME 24* (*UBC24*) gene, accumulates excessive amounts of Pi in the shoot as a consequence of increased Pi uptake. Pi starvation induced accumulation of miR399 and reduced *PHO2*/*UBC24* expression (Chiou et al. 2006). Overexpression of microRNA399 (miR399) suppressed accumulation of the *PHO2*/*UBC24* transcripts and increased Pi uptake. These results showed that miR399 controls Pi homeostasis by modulating expression of the *PHO2*/*UBC24* gene (Chiou et al. 2006).

*INDUCED BY PHOSPHATE STARVATION 1*(*IPS1*) is a non-coding RNA (542nt) (Martin et al. 2000) that contains a potential ORF encoding a small peptide (four amino acids) (Franco-Zorrilla et al. 2007). *IPS1* has a 23-nt motif complementary with miR399. *IPS1* over-expression enhances accumulations of *PHO2* mRNA in wild type plants. *IPS1* over-expression attenuates reduction of *PHO2* mRNA accumulation and Pi accumulation in the miR399 over-expression lines suggesting that *IPS1* and

miR399 have antagonistic functions (Franco-Zorrilla et al. 2007). *IPS1* was not degraded in the over-expression lines of miR399, because the pairing was interrupted by several mismatched sequences at the expected miR399 cleavage site. Therefore, *IPS1* directly binds to miR399 and sequesters miR399 from *PHO2* mRNA. That resulted that *PHO2* mRNA is more intact through the "target mimicry" effect of *IPS1* (Fig. 2b).

LincRNAs were predicted to be a potential endogenous target mimics (eTMs) for 20 conserved miRNAs in *Arabidopsis* and *Oryza sativa* by computational analysis (Wu et al. 2013). Thirty-six potential eTMs for 11 *Arabidopsis* miRNAs and 189 potential eTMs for 19 rice miRNAs have been identified. Over-expression lines of potential eTMs that target miR160 and miR166 showed developmental defects. The accumulation of known target mRNAs for each miRNA was increased in the corresponding over-expression lines suggesting that these eTMs are functional target mimics. Over-expression of other eTMs for four *Arabidopsis* miRNAs, which have similar target sites as *N. benthamiana* miRNAs, repressed endogenous functions of miRNAs in *N. benthamiana*. These results suggested that target mimicry for miRNAs is a conserved function of lincRNAs and can influence expression of target mRNAs in plants (Wu et al. 2013).

## Alternative splicing regulation by lincRNAs

Nuclear speckles are regions that are enriched for pre-mRNA splicing factors. Two *Arabidopsis* nuclear speckle RNA-binding proteins (AtNSRs), called NSRa and NSRb, are co-localized with one of the splicing factors in the nuclear speckles (Bardou et al. 2014). The *NSRa* and *NSRb* genes have been known to be related to the genes encoding the Serine/Arginine (SR) rich proteins, which are required for alternative and/ or constitutive splicing (Schindler et al. 2008). Auxin treatments induced *NSRb* in addition to increased lateral root formation in wild type seedlings. However, a double mutant of the *NSRa* and *NSRb* genes had very few lateral roots and was impaired in lateral root formation even after auxin treatment. Accumulation of alternatively spliced isoforms for 85 of 288 genes were significantly changed in the double mutant of *NSRa* and *NSRb* compared with wild type plants after auxin treatment. Some of pre-mRNAs of the 85 genes were shown to directly bind to NSRa and NSRb proteins in UV crosslinked RNA immunoprecipitation assays (RIP). Transcriptome analysis in the double mutant of the *NSRa* and *NSRb* genes after auxin treatment revealed that expression of 11 ncRNAs were significantly changed. Npc351, which has been previously identified as a ncRNA in *Arabidopsis* (Ben Amor et al. 2009) and renamed as the *Alternative Splicing Competitor* long noncoding RNA (*ASCO*-lncRNA) (Bardou et al. 2014), was shown to physically bind to AtNSRs *in vivo* using a RIP assay. *ASCO*-lncRNA and the target pre-mRNAs of AtNSRs compete for binding to the AtNSR proteins. Specific alternatively spliced isoforms were also accumulated in over-expression lines of *ASCO*-lncRNA. These experiments showed that *ASCO*-lncRNA modulates alternative splicing by binding to AtNSRs after receiving an auxin signal (Fig. 2c) (Bardou et al. 2014).

## Conclusions and future perspective

Cell type specific transcriptome analysis revealed that most of the lincRNAs are accumulated in specific cells of the *Arabidopsis* root (Li et al. 2016). However, it is still difficult to identify functionally important lincRNAs from them. Co-expression analysis between lincRNAs and protein coding genes in specific cells has suggested their roles in regulation of gene expression in specific cell types. These co-expression data could be useful in identifying the exact functions of lincRNAs in specific cell types. Further experimental analysis of these potential candidate lincRNAs may reveal their function.

Knockdown of lincRNAs expressed in mammal ES cells as well as mutants of the PRC2 components, resulted in premature differentiation suggesting that gene silencing in ES cells by PRC2 and lincRNAs is required for maintenance of stem cells (Aloia et al. 2013; Guttman et al. 2011). In plants, it has been shown that PRC2 component mutants form larger root and shoot meristems in *Arabidopsis* (Aichinger et al. 2011; Xu and Shen 2008). In *Arabidopsis* PRC2 component mutants, the genes required for stem cell maintenance of root or shoot meristem are highly expressed. This suggests that PRC2 may negatively regulate expression of the genes required for stem cell development in the differentiated cells. In mammals, PRC2 binds to many lincRNAs, however it is unknown whether there are physical interactions in plants. Binding assays between PRC2 and lincRNAs will identify potential candidates of lincRNAs that control gene expression through histone tri-methylation by PRC2. (Wang et al. 2014) showed that *HID1* interacts with the promoter site of the *PIF3* gene to negatively control expression of the gene, but it is still unknown if *HID1* regulates *PIF3* expression through PRC2 (Fig. 2a). Further analysis will further the understanding of how *HID1* negatively regulates expression of the *PIF3* gene.

In plants, many lincRNAs are induced by various stresses. *IPS1*, for example, was induced by Pi starvation (Martin et al. 2000). In *Arabidopsis* and rice, 36 and 189 potential eTMs for miRNAs have been identified suggesting that control of gene expression by eTM is a conserved function of lincRNAs. However only two eTMs were

confirmed to regulate plant development and target mRNA expression by over-expression of these eTMs in *Arabidopsis* (Wu et al. 2013). Further transcriptome analysis under many stress conditions in specific cell types will discover more functional eTMs. Plant miRNAs have a relatively small number of target mRNAs, thus biological effects and target mRNAs expression of eTMs will be easy to validate in plants. The expression and mutant phenotypes of the miRNA target genes will be validated using over-expression lines of potential candidates of eTMs under stress conditions. It has been demonstrated that artificial TMs are able to control endogenous gene expression (Franco-Zorrilla et al. 2007). Thus engineering of artificial TMs for crop production may be beneficial to increase stress tolerance in agriculturally relevant plants.

Many stresses induce alternatively spliced isoforms and lincRNAs in plants. (Bardou et al. 2014) showed that auxin inducible *ASCO*-lncRNA binds to AtNSRs and modulates alternative splicing. Eighteen *SR* genes have been identified in *Arabidopsis* (Reddy and Shad Ali 2011) and each *SR* has multiple alternatively spliced isoforms. It is known that the splicing isoform for each SR changes under stress suggesting that the protein products of each *SR* gene are also changed during stress. It is possible that other stress inducible lincRNAs bind to SR proteins in order to modulate alternative splicing. Cross-linking immunoprecipitation (CLIP) between SR protein and RNAs under stress conditions will find the specific target sequences of lincRNAs and mRNAs.

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