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Feature Review

SMARTER De-Stressed Cereal Breeding

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In cereal breeding programs, improved yield potential and stability are ultimate goals when developing new varieties. To facilitate achieving these goals, reproductive success under stressful growing conditions is of the highest priority. In recent times, small RNA (sRNA)-mediated pathways have been associated with the regulation of genes involved in stress adaptation and reproduction in both model plants and several cereals. Reproductive and physiological traits such as flowering time, reproductive branching, and root architecture can be manipulated by sRNA regulatory modules. We review sRNA-mediated pathways that could be exploited to expand crop diversity with adaptive traits and, in particular, the development of high-yielding stress-tolerant cereals: SMARTER cereal breeding through 'Small RNA-Mediated Adaptation of Reproductive Targets in Epigenetic Regulation'.

Epigenetic Adaptation to Stress: Beyond the Genes

Abiotic stresses including drought, salinity, and nutrient deficiency threaten plant growth and development, dramatically reducing crop production and quality. Climate change will also impact on the yield potential of key cereal crops such as wheat (*Triticum* spp.), maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*) [1]. Numerous signal transduction pathways prompt adaptive responses at all levels (morphological, physiological, molecular) to help the plant to survive and achieve reproductive success in hostile environments. Reprogramming of gene expression via mechanisms such as epigenetic modification may allow the production or repression of proteins to enable stress adaptation.

Epigenetic modification refers to heritable and transient changes in gene activity and function associated with biochemical modifications of chromatin and **RNA interference** (RNAi) (see Glossary) but does not entail any changes in nucleotide sequence [2–5]. The web of epigenetic regulatory pathways and the interactions therein partially rely on **small RNAs** (sRNAs) to precisely reprogram the expression of stress- or development-associated genes through **transcriptional gene silencing** (TGS) and **post-transcriptional gene silencing** (PTGS) [6–8]. Although other regulatory components (such as long non-coding RNAs and histone modifiers) [9–11] also cause epigenetic modifications associated with stress responses, they are not the focus of this review. Compared with other mechanisms, sRNAs can rapidly respond to different environmental conditions, and act as mobile signal molecules to modulate gene expression during plant development [12–14]. Differential expression of certain sRNAs in response to abiotic stress contributes to the dynamic spatiotemporal patterns of downstream target gene expression and is related to adaptive physiological and/or reproductive traits including altered reproductive timing and alleviation of cellular damage induced by stress in reproductive organs [15–19]. sRNA-mediated regulation may also provide tolerance to recurring abiotic stress through heritable stress memory [20]. Furthermore, responses of the key components in the RNAi mechanism, such as which sRNA families are expressed, appear to be genotype-dependent, thus potentially explaining genotypic differences in their physiological and

Trends

Transcriptome reprogramming and translational regulation involved in plant stress adaptation largely depend on sRNA regulatory pathways, such as transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS).

Crosstalk between sRNA-mediated pathways involved in stress signalling and reproduction has been extended from model plant species to cereal crops.

Desirable reproductive traits such as enhanced panicle branching and more efficient grain filling, and other traits including optimal root architecture in cereals, can be manipulated using RNA interference (RNAi) to maintain/improve yield under challenging conditions.

Newly developed RNAi technologies, such as artificial sRNAs and target mimicry of multifunctional sRNAs, provide new opportunities for stress tolerance improvement in cereals and the intelligent design of high-yielding varieties in molecular breeding.

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morphological stress responses [15,21–24]. Therefore, elucidation of sRNA-mediated epigenetic pathways could be exploited to expand crop phenotypic diversity with favourable physiological and reproductive traits. In this review we discuss the contribution of sRNA regulatory mechanisms to stress adaptation and reproduction in plants, highlighting recent related progress in cereal crops, and evaluate the potential of applying RNAi technologies to developing high-yielding elite cereal varieties.

sRNAs: The Epigenetic Commander Under Stress

Plant small RNAs, mainly **microRNAs** (miRNAs) and **small interfering RNAs** (siRNAs), function as negative regulators in distinct but overlapping epigenetic silencing pathways. The biogenesis of plant miRNAs and siRNAs is relatively well understood, as reviewed recently in [25]. Generally, mature single-stranded miRNAs are processed from precursor miRNAs that originate from hairpin primary-miRNAs, which are transcribed from *MIR* genes. miRNAs can also be produced from intronic or exonic regions of protein-coding genes and transposons [26,27]. siRNAs are derived from long double-stranded (ds) RNA precursors, which originate from DNA repeats, transposons, non-coding loci, and protein-coding genes (exonic and intronic regions) [25,28]. Mature miRNAs and some siRNAs, such as **trans-acting siRNA** (ta-siRNAs), are loaded into the **RNA-induced silencing complex** (RISC) in association with **Argonaute** (AGO) proteins [25]. When bound, RISCs cause sequence-specific cleavage of the complementary target mRNAs and/or translational inhibition, resulting in PTGS [29,30]. Stress-induced, untranslated region (UTR)-derived siRNAs (sutr-siRNAs) could also be functional in the PTGS mechanism through regulation of alternative precursor mRNA (pre-mRNA) splicing [31]. However, siRNAs and, in some cases, miRNAs can reversibly modify chromatin via DNA methylation or histone modification [8] affecting accessibility of chromatin, thus determining whether a particular locus is transcriptionally silent or active [7,32]. Under unfavourable conditions, sRNAs can rapidly respond to different environmental cues and reprogram the expression of downstream genes that provide stress adaptation and heritable stress memory [20]. Sitting at the crossroads of TGS and PTGS pathways, sRNAs are therefore crucial regulators in plant acclimatisation to abiotic stresses (Figure 1).

sRNAs in TGS: Stress-Adaptive Chromatin

In response to environmental and developmental cues, sRNAs help to shape the genotype into the phenotype via stress-responsive regulation of TGS mechanisms such as histone modification and DNA methylation [7,25,33]. sRNAs coordinate histone modification by recruiting enzymes that catalyse the methylation and deacetylation of specific lysine or arginine residues in histones, causing them to be more closely associated to chromatin. Thus the binding of transcription factors to template DNA is limited, leading to suppression of transcription [34,35]. Gene transcription is regulated in this manner in many stress-related processes [35] and, particularly for ABA signalling, cold adaptation, drought adaptation, and the FLC flowering pathway [36–39].

DNA methylation inhibits the transcription of protein-coding genes and transposon movement, which could affect the transcription of neighbouring genes [5,40]. During **RNA-directed DNA methylation** (RdDM), dsRNAs are processed to 21–24 nt siRNAs, which recruit DNA methyltransferases and guide *de novo* methylation by sequence complementarity [25,33]. RdDM machinery has been reported to regulate developmental processes including flowering, ovule development, and male fertility, contributing to reproductive success [33,41,42] and stress-responses to drought and salinity in plants [43,44]. Furthermore, siRNAs appear to contribute to stress tolerance through directing RdDM and modulating DNA methylation in a genotype-dependent manner in rice [43]. Given that the DNA methylation state appears to be heritable [32], the manipulation of siRNAs therefore has potential for breeding stress tolerance.

Glossary

ABCE model: a floral development model. Activity of A genes alone, such as *APETALA2* (*AP2*), leads to sepal formation. Joint activity of A and B genes, such as *AP3* and *PISTILLATA* (*PI*), leads to petal development. Joint activity of B genes with C genes, such as *AGAMOUS* (*AG*), leads to stamen formation but C gene activity alone allows carpel formation. The E genes, or *SEPALLATA* (*SEP*) family, contribute to formation of all floral organs while A and C genes are antagonistic to each other.

Argonaute (AGO): essential catalytic components of the RNA-induced silencing complex (RISC) that bind to different classes of sRNAs and coordinate downstream gene-silencing events.

Artificial microRNA (amiRNAs): artificial sRNAs (21 nt) made using modified backbones of endogenous precursor miRNAs.

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated nuclease 9): the prokaryotic immune system modified for genome editing. CRISPR are short palindromic repeats of DNA sequences acquired from previous exposure to bacterial, virus, or plasmid invasion. Cas9 is a DNA endonuclease associated with CRISPR that edits the genome with the help of guide RNA which contains user-defined targeting sequences. CRISPR/Cas9-based miRNA knockdown has the exclusive benefit of single-nucleotide precision to differentiate miRNA isoforms in the same family.

MicroRNAs (miRNAs): non-coding, single-stranded RNAs (20–25 nt) transcribed from *MIR* genes. miRNAs induce mRNA cleavage and translational inhibition in a sequence-specific manner.

Post-transcriptional gene silencing (PTGS): repression of gene activity at the post-transcriptional level.

RNA-directed DNA methylation (RdDM): the major epigenetic process involved in biogenesis of small interfering RNA (siRNAs) and DNA methylation. During RdDM, siRNAs are processed from long double-stranded (ds) RNAs.

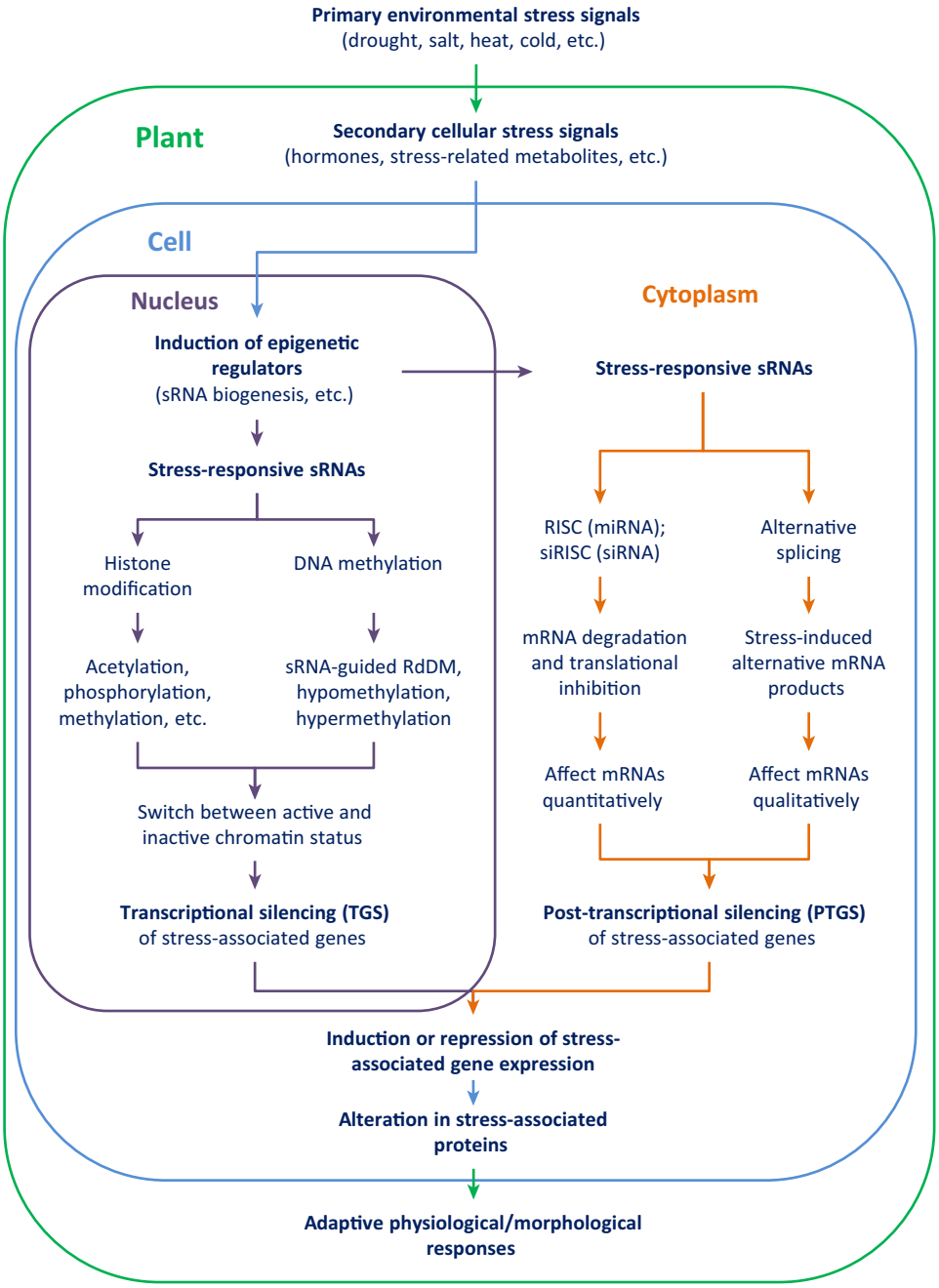


Figure 1. A Systematic View of Small RNA (sRNA)-Mediated Epigenetic Changes Contributing to Gene Expression Reprogramming in Response to Abiotic Stresses. In unfavourable environmental conditions, stress signals are perceived and transduced to plant cells. A network of epigenetic regulation pathways is triggered by cellular stress signals to reprogram gene expression. Stress-responsive sRNAs are induced or repressed in response to various abiotic stresses. Some sRNA families, mainly small interfering RNAs (siRNAs), regulate DNA methylation and histone modification activities which affect chromatin status, leading to transcriptional gene silencing (TGS). MicroRNAs, sometimes siRNAs, are transported into the cytoplasm to guide RISC (RNA-induced silencing complex), leading to post-transcriptional gene silencing (PTGS). Gene expression of functional proteins is downregulated or upregulated through the switching on/off of the TGS and PTGS mechanism under the control of stress-responsive sRNAs. Reprogrammed gene expression leads to downstream physiological or morphological changes in plants contributing to stress adaptation.

RNA-induced silencing complex (RISC): the multi-protein heterogeneous complex that incorporates Argonaute proteins and one guiding strand of a siRNA or miRNA. The guide RNA strand functions as the template in RISC for binding to mRNAs based on sequence complementarity during PTGS.

RNA interference (RNAi): a natural gene-silencing mechanism in which gene expression is repressed by sRNAs through mRNA degradation or inhibition of translation.

Short tandem TM (STTM): STTM is similar to target mimicry but only two miRNA binding sites are linked with a short spacer to deplete and degrade miRNAs in the STTM system.

Small interfering RNAs (siRNAs): sRNA molecules (21–24 nt) processed from long dsRNAs that are mainly generated from the transcription of DNA repeats and transposable elements.

Small RNAs (sRNAs): a large family of small regulatory non-coding RNA molecules (20–50 nt). In plants, sRNAs are integral components of development patterning, maintenance of genome integrity, and plant responses to abiotic and biotic stresses.

Synthetic trans-acting siRNAs (syn-tasiRNAs): siRNAs (21 nt) artificially made by using the modified backbone of an endogenous trans-acting siRNA (tasiRNA) precursor such as TAS1 or TAS3.

Transcription activator-like effector nucleases (TALENs): restriction enzymes containing a TAL effector domain and a specific DNA-binding domain. DNA-binding domain structure can be engineered to bind specifically to target sequences. TALEN-based miRNA knockdown has the advantage of being able to mutate multiple bases.

Target mimicry (TM): a mechanism whereby endogenous non-coding RNAs mimic miRNA-targeted mRNAs and sequester mature miRNAs, relieving their *bona fide* targets from the RNAi machinery.

Transacting siRNA (ta-siRNAs): plant-specific secondary siRNAs produced from transcripts of *TAS* genes with the help of specific miRNAs.

Transcriptional gene silencing (TGS): suppression of gene

transcription through modification of chromatin.

Virus-based miRNA silencing (VbMS): the silencing of endogenous miRNAs using plant viral vectors such as barley stripe mosaic virus to drive TM.

sRNAs in PTGS: Kill the Messenger under Stress

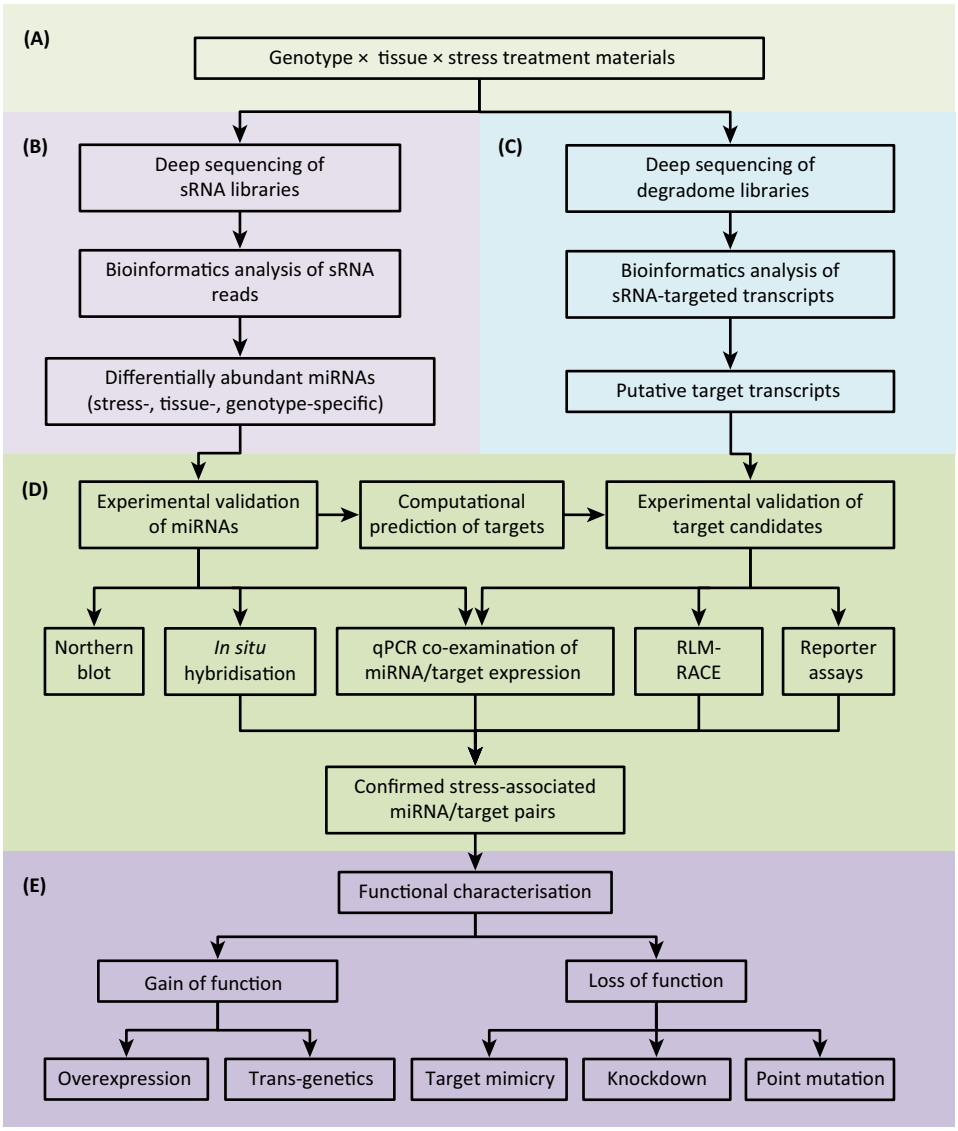
The role of sRNAs (especially sutr-siRNAs and miRNAs) in PTGS during plant stress responses and development has received significant attention. sRNAs act as negative regulators at the post-transcriptional level by affecting the mRNA population both qualitatively and quantitatively via alternative splicing or mRNA degradation, and by preventing protein translation [25].

Sutr-siRNAs appear specific to stress responses and target the genomic intron regions to affect alternative splicing (AS) [31]. The AS mechanism enables the production of multiple mature mRNA isoforms from the same pre-mRNAs but is coupled with the nonsense-mediated decay (NMD) pathway to ensure that nonsense mRNAs generated by AS are degraded. In brachypodium *(Brachypodium distachyon*), a model cereal, sutr-siRNAs were produced from the 3′-UTRs of stress-responsive coding genes under heat, cold, and salt stresses [31]. Sutr-siRNAs target specific complementary *cis*-elements, providing additional splice sites rather than the major annotated splice sites in the target introns, and this ultimately leads to the production of shorter alternative transcripts. These short transcripts possess a stop codon downstream of the sutr-siRNA-targeted splice sites, making them substrates to NMD under stress [31]. Sutr-siRNAs could therefore act as a regulatory switch between non-functional and functional transcripts according to different environmental signals. However, further experimental validation will be necessary to characterise the base-pairing interactions between sutr-siRNAs and their target introns during abiotic stress.

Under environmental stress, plants need to coordinate the balance between developmental patterning and stress defence activation because of the limitation of resources. The abundance of sRNAs, especially miRNAs, regulates gene expression in a highly explicit sequence-specific manner by either causing mRNA degradation or by inhibiting mRNA translation owing to the presence of the RISC that prevents the formation of the ribosomal machinery [45,46]. Development of high-throughput sequencing technology, enhanced bioinformatics tools, and the gradual completion of plant whole-genome sequences has enabled genome-wide analysis of the sRNA transcriptome and its target transcriptome in various plant species [47,48] (Figure 2). The target repertoire of the miRNA-mediated RNAi mechanism includes protein-coding genes involved in a broad range of biological processes {e.g. phytohormone biosynthesis, protein and nucleic acid binding, carbohydrate metabolic processes, protein transport, and ROS (reactive oxygen species) scavenging} [8,49,50]. Several recent reviews have highlighted the specific regulatory roles of different miRNA families in plant defence against environmental stresses [8,49,50]. Some stress-responsive miRNA/target modules also exhibit tissue-specific patterns for their specific roles, including regulating photosynthetic activity and stomatal development in the leaves, and also modulating lateral root initiation and water/nutrient uptake in the roots [15,51,52]. In addition, some miRNA/target modules exhibit opposite regulatory patterns between stress-tolerant and -sensitive varieties or, in some cases, are only active in the stress-tolerant genotypes [15,21–24]. The genotype-dependent nature of these miRNA/target modules and their ability to control stress signal recognition, hormone signal transduction, and downstream stress-inducible regulatory elements leading to physiologically or morphologically adaptive changes makes them promising candidates for crop improvement. Recent assessment of stress-responsive sRNA/target modules in cereal crops has provided valuable information to fully understand the molecular mechanism underlying stress tolerance (Table 1).

sRNA Control of Reproduction: Flourishing Under Stress

Epigenetic regulation coordinated by sRNAs appears to be involved in almost all reproductive processes including phase transition, flowering initiation, inflorescence branching, floral organ development, gametophyte development, and seed/fruit setting [19,21,53–58] (Figure 3, Key Figure). The manipulation of specific sRNA-mediated modules to alter floral initiation, development,



Trends in Plant Science

Figure 2. In Search of Functional Small RNAs (sRNAs) and Their Targets in Plants. (A) Plant materials with different combinations of genotypes, tissue types, developmental stages, and stress-treatments provide multiple options to compare and analyse sRNAs and their targets. (B) High-throughput sequencing of sRNA libraries produced from these materials enable the genome-wide identification of genotype-, tissue-, development-, and stress-dependent functional sRNAs. (C) Degradome sequencing of the 5'-end uncapped RNA fragments is an efficient approach for profiling sRNA-cleaved targets on a large scale. Both sequencing approaches must be integrated with powerful bioinformatics tools to decipher sequencing data, identify valid sRNA/target reads, and characterise the sRNA/target transcriptome profiles. (D) The bioinformatic predictions require experimental validation of sRNA/target candidates to confirm their interactions and (E) characterise their functional relevance. For example, co-examination of sRNA/target pairs using qPCR helps to validate the suppression of mRNAs as a result of changes in sRNA abundance. RLM (RNA ligase mediated)-5' RACE (rapid amplification of cDNA ends) confirms the truncated site in mRNAs resulting from the post-transcriptional gene silencing (PTGS) cleavage guided by sRNAs. Gain-of-function and loss-of-function studies further characterise the roles and relevance of sRNA and their targets in response to stress.

Table 1. Stress-Responsive miRNAs and Their Functional Targets in Cereal Crops

miRNA[a]	The Response of miRNA to Abiotic Stresses[b,c]				Target of miRNA	Pathways Involved	Refs
	Drought	*Salinity*	*Heat*	*Cold*			
miR156	Osa↓, Tae↑, Ttu↑↓, Zma↑	Tae↑↓, Zma↓	Osa↓, Tae↑	Osa↑, Tae↑	Squamosa promoter binding protein-like (SPL) transcription factors	Gibberellin signalling; flavonoid biosynthesis; anthocyanin metabolism	[15,16,18,21,80, 90,137–141]
miR159	Osa↑↓, Tae↑↓, Ttu↓, Zma↑	Hvu↑↓, Osa↓, Tae↓	Osa↓, Tae↓	Tae↑	MYB family transcription factors	Gibberellin signalling	[16,18,21,22,86, 90,137,139, 141–143]
miR160	Osa↑, Tae↑↓, Ttu↑↓	Osa↓, Tae↑↓	Hvu↑, Osa↑, Tae↓	Osa↑, Tae↑	Auxin response factors	Auxin signalling	[15,16,21,90,137, 139–141,144]
miR162	Ttu↑↓	—	Osa↑	—	DICER LIKE 1	Small RNA biogenesis	[22,90,145]
miR164	Osa↑, Tae↑↓, Ttu↑↓	Hvu↑↓, Osa↓, Tae↑↓	Osa↓	Tae↓	NAC domain transcription factors	Hormone signalling	[15,21,22,90,137, 139,141,143,146]
miR166	Osa↓, Tae↑↓, Ttu↑↓, Zma↓	Osa↓, Zma↑	Hvu↑, Osa↑	Osa↓	Homeodomain–leucine-zipper (HD-Zip) transcription factors	Jasmonic acid signalling; ethylene pathways	[15,16,18,21,90, 137,138,144]
miR167	Osa↓, Tae↑, Ttu↑↓, Zma↑	Hvu↑↓, Osa↓, Tae↑, Zma↓	Hvu↑, Osa↓, Tae↑	Osa↑, Tae↓	Auxin response factors	Auxin signalling	[15,16,18,21,90, 137–141,143,144]
miR168	Hvu↓, Osa↓, Tae↑, Ttu↑↓, Zma↓	Hvu↑↓, Tae↑, Zma↑		Tae↑	Argonaute 1	RISC loading; ABA signalling	[15,18,21,137,138, 141–143,147]
miR169	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Tae↑↓, Zma↑	Osa↓	Tae↓	NF-YA transcription factors	ABA biosynthesis; ABA signalling	[21,22,90,138,139, 141–143,147]
miR171	Hvu↓, Osa↑↓, Tae↓, Ttu↑↓	Hvu↑↓, Tae↑	Tae↓, Osa↑↓	Tae↑	SCARECROW-like (SCL) transcription factors	Gibberellin signalling	[15,21,22,90, 140–143,147]
miR172	Hvu↓, Osa↓, Tae↑↓	Hvu↑↓, Tae↑	—	—	APETALA2 (AP2) and AP2-like transcription factors	ABA biosynthesis and signalling; meristem establishment	[21,139,142, 143,147]
miR319	Osa↑↓, Tae↑↓, Ttu↓, Zma↑	Hvu↑↓, Osa↓, Tae↑	Tae↓	Tae↑	TCP family transcription factors	Jasmonate biosynthesis and senescence	[16,18,21,22,137, 139–143]
miR393	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Osa↑, Tae↑	Osa↑	Osa↑, Tae↑	TIR1 (transport inhibitor response 1) proteins, AFB (auxin signalling F-box) proteins	Auxin signalling; auxin homeostasis	[16,21,22,90,137, 141,143,147]
miR394	Osa↑, Ttu↓↑	Osa↓	—	Osa↑	F-box domain-containing proteins	ABA signalling	[15,16,137]
miR395	Osa↑, Tae↓, Ttu↑↓, Zma↓	Hvu↓, Tae↑, Zma↑	Tae↑	Tae↓	ATP sulfurylase genes, SULTR2;1 (sulfate transporter 2;1) protein	Sulfate transport and assimilation	[15,18,21,138–143]
miR396	Hvu↓, Osa↑↓, Ttu↑↓, Tae↑↓, Zma↓	Hvu↑↓, Osa↓, Tae↑↓, Zma↓	Osa↑	Osa↑, Tae↑	GRF (growth-regulating factor) proteins, bHLH74 (basic helix-loop-helix transcription factor 74)	Cell proliferation	[15,16,18,21,22,90, 137–139,141–143, 147–149]
miR397	Hvu↓, Osa↑↓, Ttu↑	Tae↑	Osa↑	Tae↑	Laccase (LAC) genes	Brassinosteroid sensitivity; cell wall biosynthesis	[15,24,90,141,147]
miR398	Osa↑↓, Ttu↑↓, Zma↑	Tae↓, Zma↑	Osa↑, Tae↑	Tae↓	CSDs (Cu/Zn superoxide dismutases)	Reactive oxygen species (ROS) scavenging	[15,18,24,90,138, 140,141]

Table 1. (continued)

miRNA[a]	The Response of miRNA to Abiotic Stresses[b,c]				Target of miRNA	Pathways Involved	Refs
	Drought	*Salinity*	*Heat*	*Cold*			
miR399	Osa↑, Ttu↑↓, Zma↓	Tae↑, Zma↓	Osa↓	Tae↑	Ubiquitin-conjugating (E2) enzymes	Cellular phosphate homeostasis; phosphate remobilisation	[15,18,90,137,141,148]
miR408	Osa↑↓, Ttu↑↓	Hvu↓, Tae↑	Tae↑	Tae↑	Plastocyanin-like (basic blue) proteins, TOC1	Copper homeostasis; cell-to-cell signalling	[15,23,140,141,143]
miR444	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Tae↓	—	Tae↑	MADS-box transcription factors	Cellular nitrate signalling	[15,21,137,139,141, 143,147]
miR528	Osa↑↓, Ttu↑↓, Zma↓	Osa↓, Zma↑	—	Osa↓	AAO (ascorbic acid oxidase), laccase precursor proteins, CSDs	Oxidation/reduction processes	[15,16,18,24,137,148]
miR529	Osa↑↓	Osa↑, Tae↓	Osa↓	Osa↑	SPL transcription factors	Gibberellin signalling	[16,90,137,139,142]
miR827	Hvu↓, Osa↓, Tae↑↓, Ttu↑↓, Zma↑	Hvu↑↓, Zma↓	—	—	SPX-MSF genes	Cellular phosphate homeostasis	[15,18,21,137,143, 147,148]
miR1029	Tae↑	—	Tae↑	Tae↓	DRE-binding factors, AP2-like transcription factors	Gibberellin biosynthesis; ABA signalling	[150]
miR1030	Hvu↓, Osa↓	Tae↑	—	—	—	—	[141,142,147]
miR5048	Hvu↓, Ttu↑↓	Hvu↑↓	—	—	Cysteine-rich receptor-like protein kinases	—	[15,143,147]
miR5049	Hvu↑↓, Ttu↑↓	Tae↓	—	—	Ubiquitin-conjugating (E2) enzymes	—	[15,139,147]
miR5064	Hvu↓, Ttu↑↓	—	—	—	—	—	[22,147]
miR5072	Hvu↓	Hvu↑↓	—	—	Anthocyanidin reductase	—	[143,147]
miR6300	Hvu↑, Ttu↑↓	—	—	—	—	—	[15,147]

[a]miRNAs that are also discussed in this review for their role in the regulation of plant development are in italic font.
[b]Abbreviations: Hvu, *Hordeum vulgare*; Osa, *Oryza sativa*; Tae, *Triticum aestivum*; Ttu, *Triticum turgidum*; Zma, *Zea mays*.
[c]Symbols: ↑, upregulated; ↓, downregulated; —, not determined; ↑↓, opposite regulatory patterns observed in different studies; ↑↓, opposite regulatory patterns observed between stress-tolerant and stress-sensitive genotypes in the same study.

and grain fill therefore holds great potential as a tool to facilitate reproductive success and yield improvement under different environmental conditions (Figure 3).

Flowering Time and Floral Development

Regulatory networks controlling the vegetative to reproductive phase transition are highly complex and regulated strongly by environmental cues [59,60]. These networks have been well studied in arabidopsis (*Arabidopsis thaliana*), as reviewed recently [59], to reveal the importance of a key set of genes that integrate pathways to initiate flowering: *SUPPRESSOR OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), and *AGAMOUS-LIKE 24* (*AGL24*). These genes then switch on a number of floral meristem (FM) identity genes including *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *FRUITFULL* (*FUL*) leading to FM development. Both sets of genes have been shown to be regulated by various genes associated with environmental cues, including *TIMING OF CAB EXPRESSION 1* (*TOC1)* (circadian clock), *FLOWERING LOCUS* C (*FLC)* (vernalisation), *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, *TARGET OF EAT1* (*TOE1*) (age), and *DELLA* (gibberellins). The FM then gives rise to various floral organ primordia, a process directed by floral organ identity genes often represented in the **ABCE model** [60,61].

Key Figure

Key MicroRNA (miRNA)-Mediated Regulatory Modules Involved in Plant Development and Reproduction

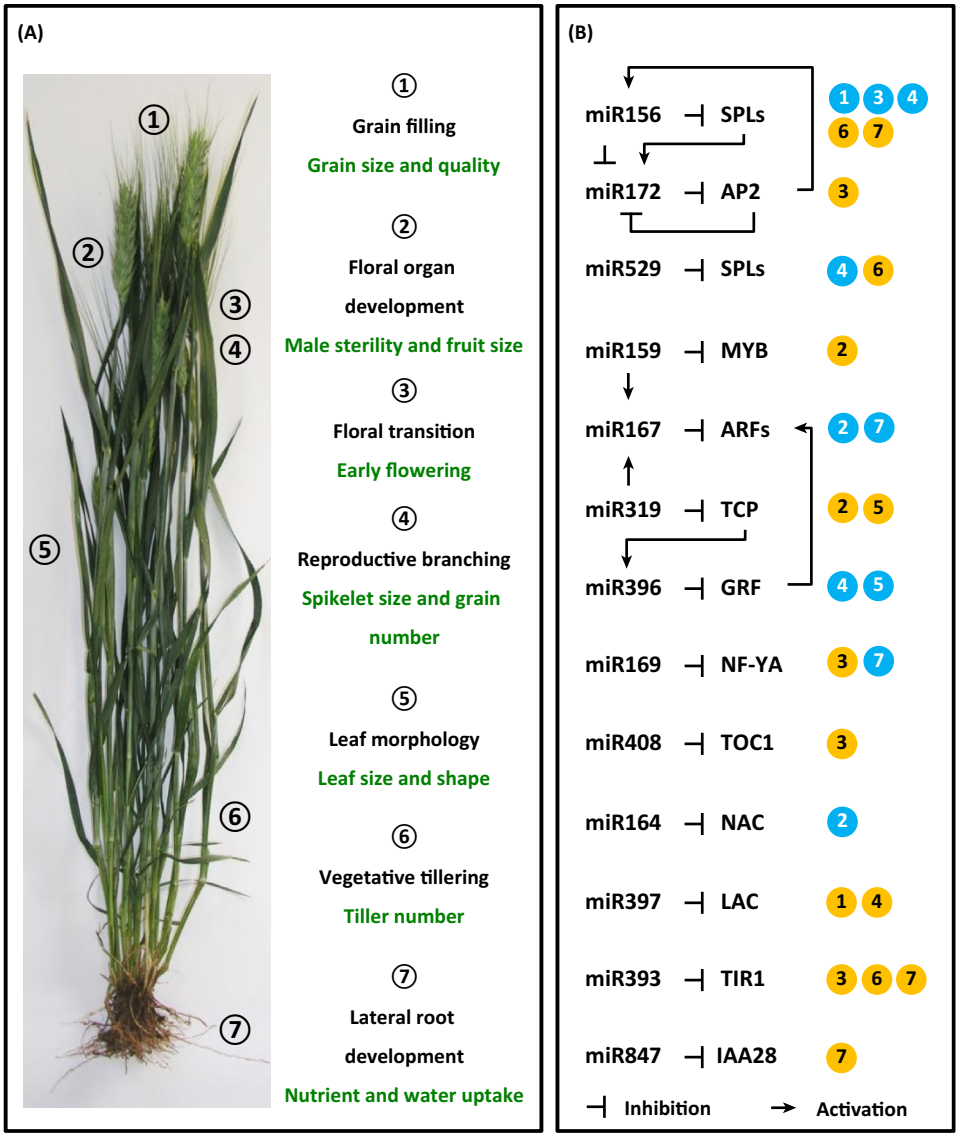


Figure 3. For a Figure360 author presentation of Figure 3, see the figure online at http://dx.doi.org/10.1016/j.tplants.2016.07.006#mmc1.

(A) Plant development stages and potential breeding targets. Seven development-associated events are indicated and numbered. Green text refers to favourable physiological and reproductive traits in breeding that could potentially be engineered during plant development. (B) Key miRNA/target regulatory modules discussed in the review that could be used as a tool to manipulate plant development and reproduction. Numbered plant developmental stages highlighted in blue indicate the positive regulation of development by miRNA-targeted genes. Numbered plant development stages highlighted in orange indicate the negative inhibition of development by miRNA-targeted genes.

Knowledge of the gene network responsible for floral timing and development is equally important in cereal breeding where flowering time affects pollination, seed quality, yield, harvest time, and stress avoidance. Although many of the regulatory network components, primarily transcription factors, described in arabidopsis have also been identified in cereals [59], no functional *FLC* orthologues have been validated in cereals thus far. In arabidopsis, vernalisation or the acceleration of flowering via cold temperatures requires epigenetic silencing of the floral repressor gene *FLC* [62,63] by sRNAs, histone modifiers, and long non-coding RNAs [11,62–64]. Major crops such as winter wheat and barley respond to vernalisation by increasing the expression of *VRN1* (similar to *AP1/FUL*), which usually represses *VRN2*. Because *VRN2* (a zinc-finger CCT domain-containing gene) usually represses the *FT* orthologue, *VRN3*, flowering is initiated. Even though a TamiR1123 (previously named miR507) was identified to originate from miniature inverted-repeat transposable elements in the *VRN*-A1a promoter, the role of cereal miRNAs in these networks remains unknown [59,65] (see Outstanding Questions).

The miR156/157 and miR172 families are probably the best-characterised flowering time regulators because their functions are highly conserved across dicots and monocots. These two miRNA families exhibit temporally opposite expression patterns and have inverse functions in regulating floral time [66]. miR156 is highly expressed in the vegetative stage and its abundance gradually declines as the plant ages, whereas miR172 accumulates over time in leaves and floral organs from the vegetative to reproductive stage [67]. The main targets of the miR156/157 family are *SPL* transcription factors and, in arabidopsis, rice, maize, and brachypodium, they appear to regulate vegetative/reproductive phase transition, inflorescence branching, and axillary meristem boundary establishment [66,68–70]. Indeed, overexpression or upregulation of miR156 (and therefore decreased *SPL* expression) led to delayed flowering and a prolonged vegetative phase in several species (reviewed in [53]). miR172 has been shown to target the 'A' gene *AP2 (APETALA2)* [71], and *AP2*-like genes including *TOE1*, *TOE2*, *TOE3*, *SMZ*, and *SNZ* [72–74]. In arabidopsis, overexpressing miR172 led to increased cleavage of *TOE1, TOE2*, and *AP2* [74], and caused early flowering [73]. Overexpression of rice miR172 also significantly reduced flowering time through its repression of two *AP2* genes, *SUPERNUMERARY BRACT* (*SNB*) and *INDETERMINATE SPIKELET 1* (*IDS1*) [71]. Interestingly, the AP2 transcription factor negatively regulates miR172 expression and positively regulates miR156 expression, forming a well-coordinated feedback loop [75]. Moreover, miR156 indirectly regulates miR172 abundance because some *SPL* genes, such as *SPL9* and *SPL10*, induce transcription of miR172 [66]. The temporal pattern of miR172 increasing with age could therefore be the direct consequence of reducing miR156 and increasing *SPL*s [66]. Nutrient availability is also involved in this developmental timing feedback loop through sugar-mediated repression of miR156 [76], and miR156-mediated responses to phosphate starvation [77]. Furthermore, environmental factors such as drought and heat affect flowering time through increased biogenesis of miR172 induced by GIGANTEA and FCA proteins, respectively [78,79], as well as miR156-regulated stress response and memory [20,80]. The regulatory circuit between miR156/157, miR172, and their targets therefore appears crucial for floral transition. Furthermore, because *SPL*s have diverse functions across plant development, miR156 appears to be important in other aspects including inflorescence development. In rice, *SPL14* is encoded by the quantitative trait locus, *IPA1* (*IDEAL PLANT ARCHITECTURE 1*) [81,82]. Interruption of miR156-directed binding of *IPA1* via a point mutation in *OsSPL14* caused a marked accumulation of *IPA1*, leading to denser panicles with more primary and secondary branching, and therefore more grain [81]. IPA1 affects inflorescence development by activating transcription of *TB1* (*TEOSINTE BRANCHED 1*, a negative regulator of tiller bud outgrowth) and *DEP1* (*DENSE AND ERECT PANICLE 1*, a positive regulator of panicle architecture and panicle length) [83]. In rice, another miRNA (miR529) also targets the *SPL* family, affecting panicle size. Rice plants overexpressing miR156 or miR529 exhibited significantly increased tillers and smaller panicles but with less reduction caused by miR529 [57]. Interestingly, miR529 appears to be specific to

monocots [84]. However, in arabidopsis, *AtSPL9* and *AtSPL15* retain the target site of miR529 and were still responsive to regulation by osa-miR529. The evolutionary relatedness of miRNA/target modules could therefore be used when considering their transfer between dicots and monocots for floral engineering (see Outstanding Questions).

Another characterised miRNA regulatory circuit in floral development involves miR159, miR319, and miR167 [85]. miR159-regulated MYB transcription factors and miR319-regulated TCP transcription factors have overlapping functions in floral organ development [85,86], and can independently induce the expression of miR167, which in turn represses *AUXIN RESPONSE FACTOR 6* (*ARF6*) and *ARF8*. Both of these participate in auxin signalling, cytokinin activity, and the activation of jasmonic acid biosynthetic enzymes [85]. Impairment of miR159 and miR319 through target mimicry led to defects in sepals, petals, stamen, and anthers, which interestingly resembled the defects caused by the reduced activity of *ARF6/8* when miR167 was enhanced [85]. In addition, overexpressing tae-miR159 in rice resulted in delayed heading time and male sterility [86], probably owing to the role that the target MYBs play in stamen and anther development [85,87]. Furthermore, in the maize *dicer-1 like* mutant, *fuzzy tassel*, downregulation of miR159 and subsequent misregulation of its target mRNA, gibberellin (GA)-induced MYB, led to male sterility [87]. The modulation of the miR159–miR319–miR167 regulatory circuit might therefore be useful when considering the creation of male-sterile lines for F_1 hybrid production in breeding programs.

Because plants will flower earlier in response to stress [88], miRNAs identified as being upregulated during abiotic stress might also control flowering. These include the previously discussed miR156–miR172 regulatory circuit, as well as miR169 and miR408 family members which target key components of the floral regulatory network. The miR169 family targets the universal transcription factor subunit NF-YA (nuclear factor Y subunit A), which binds to the promoter and first intron of the *FLC* gene and induces its transcription [19] while miR408 appears to target the circadian clock gene *TOC1* [54]. In arabidopsis and wheat, most members of the miR169 family are upregulated in response to abiotic stress [19,21]. However, in maize roots and rice panicles, miR169 showed decreased abundance under abiotic stress [89,90]. Therefore, the miR169/NF-YA module may not necessarily be ideal for the control of stress-induced flowering. However, miR408 overexpression in wheat has shown some promise for future application [54], with knockdown of *TOC1* expression leading to an early-heading wheat phenotype [54], and therefore the possibility of avoiding the usual stresses that occur during grain development such as water deficit stress [15] and heat stress [91]. Interestingly, bioinformatics analysis indicated that the miR408 targeting site in *TOC1* also exists in barley, but could not be found in rice, maize, brachypodium, soybean (*Glycine max*), or arabidopsis. Furthermore, in arabidopsis, overexpressing tae-miR408 did not repress *TOC1* [54]. Consequently, the manipulation of this miRNA regulatory module in adjusting heading time may be applicable only in particular cereal species.

The cautionary tale of understanding multiple functions of specific miRNA modules to avoid undesirable side effects continues with miR164 which appears to be crucial for defining morphogenetic floral organ boundaries in developing flowers [55,56,92] through its ability to downregulate various NAC-domain transcription factor families [56,93]. However, miR164-targeted NAC genes also negatively regulate drought resistance in rice and stripe rust resistance in wheat [93,94]. Therefore, enhancement of miR164 expression in these crops could contribute to stress resistance, but might cause undesirable reproductive defects.

The miR396 and miR397 families are also influenced by abiotic stresses (Table 1), but both appear to integrate inflorescence development, auxin biosynthesis, and hormone signalling pathways [58,95]. For example, osa-miR396 targets *GROWTH REGULATING FACTOR 6*

(*OsGRF6*), which functions in auxin biosynthesis and activates auxin response factors and branch/spikelet development-related transcription factors [95]. Increased grain yield occurs in rice plants with knocked-down miR396 because enhanced expression of *OsGRF6* promotes the formation of axillary branches and spikelets [95]. Likewise, increased grain yield occurs in rice plants overexpressing osa-miR397, but this is due to enhanced panicle branching and larger grain size. In the case of osa-miR397, it represses *LACCASE-LIKE PROTEIN* (LAC) which is involved in brassinosteroid sensitivity and cell wall biosynthesis [58]. Clearly, miRNAs such as these are therefore not only important in controlling floral development but also in modulating events downstream of fertilisation such as embryo and endosperm development, often referred to as grain filling in cereals.

Grain Filling

sRNA profiling in rice, wheat, barley, and maize has demonstrated that various sRNA families, especially miRNAs, exhibit spatiotemporal patterns of expression during grain development [17,96–99]. These differentially expressed miRNAs and their targets are mostly involved in multiple signalling and biosynthetic pathways such as hormone homeostasis and starch biosynthesis, which could contribute to coordinated nutrient accumulation in the growing endosperm. For example, in rice, a quantitative trait locus *GW8* (synonymous with the miR156-targeted *OsSPL16*) encodes a protein that is a positive regulator of cell proliferation [100]. Increased expression of *OsSPL16* promoted cell division and grain filling, and this led to enlarged endosperm size, grain width, and increased yield in rice. As mentioned earlier, the manipulation of miR397 in rice also enhanced grain filling and generated larger grains, ultimately contributing to a 25% increase of grain yield in field trials [58]. Some miRNA families, including miR156, miR164, miR167, miR397, miR1861, and miR1867, have higher abundance in superior spikelets (earlier flowering, faster grain fill) [96,101]. By contrast, 24 nt siRNAs showed higher abundance in inferior spikelets (later flowering, slower grain fill) [101]. These 24 nt siRNAs were more likely to be involved in the RdDM pathway, or to more effectively compete for 2'-OH methylation to enable stabilisation [102,103], such that miRNAs will degrade more quickly and therefore lead to a lower abundance of miRNAs in the inferior spikelets [101]. Hence, repression of 24 nt siRNAs could contribute to miRNA accumulation, which might enhance the grain filling rate in inferior spikelets and produce better-quality grains.

sRNA Engineering in Crops: Leap-Frogging Through the Field

Achieving high yield in crops not only relies on adaptive reproductive traits under unfavourable environments but also on agronomic traits such as leaf morphology, root architecture, and tiller branching/number. Leaves with increased photosynthetic efficiency contribute greatly to nutrient accumulation and grain setting rate during reproduction, while a well-developed, well-adapted root system spatially deploys lateral roots and primary roots to optimise water and nutrient uptake. Tiller dynamics, including density and spatial distribution, could affect plant gas exchange, canopy temperature, and also light interception. Most importantly, the fertile tiller ratio and the development of grain-bearing tillers can directly determine the final yield in cereal crops. The involvement of sRNAs in these traits provides new options for researchers to engineer crop architecture, leading to improved plant fitness, subsequent reproductive success, and high grain yield (Figure 3).

A regulatory miRNA circuit involving miR319, miR396, and their respective targets – the *TCP4* and *GRF* genes – appears to play a conserved role in leaf development. In arabidopsis, *TCP4* has been shown to repress cell proliferation, causing a negative impact on leaf size as a result of reduced leaf cell number [104]. However, GRF proteins promote cell proliferation in the meristem and developing leaves [105,106]. The accumulation of *TCP4* induces the expression of miR396, leading to downregulation of *GRF*s and subsequent repression of cell proliferation [107], as does overexpressing miR396 family members in arabidopsis and rice [106–108]. The upregulation of

miR319 could therefore repress the expression of miR396 and alleviate its negative impacts on GRF proteins and leaf development. In rice, the overexpression of two miR319 family members led to increased longitudinal leaf veins and wider leaf blades, and also enhanced cold tolerance [109]. Similarly, overexpression of osa-miR319 in creeping bentgrass (*Agrostis stolonifera*) caused formation of thicker and more-expanded leaves with increased leaf wax, which contributed to enhanced salt and drought tolerance [110]. Given their conserved functions across plant species, the miR319/TCP and miR396/GRF modules could serve as evolutionary RNAi targets to modify leaf morphology.

Several sRNA-mediated pathways also regulate root development through their roles in auxin signalling and can impact on nutrient and water uptake. Overexpressing miR393 and the knockdown of its targets (the auxin receptor genes *AUXIN-BINDING F-BOX 2* and *TIR1*) in rice plants produced similar phenotypes, with significantly longer primary roots and reduced crown roots, typical root traits associated with altered auxin signalling [111]. However, overexpression of miR393 increased grain-bearing tillers and early flowering in rice, but led to reduced tolerance to salinity and drought [112]. Arabidopsis plants overexpressing miR156 produced more lateral roots, whereas reducing miR156 abundance led to less lateral roots through regulation of *SPL*s involved in auxin signalling [113]. In rice, overexpression of miR156 also increased tiller number and reduced plant height [57,70], but ectopic expression produced a higher fertile tiller ratio, larger panicles, increased grain setting rate, and significant grain yield improvement through the regulation of *SPL*s as mentioned previously [70,81]. Likewise, miR167 overexpression in soybean to downregulate *ARF6* and *ARF8*, and overexpression of miR847 in arabidopsis to downregulate IAA28 (which normally represses *ARF* expression), increased total lateral root number and increased lateral root length [114,115]. Furthermore, the alleviation of miR169-directed repression of NF-YA increased lateral root initiation in arabidopsis [116]. Because miR169 is downregulated under low nitrogen (N) and phosphorous (P) conditions [117,118], knockdown of miR169 may allow increases in N and P uptake through enhanced lateral root development, ultimately leading to improved grain yield and quality. Indeed, overexpressing NF-YA in wheat significantly increased both N and P uptake [119]. Similarly in rice, the miR166-targeted transcription factor RDD1 promotes the uptake and accumulation of various nutrient ions in the roots [120]. The impairment of miR166/RDD1 binding through nucleotide substitution within the miR166 target recognition site produced constitutive RDD1 expression, which ultimately increased nitrogen responsiveness and grain production in rice [120]. Therefore, several candidate regulatory RNAi/target modules exist for improvement of grain yield and quality through the manipulation of leaf morphology, tillering, and root architecture. However, care must be taken to avoid undesirable effects on other traits.

Significant Potential of sRNA Technologies

As a natural mechanism for genetic reprogramming, sRNA-directed RNAi has emerged as a powerful biotechnological tool for gene silencing studies in functional genomics. The use of various RNAi methods has assisted researchers to modify stress responses and reproductive processes in plants, and also expands the power of RNAi in developing high-yielding superior crop varieties.

Several RNAi approaches such as **artificial microRNAs** (amiRNAs) [121–123], **synthetic ta-siRNAs** (syn-tasiRNAs) [124], and the overexpression of *MIR* genes [54,58,109,112] are powerful tools to activate gene silencing through inducing endogenous or exogenous sRNAs. Conversely, the activity of miRNAs can be sequestered using approaches such as sRNA **target mimicry** (TM) [95,125–127], **short tandem TM** (STTM) [128–130], **virus-based miRNA silencing** (VbMS) [131], and **transcription activator-like effector nuclease** (TALEN)-based or **CRISPR/Cas9**-based knockdown of sRNAs [132,133]. TM-based approaches, amiRNAs, and miRNA overexpression, which can all directly modify mature miRNA abundance, are so far the most promising for manipulating reproduction and stress tolerance in crops (Table 2).

Table 2. Current Progress of RNAi Applications in Crop Improvement

RNAi Method	Advantages	Disadvantages	Examples	Refs
Artificial miRNAs	Very effective in knock-down/knock-out studies Few off-target effects Customised to silence both coding and non-coding genes	Not applicable at the DNA level Needs to be combined with tissue-specific promoters to improve efficiency	Improvement of plant height and panicle exsertion to facilitate hybrid rice production	[121–123]
			Control of root architecture through targeting *ETHYLENE RESPONSE FACTOR* genes (*ERF*s)	
			Resistance to Wheat dwarf virus (WDV) through targeting conservative WDV sequences in barley	
Overexpression of miRNAs	Easy to reveal miRNA function through gain-of-function Does not need artificial sRNA constructs	Not very effective for miRNA family members with functional redundancy Needs to be combined with tissue-specific promoters to improve efficiency	Overexpression of miR319 promoted leaf morphogenesis and improved cold tolerance in rice	[54,58,109,112]
			Overexpression of miR393 improved salt and drought tolerance in rice	
			Overexpression of miR397 promoted panicle branching and increased grain size in rice	
			Overexpression of miR408 promoted early heading in wheat	
Target mimics	Easy to generate for their simple structure Very effective in attracting endogenous miRNAs intended to be knocked down	Do not completely degrade mature RNAs Not very effective on highly abundant miRNAs or miRNA family members with functional redundancy	Target mimic of miR156 increased *OsSPL13* to control grain size in rice	[95,125–127]
			Target mimic of miR396 generated higher root biomass and highly-efficient colonization in *Medicago truncatula*	
			Target mimic of miR396 increased secondary branches and spikelets and improved yield in rice	
			Target mimic of miR5200 regulated photoperiod-mediated flowering time in brachypodium	
Short tandem target mimics (STTM)	Effective degradation of mature RNAs through the small degrading nucleases	Not very effective on miRNAs with low abundance	STTM degradation of miR1848 modulated phytosterol and brassinosteroid biosynthesis during plant development and stress response in rice	[128–130]
			STTM degradation of miR396 generated larger reproductive organs and increased fruit yield in tomato	
			STTM blockage of miRNA858 induced anthocyanin biosynthesis in tomato	

Exogenous amiRNAs function in PTGS similar to endogenous miRNAs, but their sequence complementarity can be custom-made to target almost any gene. For example, in rice, the role of *Ghd7* (*Grain number, plant height, and heading date 7*) in regulating heading date, reproductive development, and stress response was revealed by introducing ami-Ghd7, an amiRNA designed complementary to *Ghd7* [134]. Apart from assessing gene function, modifying sRNA could also directly improve agronomically valuable traits. For example, overexpression of amiRNAs results in 80% reduction in the expression level of its target *BETAINE ALDEHYDE DEHYDROGENASE* 2 (*BADH2*), which led to increased 2-acetyl-1-pyrroline, the major compound generating grain fragrance in rice that brings high market value [135]. However, as mentioned earlier, constitutive expression of amiRNAs or overexpression of endogenous miRNAs may also generate undesirable phenotypes. Utilisation of suitable tissue-specific and stress-inducible promoters could adjust gene activity in a more controlled manner, thus minimising undesirable side effects. For example, while flowering can be delayed by silencing *FT* with ami-FT, if an alcohol-inducible promoter is used, flowering could be induced synchronously upon exogenous application of ethanol [136]. However, the design of successful tissue-specific or stress-inducible promoters is challenging. Given that amiRNA-mediated RNAi is a quantitatively effective approach, future development and careful selection of transgenic promoters is very important to fully unlock the potential of amiRNA in crop improvement.

Concluding Remarks and Future Perspectives

Small RNA-mediated epigenetic regulation is involved in almost all biological and metabolic processes during the plant life cycle. Many of these processes are crucial to the maintenance of plant fitness and reproductive success under stressful environmental conditions. Recently characterised sRNA-regulated modules playing decisive roles in reproductive events such as flowering time, panicle branching, and grain development have emerged as a resourceful genetic reservoir for manipulating these challenging breeding targets. However, the contribution of some sRNA families to stress responses and plant development, as well as their trans-generational inheritance and the stability of acquired sRNA-mediated responses, remains unclear (see Outstanding Questions). Furthermore, most of our understanding of stress-induced epigenetic regulation and its adaptive value has been generated from laboratory studies with arabidopsis and rice. Under these conditions plants are often exposed to acute and controlled levels of one single stress, whereas in the field combinations of different abiotic stresses occur simultaneously. The systematic study of sRNA-mediated regulatory mechanisms, and their function, under field conditions for commercial crop cultivation is therefore necessary. Inheritable epigenetic changes, such as DNA methylation and histone variants, could also be exploited, but trans-generational memory of epigenetic variation induced by sRNAs differs according to the environment [7]. Therefore, the benefits and risks of these stress-induced adaptations must be examined in the progenies under different conditions based on their intended regions of cultivation. Together with the identification and characterisation of suitable sRNA/target modules, crops could be manipulated using the various RNAi-based approaches discussed earlier to modify gene expression associated with stress responses and plant reproduction in a controllable manner. These sRNA-associated approaches, together with the development of suitable constitutive, stress-inducible, and tissue-specific RNAi promoters in crop species, could become a sustainable strategy. Furthermore, once whole-genome sequences are available for all species, the full potential of RNAi should be unlocked. SMARTER breeding, through the utilisation of 'Small RNA-Mediated Adaptation of Reproductive Targets in Epigenetic Regulation', could be one of the most promising solutions to improving agricultural productivity by engineering elite crop varieties with enhanced stress tolerance and increased grain yield.

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Outstanding Questions

During domestication, the selection process focusing on high yield performance has considerably limited the genetic diversity of modern crop cultivars. Has this process caused differences in the sRNA mechanisms between cultivars, landraces, and wild relatives? Given the importance of epigenetic regulation in stress adaptation, what are the best ways to exploit sRNA mechanisms among the diverse gene pool available to modern cereal breeding?

Even though *FLC*-like genes have been identified in wheat and barley, to date there is no functional *FLC* validation in cereals. Despite characterisation of the vernalisation-associated *VRN1*, *VRN2*, and *VRN3* (homologue of *FT*) genes in wheat and barley, the epigenetic regulatory mechanism underlying vernalisation is poorly understood. What roles do cereal sRNAs play in this alternative flowering regulatory mechanism governed by the VRN genes?

How did species-specific miRNA regulatory modules such as miR156/529 and their targets (*SPL*/*SBP*-box genes) evolve differently for dicots and monocots? What role do these miRNA regulatory circuits play in the phenotypic changes and speciation that differentiate dicots and monocots? With answers to these questions, could the natural loss of functionality of crucial sRNA regulators be compensated by RNAi manipulation of their evolutionary orthologues, which would concomitantly provide increased options to alter morphological and reproductive traits across dicot and monocot species?

Given the intricacy of complex cereal genomes, would researchers be able to regulate RNAi activity at a chromosome-specific level? Can the accuracy of RNAi-based approaches be improved when targeting individual homoeologous genes with high sequence conservation?

What is the best way to minimise the undesirable pleiotropic effects in RNAi-engineered crops? What is the best way to modify a single trait when a master regulator sRNA controls multiple changes in plant morphology and development?

How can RNAi-conferred stress tolerance in progenies be efficiently and

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accurately evaluated under field conditions given the complex nature of genotype × environment interactions?

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