Chapter 9
Regulation of High-Temperature Stress Response by Small RNAs

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Abstract Temperature extremes constitute one of the most common environmental stresses that adversely affect the growth and development of plants. Transcriptional regulation of temperature stress responses, particularly involving protein-coding gene networks, has been intensively studied in recent years. High-throughput sequencing technologies enabled the detection of a great number of small RNAs that have been found to change during and following temperature stress. The precise molecular action of some of these has been elucidated in detail. In the present chapter, we summarize the current understanding of small RNA-mediated modulation of high-temperature stress-regulatory pathways including basal stress responses, acclimation, and thermo-memory. We gather evidence that suggests that small RNA network changes, involving multiple upregulated and downregulated small RNAs, balance the trade-off between growth/development and stress responses, in order to ensure successful adaptation. We highlight specific characteristics of small RNA-based temperature stress regulation in crop plants. Finally, we explore the perspectives of the use of small RNAs in breeding to improve stress tolerance, which may be relevant for agriculture in the near future.

Keywords Plants · Heat stress · RNA silencing · Thermomemory · Adaptation

9.1   Introduction

Temperature is a primary environmental factor that is continuously monitored by living organisms to adjust their growth and reproduction accordingly. Due to climate change, the global temperature is constantly increasing. Besides this, the frequency and amplitude of extreme temperature events are also elevated. Extreme temperatures exceeding the optimal range of a given organism have an adverse impact on almost every aspect of life including growth and reproduction. The extreme heat exposure damages lipid membranes, proteins, and nucleic acids, etc. Being sessile organisms,

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plants are especially exposed to extreme temperature fluctuations and heat stress (Luria et al. 2019; Mazzeo et al. 2018; Mittler et al. 2012; Ohama et al. 2017; Scharf et al. 2012). To mitigate the negative effects, heat stress response (HSR) pathways are initiated. HSR is a multilayered complex set of physiological and phenological alterations that help plants to cope with the extreme temperatures and sudden temperature changes and enables survival and reproduction (Mittler et al. 2012; Ohama et al. 2017; Scharf et al. 2012). HSR acts on epigenetic, transcriptional and post-transcriptional levels (Ohama et al. 2017).

9.2 Regulation of HSR

Plant heat stress response is initiated by a number of signaling mechanisms. One central role of heat stress signaling is the activation of Heat Stress Factors (HSFs). HSFs are pioneer transcription factors (TFs) that recognize and bind to tandem inverted repeats of nGAAn consensus sequence within the target gene loci to activate transcription (Maruyama et al. 2017). HSF family comprises multiple members (for example *Arabidopsis thaliana* has 21, *Brassica napus* 64, soybean 52, maize 30 members) (Scharf et al. 2012; Zhu et al. 2017). HSF members show high functional diversification and partial genetic redundancy. HSFs might be induced during other stresses or combined stress conditions as well. The master regulators of HSR are the Heat Stress Transcription factors HSFA1 family proteins (in *A. thaliana* HSFA1A, HSFA1B, and HSFA1D) (Liu and Charng 2013; Liu et al. 2011; Ohama et al. 2017; Yoshida et al. 2011). In the absence of these factors, induction of a large number of downstream HS-inducible genes is absent, and plants become HS-sensitive. Temperature-sensitivity of plants becomes apparent already at high ambient temperatures in *hsfa1*-mutant plants, suggesting that the HSF-mediated stress pathways are initiated at these temperatures (Cortijo et al. 2017). *HSFA2* is a direct target of HSFA1s and is a major enhancer of thermotolerance. HSFA1 and HSFA2 proteins form a heteromultimer TF complex (superactivator complex) and strongly induce expression of downstream HSFs, transcription factors, heat shock proteins (HSPs), non-chaperone proteins or non-coding RNAs (Chan-Schaminet et al. 2009; Scharf et al. 1998, 2012). If plants encounter mild temperature stress they become acclimated. This, so-called acquired thermotolerance, allows plants to survive later stronger heat stress exposures (Lin et al. 2014; Liu and Charng 2012). Acquired thermotolerance may be actively maintained for several days post-stress through a process called heat stress memory (Brzezinka et al. 2016; Charng et al. 2007; Lamke et al. 2016; Liu et al. 2018; Stief et al. 2014). Transcriptional memory allows a faster or stronger stress response to be initiated when the same stress is re-encountered. HSFA2 is a central component of thermomemory. Transient binding of HSFA2 induces deposition of Histone 3 lysine 4 dimethylation and trimethylation (H3K4me2, H3K4me3) at memory loci (Lamke et al. 2016). These histone modifications persist after active transcription was subsided, marking them as recently transcriptionally active. Upon recurring heat stress, transcription of these memory

loci is hyperinduced, and thus enables a quicker and more efficient HSR. Besides HSFA2, a growing number of stress memory factors have been discovered (Brzezinka et al. 2018; Charng et al. 2006; Lin et al. 2014; Liu et al. 2018; Meiri and Breiman 2009). Thermomemory was shown to be transmitted transgenerationally (Liu et al. 2019). Micro RNAs (miRNA) and small interfering RNAs (siRNA) are intimately involved in high-temperature stress response and thermomemory.

9.3 Abiotic Stresses Alter sRNAs' Level

The central molecules of RNA silencing pathways are short non-coding regulatory RNAs (small RNAs, sRNA), with the size of 21–24 nucleotides (Axtell 2013; Borges and Martienssen 2015; Rogers and Chen 2013; Vaucheret 2006). There are several classes of sRNAs including miRNA and small interfering RNAs (trans-acting siRNAs or ta-siRNAs; heterochromatic siRNAs or het-siRNA, repeat-associated siRNAs or ra-siRNA, etc.). miRNAs and siRNAs differ in their biogenesis. miRNAs are encoded on distinct genomic loci; their precursors (pri-miRNA) are transcribed by RNA Polymerase II, capped, and polyadenylated. In *A. thaliana*, DICER-LIKE 1 (DCL1), together with SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1) forms the Microprocessor complex. Microprocessor complex processes pri-miRNAs in two subsequent steps to generate pre-miRNA then mature miRNAs. miRNAs are mostly 21-nt long; they are loaded into RNA-induced Silencing Complex (RISC) effector protein ARGONAUTE (AGO1 in *A. thaliana*) and guide sequence-specifically the cleavage or inhibit translation of complementary target mRNAs through a process called Post-Transcriptional Gene Silencing (PTGS) or RNA silencing. 22-nt-long (or in special cases, 21-nt-long) miRNAs not only cleave their targets but also trigger the biogenesis of secondary siRNAs. Secondary siRNA biogenesis requires the activity of DCLs, AGOs and RNA-DEPENDENT RNA POLYMERASES (RDRs). In *A. thaliana*, DCL4, AGO1 or AGO7, and RDR6 are the key enzymes involved in secondary siRNA biogenesis (Dalmay et al. 2000; Martinez de Alba et al. 2013). The secondary siRNA further represses target RNAs *in trans* (at PTGS level), therefore, are called trans-acting siRNAs. miRNAs and ta-siRNAs are involved in almost every aspect of plant growth and development.

The 24-nt-long siRNAs (het-siRNA, ra-siRNA) originate from long double-stranded RNA molecules and serve to silence specific regions of the genome through guiding DNA methylation (RNA-directed DNA methylation, RdDM) and subsequent histone modifications (Borges and Martienssen 2015). This affects gene expression at the transcriptional level; therefore, the process is called Transcriptional Gene Silencing (TGS). During TGS, stable repression of transcription is established at transposons, chromosomal repeats, transgenic inserts but also may occur at certain protein-coding genes. The main function of TGS is the preservation of genome stability and epigenetic regulation of certain genes.

To date, thousands of sRNA have been identified through bioinformatic predictions, cloning and deep sequencing, or hybridization methods both from model plants

and numerous crops (Baksa et al. 2015; Barciszewska-Pacak et al. 2015; Beckers et al. 2017; Gyula et al. 2018; Kruszka et al. 2014; Liu et al. 2017; May et al. 2013; Mohorianu et al. 2013; Zhou et al. 2016).

Besides their role in growth, development and genome stability, sRNAs are also important components of stress responses. Environmental alteration of sRNAs was observed in a variety of species like *A. thaliana*, *Brassica rapa*, *Populus euphratica*, *Triticum aestivum, Oryza sativa*, etc. (Barciszewska-Pacak et al. 2015; Chen et al. 2012; Kumar et al. 2015; Mangrauthia et al. 2017; Sunkar and Zhu 2004; Xin et al. 2011; Yu et al. 2012). Different environmental conditions including heat, drought, salt, oxidative stress, hypoxia, cold, nutrient deprivation, UV-B, strong light, etc. caused severe alteration of sRNA levels or biosynthesis of novel sRNAs (see Table 9.1). Stress-responsiveness suggests regulatory roles during stress or recovery period. This is supported also by the differences between sRNAs' stress-response observed between stress-sensitive and -tolerant varieties or cultivars (Liu et al. 2017; Mangrauthia et al. 2017).

The fact that stress-induced changes have similar trends, occur in multiple species and correlate with the stress-sensitivity, points to a conserved mechanism involving sRNAs as active players. However, some sRNAs' stress response was found to be very specific. For example, miR159, miR160, miR164, miR166, miR319 or miR398 were oppositely regulated in different species (Kumar et al. 2015; Liu et al. 2017; Xin et al. 2010): miR156 family members are induced in response to HS in *A. thaliana, Brassica rapa,* and wheat (Stief et al. 2014; Xin et al. 2010; Yu et al. 2012), but repressed in cassava, or rice (Ballen-Taborda et al. 2013; Liu et al. 2017); miR164 was upregulated and miR164-target NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX1 (NAC1) was suppressed in *Arabidopsis* (May et al. 2013); oppositely to this, tae-miR164 was downregulated and its target HSP17 upregulated in wheat (Kumar et al. 2015); miR164, miR160, miR319, and miR398 were upregulated in *Arabidopsis* but downregulated in wheat (Kumar et al. 2015). Some miRNAs possess tissue-specific changes characteristic to roots, leaves or flowers (Gyula et al. 2018; Liu et al. 2017; Mangrauthia et al. 2017). In specific cases, novel sRNA-target interactions have evolved from conserved miRNA and mRNA target components: for example, in sunflower, the HaWRKY6 transcription factor recently evolved to be the target of the highly conserved miR396 (Giacomelli et al. 2012). These findings warn that sRNA-based stress response regulation is complex. Detailed studying of unique sRNA-target pairs and characterization of their putative roles during HSR response in every specific case is required before generalization is made about their role in non-model plants.

A handful of sRNA::target pairs have been studied in detail. Accumulating evidence shows that sRNA-mediated regulation plays essential roles in plant responses to abiotic stresses, including high-temperature stress response (Cui et al. 2014; Guan et al. 2013; He et al. 2018; Ma et al. 2015; Stief et al. 2014) (see Table 9.1). sRNAs were shown to be required for adaptation to the mild elevation in ambient temperatures, some sRNAs are involved in the establishment of acute stress response, acquired thermo-tolerance or thermomemory in response to heat, while others indirectly alter development in the post-stress period. Importantly, changes in sRNA

Table 9.1 sRNA changes during high-temperature stress response in plants

sRNA	Known targets	Species	Direction of change	References
miR156	SPLS (SQUAMOSA PROMOTER BINDING PROTEIN LIKE)	*Arabidopsis thaliana*	Up	Stief et al. (2014)
		Brassica rapa	Up (miR156h, g)	Yu et al. (2012)
		Manihot esculenta	Down	Ballen-Taborda et al. (2013)
		Oryza sativa	Down	Sailaja et al. (2014), Liu et al. (2017)
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR159	MYBS (MYB DNA-BINDING DOMAIN CONTAINING PROTEINS)	*Cucumis sativus*	Down (if +ABA or +Luffa rootstock)	Li et al. (2016a)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Triticum aestivum L.	Up	Xin et al. (2011), Wang et al. (2012)
miR160	ARF10, ARF16, ARF17 (AUXIN RESPONSIVE FACTORS)	*Apium graveolens* L.	Up	Li et al. (2014a)
		Arabidopsis thaliana	Up	Lin et al. (2018), May et al. (2013)
		Helianthus annuus	Up (1,5h HS) Down (3–6h HS)	Ebrahimi Khaksefidi et al. (2015)
		Hordeum vulgare	Up (miR160a)	Kruszka et al. (2014)
		Oryza sativa	Up (shoot) Down (root)	Sailaja et al. (2014)
		Panicum virgatum	Up	Hivrale et al. (2016)

(continued)

Table 9.1 (continued)

sRNA	Known targets	Species	Direction of change	References
		Populus tomentosa	Down (miR160a, b, c)	Chen et al. (2012)
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Down	Kumar et al. (2015)
miR164	NAC1 (NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX1), HSP17 (HEAT SHOCK PROTEIN 17)	*Apium graveolens* L.	Up	Li et al. (2014a)
		Arabidopsis thaliana	Up	May et al. (2013)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Triticum aestivum L.	Down	Kumar et al. (2015)
miR166	PHV (PHAVOLUTA), REV (REVOLUTA), HOX9 (HOMEOBOX LEUCINE ZIPPER PROTEIN 9)	*Arabidopsis thaliana*	Up	May et al. (2013)
		Hordeum vulgare	Up (miR166a)	Kruszka et al. (2014)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Triticum aestivum L.	Up (miR166d)	(Xin et al. 2011)
miR167	ARFS (AUXIN RESPONSIVE FACTORS), IAA-ALA RESISTANT3 (IAR3)	*Brassica rapa*	Up (endosperm, pollen)	Bilichak et al. (2015)
		Helianthus annuus	Up (1,5h HS), Down (3–6h HS)	Ebrahimi Khaksefidi et al. (2015)
		Hordeum vulgare	Up (miR167 h)	Kruszka et al. (2014)
		Oryza sativa	Down	Sailaja et al. (2014)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Populus tomentosa	Down (miR167c, d)	Chen et al. (2012)

(continued)

Table 9.1 (continued)

sRNA	Known targets	Species	Direction of change	References
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR168	AGO1 (ARGONAUTE 1)	*Apium graveolens* L.	Up	Li et al. (2014a)
		Brassica rapa	Up (endosperm)	Bilichak et al. (2015)
		Oryza sativa	Down	Sailaja et al. (2014)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Populus tomentosa	Down (miR168a, b)	Chen et al. (2012)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR169	NF-YS (NUCLEAR TRANSCRIPTION FACTOR Y)	*Arabidopsis thaliana*	Up	Guan et al. (2013)
		Oryza sativa	Up (shoot), Down (root)	Sailaja et al. (2014)
		Panicum virgatum	Down	Hivrale et al. (2016)
		Populus tomentosa	Up (miR169j) Down (miR169a, b, n)	Chen et al. (2012)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR171	SCL6 (SCARECROW-LIKE6)	*Arabidopsis thaliana*	Up	Mahale et al. (2013)
		Panicum virgatum	Down	Hivrale et al. (2016)
		Populus tomentosa	Down	Chen et al. (2012)
		Populus trichocarpa	Down	Lu et al. (2008)
miR172	AP2 (APETALA2)	*Arabidopsis thaliana*	Down	May et al. (2013)

(continued)

Table 9.1 (continued)

sRNA	Known targets	Species	Direction of change	References
		Helianthus annuus	Down	Ebrahimi Khaksefidi et al. (2015)
		Oryza sativa	Down	Mangrauthia et al. (2017)
		Triticum aestivum L.	Down	Xin et al. (2011)
miR319	TCPS (TEOSINTE BRANCHED1, CYCLOIDEA, AND PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR), MYBS (MYB DNA-BINDING DOMAIN CONTAINING PROTEINS)	*Arabidopsis thaliana*	Up	Li et al. (2014b)
		Oryza sativa	Up	Mangrauthia et al. (2017)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Triticum aestivum L.	Down	Kumar et al. (2015)
miR390	ARFS (AUXIN RESPONSIVE FACTORS)	*Panicum virgatum*	Up	Hivrale et al. (2016)
		Brassica rapa	Up (endosperm, pollen)	Bilichak et al. (2015)
miR393	TIR1 (TRANSPORT INHIBITOR RESPONSE 1)	*Arabidopsis thaliana*	Down	Guan et al. (2013)
		Oryza sativa	Up	Mangrauthia et al. (2017)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR394	LCR (LEAF CURLING RESPONSIVENESS)	*Apium graveolens* L.	Up	Li et al. (2014a)

(continued)

Table 9.1 (continued)

sRNA	Known targets	Species	Direction of change	References
		Populus tomentosa	Down (miR394a, b)	Chen et al. (2012)
miR395	APSS (ATP SULFURYLASES), SULTR2;1 (SULFATE TRANSPORTER 2;1)	*Apium graveolens* L.	Up	Li et al. (2014a)
		Panicum virgatum	Up	Hivrale et al. (2016)
		Populus tomentosa	Down	Chen et al. (2012)
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Up	Kumar et al. (2015)
miR396	GRFS (GROWTH-REGULATING FACTOR), BHLH74 (BASIC HELIX-LOOP-HELIX 74), HAWRKY6	*Helianthus annuus*	Up (1,5h HS) Down (3–24h HS)	Giacomelli et al. (2012)
		Oryza sativa	Down	Liu et al. (2017), Mangrauthia et al. (2017)
		Panicum virgatum	Up	Hivrale et al. (2016)
miR397	LACCASES	*Arabidopsis thaliana*	Down	Mahale et al. (2013)
		Oryza sativa	Up	Jeong et al. (2011)
miR398	CSD1 (COPPER/ZINC SUPEROXID DISMUTASE1), CSD2, CCS (COPPER CHAPERONE FOR SOD), COX5 (SUBUNIT OF THE MITOCHONDRIAL CYTOCHROME C OXIDASE)	*Arabidopsis thaliana*	Up	Guan et al. (2013), Fang et al. (2019)
		Brassica rapa	Down (miR398a, b)	Yu et al. (2012)
		Helianthus annuus	Up	Ebrahimi Khaksefidi et al. (2015)
		Oryza sativa	Down	Sailaja et al. (2014)

(continued)

Table 9.1 (continued)

sRNA	Known targets	Species	Direction of change	References
		Panicum virgatum	Up	Hivrale et al. (2016)
		Populus tomentosa	Down (miR398a, b)	Chen et al. (2012)
		Triticum aestivum L.	Up	Xin et al. (2011)
		Zea mays	Up	Guan et al. (2013)
miR399	PHO2 (PHOSPHATE 2)	*Arabidopsis thaliana*	Down	Kim et al. (2011)
		Brassica rapa	Down	Yu et al. (2012)
		Oryza sativa	Down	Liu et al. (2017)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR400	PPRS (PENTATRICOPEPTID REPEAT PROTEINS)	*Arabidopsis thaliana*	Down	Yan et al. (2012)
miR403	AGO2 (ARGONAUTE 2)	*Helianthus annuus*	Up	Ebrahimi Khaksefidi et al. (2015)
miR408	LACCASES, PLASTOCYANIN-LIKE FAMILY PROTEINS	*Apium graveolens* L.	Up	Li et al. (2014a)
		Oryza sativa	Up	Mangrauthia et al. (2017)
		Panicum virgatum	Down	Hivrale et al. (2016)
		Populus tomentosa	Down	Chen et al. (2012)
		Saccharina japonica	Down	Liu et al. (2013)
		Solanum pimpinellifolium L.	Up	Zhou et al. (2016)
		Triticum aestivum L.	Up	Kumar et al. (2015)

Table 9.1 (continued)

| sRNA	Known targets	Species	Direction of change	References
miR529	SPLS (SQUAMOSA PROMOTER BINDING PROTEIN LIKE)	*Panicum virgatum*	Up	Hivrale et al. (2016)
miR827	SPX (SYG1/PHO81/XPR1)-DOMAIN-CONTAINING GENES, NLA (NITROGEN LIMITATION ADAPTATION), PHT5 (PHOSPHATE TRANSPORTER 5)	*Panicum virgatum*	Up	Hivrale et al. (2016)
		Triticum aestivum L.	Up	Xin et al. (2011)
miR842	JACALIN LECTIN FAMILY MEMBERS	*Helianthus annuus*	Up	Ebrahimi Khaksefidi et al. (2015)
miR1884	–	*Oryza sativa*	Up (shoot) Down (root)	Sailaja et al. (2014)
miR1885b.3	–	*Brassica rapa*	Down	Yu et al. (2012)
miR2005	–	*Triticum aestivum* L.	Up	Xin et al. (2011)
miR5175	–	*Hordeum vulgare*	Up (miR5175a)	Kruszka et al. (2014)
miR5714	–	*Brassica rapa*	Up	Yu et al. (2012)
miR5716	–	*Brassica rapa*	Down	Yu et al. (2012)
miR5736	–	*Brassica rapa*	Up	Yu et al. (2012)
TAS1-derived ta-siRNAs	HTT1, HTT2 (HEAT-INDUCED TAS1 TARGET1 AND 2)	*Arabidopsis thaliana*	Up	Li et al. (2014b)
Ta-miR2002	–	*Triticum aestivum* L.	Up	Xin et al. (2011)
SiRNA 002061_0636_3054.1	–	*Triticum aestivum* L.	Down	Yao and Sun (2012)
SiRNA 005047_0654_1904.1	–	*Triticum aestivum* L.	Down	Yao and Sun (2012)
SiRNA 080621_1340_98.1	–	*Triticum aestivum* L.	Down	Yao and Sun (2012)
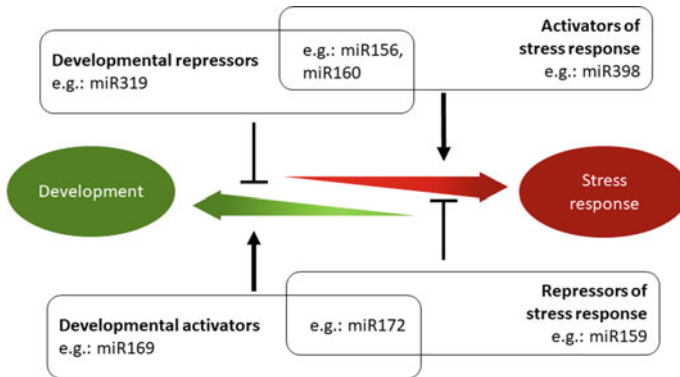
Fig. 9.1 Small RNAs act in a complex network to regulate the balance between developmental pathways and stress responses. Examples are given for each category (for activity of specific sRNAs, please see Table 9.1)

or/and target act in a complex, interconnected network that fine-tunes the balance between growth and stress response (Fig. 9.1).

9.4 sRNAs' Involvement in the Ambient Temperature Response

Plants substantially alter their developmental program upon changes in the ambient temperature (Quint et al. 2016; Wigge 2013). It was shown recently that changes in ambient temperature affect the steady-state levels of only a small fraction of the total sRNA loci (0.6%) in *A. thaliana* (Gyula et al. 2018). One of these temperature-responsive loci can add an extra regulatory layer to the auxin-dependent thermomorphogenesis by regulating epigenetically the expression of the auxin synthesis gene *YUCCA2* (*YUC2*). This finding also suggests that the temperature-dependency of these loci is not conferred by some common siRNA biogenesis components (i.e. PolIV/V, RDR2, DCL3) (Borges and Martienssen 2015), but some other, unknown *trans* factors.

Several *Arabidopsis* miRNAs were identified in microarray and northern blot experiments to be differentially expressed (miR156, miR163, miR169, miR172, miR398, and miR399) in response to ambient temperature changes (Lee et al. 2010). The gradient of miR156 and miR172 regulates the juvenile to adult phase transition and adult to reproductive phase transitions. miR156 is highly expressed during the juvenile stage and gradually decreasing its level as the plant ages (Poethig 2009). Targets of miR156 are the SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors. SPLs regulate a number of genes involved in floral time transition (Srikanth and Schmid 2011; Wang et al. 2009). Overexpression of miR156 in *Arabidopsis* causes altered vegetative phase transition and delayed flowering (Xing et al.

2013; Yu et al. 2010). The level of miR156 is high at low ambient temperatures (16 °C) and therefore could delay juvenile to adult phase transition or floral transition. The thermodynamic properties of mir156 precursor stem-loop structure can affect the cleavage efficiency of the mature miRNA/miRNA* duplex. Structural analysis studies showed that certain positions in the mir156a precursor sequence can particularly affect the cleavage efficiency, and in turn, the flowering time at different ambient temperatures (16 °C vs. 23 °C) (Kim et al. 2016). Therefore, the mir156 precursors can serve as a temperature sensor. Furthermore, warm temperature (23 °C) enhances the expression of miR172, which plays the opposite role to miR156. Overexpression of miR172 causes early flowering by down-regulating the AP2-domain transcription factors TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (SNZ) (Aukerman and Sakai 2003; Chen 2004; Spanudakis and Jackson 2014).

Warm ambient temperature (27 °C) reduces the expression of miR169 (Gyula et al. 2018), which target mRNAs encoding subunits A of the NUCLEAR FACTOR Y (NF-Y) transcription factor complex. These TF form a conserved heterotrimeric TF complex that is composed of NF-Y subunit A (NF-YA), NF-YB and NF-YC subunits (Petroni et al. 2012). The miR169 family represses the NF-YA subunit, which affects the expression of the NF-Y -dependent genes, including the flowering time regulator *FLOWERING LOCUS T (FT),* the auxin synthesis gene *YUC2*, and could mediate the high ambient temperature-induced early flowering (Gyula et al. 2018; Siriwardana et al. 2016).

PHYTOCHROME B (PHYB) mediates red light and high ambient temperature signals through a subset of basic helix-loop-helix (b-HLH) transcription factors, the PHYTOCHROME INTERACTING FACTOR1 (PIF1), PIF3, PIF4 and PIF5 (Jung et al. 2016; Legris et al. 2016). PIF4 is a key regulator of the photo- and thermomorphogenesis. At high ambient temperature, the PIF4 alters the expression of hundreds of genes that result in morphological and physiological changes that are necessary for thermal adaptation. A recent study described a new, surprising function of this transcription factor (Sun et al. 2018). According to this, PIF4 not only regulates the transcription of some miRNA genes, but being the part of the Microprocessor complex, it affects the cleavage of the miRNA duplex from the miRNA-precursor in a red light-, and probably a temperature-regulated manner by promoting the degradation of DCL1 and HYL1 proteins. This moonlighting activity of PIF4 suggests that other bHLH proteins may also bind the DCL1 and HYL1 conveying other environmental or hormonal signals to the Microprocessor complex. Temperature affects miRNA processing at multiple levels; at low ambient temperature (16 °C), the miRNA processing machinery becomes more robust and it does not require the presence of DCL1 cofactors HYL1 and SE, for short base-to-loop processing of miRNA precursor (Re et al. 2019).

9.5 sRNAs Regulate Thermotolerance Response

Many sRNA (like miR156, miR160, miR164, miR166, miR169, miR171, miR319, miR398 or TAS1-derived ta-siRNAs) are also induced in response to heat (see Table 9.1). Heat-induced changes of miR160 contribute to thermotolerance through alteration of HSP expression. High-throughput sequencing and hybridization experiments have revealed that induction of miR160 occurs in various plant species (Kruszka et al. 2014; Kumar et al. 2015; Lin et al. 2018). HS induced miR160 transcriptionally, that caused repression of AUXIN RESPONSIVE FACTOR 10 (ARF10), ARF16 and ARF17 targets (Lin et al. 2018). Auxin decrease during heat also contributes to ARF10 and ARF16 repression through a miR160-independent pathway (Wang et al. 2005). Downregulation of ARFs was necessary for proper HSR: overexpression of miR160 improved seed germination and seedling survival in response to heat while miR160-mimicry plants became HS-sensitive (Lin et al. 2018). The HS-tolerant phenotype of *arf10*, *arf16*, and *arf17* mutant plants was similar to miR160-overexpressors. miR160's and ARFs' changes during HS alter the expression of multiple HSPs. Therefore, the intertwined activities of hormonal signals, protein homeostasis, and miR160-guided silencing contribute to basal thermotolerance in a positive manner. Some miRNAs might affect HS tolerance negatively. miR400 is a negative regulator of HS response in *Arabidopsis*. ath-miR400 is located within an intron and is co-transcribed with its host nascent RNA transcript. Under non-inductive conditions, the miR400-containing intron is spliced, and the miR400 matured from this intron/precursor. Under HS conditions, alternative splicing of host genes led to partial intron retention and abolishment of miR400 processing (the level of the host gene was not affected). Overexpression of miR400 causes HS-sensitive phenotype, therefore, downregulation of miR400 is required for proper thermotolerance response (Yan et al. 2012). Alternative splicing, therefore, regulates HS response via the miRNA pathway.

miR159 has also a negative impact on HSR. It was shown that luffa (*Luffa cylindrica*) rootstock enhanced thermotolerance in cucumber (*Cucumis sativus*) plants (Li et al. 2016a). Luffa rootstock induced a significant downregulation of a subset of miRNAs in the shoot during HS, suggesting that miRNAs might participate in luffa rootstock-induced heat tolerance. Amongst these, miR156, miR159, miR169, and miR398 were linked to HS response previously (Guan et al. 2013; Kruszka et al. 2014; Xin et al. 2010). Luffa rootstock also induced enhanced abscisic acid (ABA) accumulation in the shoot. It is known that ABA plays a role in the regulation of several miRNAs, such as miR159. miR159 targets are the MYB transcription factors that are involved in abiotic stress responses. Foliar application of exogenous ABA suppressed miR159 and induced CsMYB29-like and CsGAMYB1 target levels (Li et al. 2016a). Transgenic tae-miR159-overexpressor rice, csa-miR159-overexpressor, and *myb33;myb65* double mutant *Arabidopsis* plants were shown to be HS-sensitive (Li et al. 2016a; Wang et al. 2012). AtHSP70 transcript and protein accumulation were impaired in miR159b overexpression lines suggesting that HSP70 may be directly or

indirectly, negatively regulated by miR159 under natural conditions. miR159 downregulation, therefore, is required for efficient HSR. It was suggested that cucumber plants that originate in the temperate climate have evolved poor HSR compared to luffa plants. However, the role of the *Arabidopsis* miR159-MYB33/65 pathway in abiotic stress responses including heat was questioned by another study (Li et al. 2016b).

The decrease of ta-siRNAs is also required for proper HSR. It has been shown that targets of TAS1-derived ta-siRNA, named HEAT-INDUCED TAS1 TARGET1 (HTT1) and HTT2 regulate thermotolerance (Li et al. 2014b). HTT1 and HTT2 are strongly accumulated during HS due to the direct HSFA1s-mediated transcriptional activation. Parallel to this, HS inhibited the production of TAS1-derived ta-siRNAs, the negative regulators of HTT1 and HTT2 that contributed to the elevated level of HTT1 and HTT2 under HS conditions. Overexpression of TAS1a reduced expression of HTT genes and caused HS-sensitive phenotype while overexpression of HTT1 and HTT2 upregulated several HSF genes and improved thermotolerance of transgenic plants. HTT1 and HTT2 positively regulate HSR through transcriptional activation of HS-related targets within a transcriptional complex comprising HSP70-14, HSP40, and NF-Y complex subunit C2 (NF-YC2) factors.

9.6 Role of sRNAs in Acquired Thermotolerance and Heat Stress Memory

Stress events are recurring in nature. The encounter of nonlethal stress leads to the acquisition of thermotolerance (acquired thermotolerance or AT). AT functions to prime the plants for a quicker and/or stronger HSR during later, more severe stresses and to alter post-stress development of plants in order to be able to complete the life cycle before lethal stress (e.g. modulate growth rate, flowering time, senescence, etc.). *ago1* mutant plants are impaired in acquired thermotolerance suggesting the involvement of RNA silencing pathway also in this form of the stress response (Stief et al. 2014). AGO1 levels might be modulated in response to heat through an autoregulatory loop involving miR168 activity (Chen et al. 2012; Hivrale et al. 2016; Vaucheret et al. 2006; Xin et al. 2010).

miRNAs and ta-siRNA classes are both involved in AT. The miR156 family is highly conserved (Cui et al. 2014; Stief et al. 2014) and controls juvenile to adult transition. miR156 is strongly induced transcriptionally during heat stress and its activation persists for several days (Stief et al. 2014). miR156 family downregulates SPLs to promote sustained expression of heat-stress memory-related genes including *APX2*, *sHSP17.6A*, *HSFA2*, *HSA32*, *sHSP22*. The expression of MIR156 genes following HS is critical for acquired thermotolerance and heat stress memory. Epigenetic modifications like H3K4me2/3 are involved in transcriptional memory (Lamke et al. 2016).

The most deleterious impact of heat is caused during the reproductive phase of plant life. Flowers are the most heat-susceptible tissues: heat affects pollen viability, grain filling, and maturation. Through all these, HS compromises reproductive success (Liu et al. 2017; Stief et al. 2014). Flowering time, therefore, is regulated in a complex manner to align it to the most favorable environmental conditions. miR156 is a repressor, miR172 is an activator of flowering time through age pathway (Wang 2014). The decline of the miR156 level with aging provides a permissive environment for flowering through de-repression of SPLs that positively regulate floral activator genes including LEAFY, FRUITFULL, APETALA1, APETALA2 or FT (Kim et al. 2012; Wang et al. 2009; Yamaguchi et al. 2009). Bra-miR156 upregulation and BraSPL2 downregulation also occur in *B. rapa* (Yu et al. 2012). Heat (37–45 °C) not only induces miR156 but also represses miR172 in *Arabidopsis*, wheat, or *Helianthus annuus* (Ebrahimi Khaksefidi et al. 2015; May et al. 2013; Stief et al. 2014; Xin et al. 2010) while its targets are upregulated. Antagonistic changes of miR156-SPL and miR172-AP2 regulatory modules converge to record plant age as "time well spent" (excluding stress time) (Stief et al. 2014) and modulate flowering time accordingly.

miR319 also contributes to flowering time changes in response to HS. The miR319 family targets TEOSINTE BRANCHED/CYCLOIDEA/PCF domain proteins (TCPs). TCPs are positive regulators of flowering time: overexpression of miR319 and loss-of-function of the miR319 target TCP4 cause late flowering. miR319 is upregulated and *TCP* family genes are downregulated during HS (Hivrale et al. 2016). ta-siRNAs also affect flowering time regulation (Liu et al. 2019) (*see later*). In summary, the spectrum of sRNAome fine-tunes stress response both through positive and negative activities in order to mitigate the detrimental impact of heat and successfully accomplish reproduction.

9.7 sRNAs Are Required for Trans-Generational Stress Memory

Temperature affects the efficiency of RNA silencing (Szittya et al. 2003; Zhong et al. 2013). Moderate heat stress (30 °C) induces the epigenetic release of RNA silencing through inhibition of ta-siRNA biogenesis (Zhong et al. 2013). It was shown recently that this pathway requires the activity of HSFA2, REF6, and SGIP1 (Liu et al. 2019). HSFA2 transcriptionally activates *RELATIVE OF EARLY FLOWERING 6* (*REF6*), an H3K27-demethylase gene (Lu et al. 2011). REF6, in turn, de-represses *HSFA2*, through H3K27me3 demethylation. As a consequence, it is established a heritable feedback loop that positively affects both heat response and thermomemory. REF6-HSFA2 interplay activates SGS3-INTERACTING PROTEIN 1 (SGIP1), an E3 ubiquitin ligase. SGIP1 mediates degradation of SUPPRESSOR OF GENE SILENCING (SGS3) protein (Kumakura et al. 2009; Mourrain et al. 2000), the cofactor of RDR6. SGS3 degradation leads to the abolishment of ta-siRNA production in

the heat-stressed plants. Low levels of SGS3 are epigenetically maintained also in the non-stressed progeny (Liu et al. 2019; Zhong et al. 2013). HS-induced transcriptional activation and absence of ta-siRNAs activity converge to release HTT5, which drives early flowering (Liu et al. 2019). The basis for trans-generational adaptation is the maternally transmitted H3K27me3 demethylation of the *HSFA2* locus.

Besides the PTGS pathway, RdDM-mediated TGS also plays a role in the defense against the detrimental effects of excessive heat. It has been shown that under heat stress, the Copia-type retrotransposon ONSEN is transcriptionally activated and produces extrachromosomal DNA copies (Ito et al. 2011). HS-induction of ONSEN requires HSE *cis* motifs and HSFA1s and HSFA2 *trans* factors (Pecinka et al. 2010). Activation of ONSEN was stimulated in siRNA biogenesis mutants: a high frequency of new ONSEN insertions was observed in the progeny of stressed siRNA-deficient plants. As retrotransposition was not observed in wild-type plants subjected to the same treatment (Ito et al. 2011), siRNA pathway has a crucial role in restricting stress-triggered retrotransposition ensuring genome stability during and following heat stress. Transient derepression of ONSEN may result occasionally in new retrotransposition events. Heat-induced trans-generational trait changes via ta-siRNA and het-siRNA pathway may offer adaptive advantage and flexibility amongst warmer environments.

Trans-generational thermomemory, however, can be associated with negative consequences: the release of HTT5 in absence of ta-siRNAs attenuated plants' immunity. Abiotic resistance might also affect growth rate amongst non-stress conditions or in response to different stresses. Therefore it is conceivable that unknown mechanisms hamper full inheritance of epigenetic traits. Chromatin regulators DECREASE IN DNA METHYLATION1 (DDM1) and MORPHEUS' MOLECULE1 (MOM1) were found to redundantly prevent transgenerational inheritance of environmentally induced traits (Iwasaki and Paszkowski 2014). In accordance with this idea, ta-siRNA biogenesis and basal level of thermoresistance were reset in later generations (Liu et al. 2019), providing flexibility for adaptation in the long term.

9.8 A Complex sRNA Network Contributes to HSR

The above examples (see also Table 9.1) illustrate that under elevated ambient temperature (27–29 °C) and heat stress (32–45 °C) conditions a number of sRNAs are changed to coordinate temperature response. The altered sRNAome causes changes in the mRNA target network that affects a large spectrum of cellular and physiological processes. Under optimal conditions, resources are directed to support growth and development. Under elevated ambient temperatures, stress responses are already initiated but at the same time, the flowering transition is accelerated to ensure reproductive success: the cost of this is that fewer seeds are produced. During severe stress, growth and development are rather stalled and resources are primarily mobilized to manage the negative impacts of stress. The trade-off between development

and stress response is continuously fine-tuned based on the strength, type and duration of temperature changes encountered.

The miR156-SPL9-DFR pathway was suggested to coordinate the balance between growth and abiotic stress tolerance in *Arabidopsis* (Cui et al. 2014). Under optimal conditions, miR156 is suppressed to accelerate development, while under stress conditions it is induced to maintain juvenile state for a longer period. The mechanism is conserved also in rice. Downstream genes that take part in this include miR156-targets SPL9 and DIHYDROFLAVONOL-4-REDUCTASE (DFR) that modulate anthocyanin metabolism. miR156 is induced in response to multiple abiotic stresses including heat, salt or mannitol. SPL9 and SPL10 targets of miR156 promote transcription of miR172 that is required for the juvenile-to-adult transition. miR172 overexpression cause stress-sensitivity, further supporting the antagonistic relationship between development and stress response.

Auxin perception and signaling pathway also attenuate growth through miRNA activity. Expression of auxin-responsive genes is mediated by the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) through ARFs release from auxin-mediated heterodimerization. miR393 negatively regulates TIR1, while miR160 and miR167 negatively regulate ARFs. Upon stress, miR160 is induced, it downregulates ARF10, ARF16, and ARF17 leading to suppression of ARF-induced gene expression and thereby growth attenuation (Sunkar et al. 2012). Upregulation of miR160 and parallel downregulation of ARF targets was also observed in barley and *H. annuus* (Ebrahimi Khaksefidi et al. 2015; Kruszka et al. 2014). In wheat, however, miR160 was downregulated in response to heat (Kumar 2014). Besides these, miR390 and miR393 also participates in auxin-mediated HSR (Hivrale et al. 2016; Vidal et al. 2010).

The miR166 target PHAVOLUTA (PHV), REVOLUTA (REV), and HOME-OBOX LEUCINE ZIPPER PROTEIN 9 (HOX9) and miR319 target TCP are involved in leaf development; miR171 targets SCARECROW-LIKE6s (SCL6s) and miR164 target NAC1 regulate shoot and root development; all these miRNAs are induced and their targets are repressed in response to heat (Mahale et al. 2013; Kruszka et al. 2014; Schommer et al. 2008), further underpinning the hypothesis that developmental pathways are being retarded temporarily to allow stress recovery.

In summary, combined sRNA changes contribute to the balance between growth and stress response and aim completion of life-cycle to establish the next generation (Fig. 9.1).

9.9   Combined Stress Pathways Are Regulated by sRNAs

In nature, abiotic stresses occur mostly in combination. Multiple miRNAs, including the above-mentioned miRNAs (miR156, miR160, miR167, and miR393) are upregulated or downregulated in response to heat, cold, salt, mannitol, UV-B, drought, hypoxia or oxidative stress in diverse species (Khraiwesh et al. 2012). This suggests

that miRNAs are integrators of environmental stimuli under combined environmental stress conditions.

Exposure to high temperature causes oxidative stress as well. Signals generated during oxidative stress are absolutely required for an effective HSR (Dickinson et al. 2018). Subcellular energy organelles, the chloroplasts, drive a series of metabolic reprogramming (Wang et al. 2018) and initiate multiple retrograde signaling to regulate abiotic (light, heat, oxidative) stress responses (Dickinson et al. 2018; Fang et al. 2019). One of these pathways acts through miRNAs (Fang et al. 2019): plastid synthesized tocopherols and 3′-phosphoadenosine 5′-phosphate (PAP) inhibit nuclear exoribonucleases (XRN)-catalyzed pri-miRNA degradation. This promotes enhanced miRNA biogenesis, amongst others that of miR398 that confers thermotolerance to the plants. Efficient accumulation of miR398 also requires transcriptional induction via direct binding of HSFA1b and HSFA7b to its gene promoter region (Guan et al. 2013). As a consequence, the level of miR398 targets Cu/Zn SUPEROXIDE DISMUTASE 1 (CSD1), CSD2, and COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS) mRNAs are downregulated in response to heat (Guan et al. 2013). Transgenic plants expressing miR398-resistant forms of CSD1, CSD2, and CCS are HS-sensitive and expression of numerous HSFs and HSPs during the heat is reduced. Oppositely to this, *csd1*, *csd2*, and *ccs* mutant plants are HS-resistant and expression of certain HSFs and HSPs is induced. It was suggested that heat-induced miR398 alters cellular redox status through negative regulation of CSD1, CSD2 and CCS targets. The altered redox environment is directly or indirectly sensed by HSFs (Volkov et al. 2006) and thermotolerance pathways are initiated through HSR-regulon. miR398 family members and their target genes are highly conserved across monocots and dicots. miR398 heat-induction was found to occur also in maize, *Brassica rapa* and *Populus tomentosa* (Guan et al. 2013; Kotak et al. 2007; Yu et al. 2012).

Another miRNA, miR408, also contributes to redox changes during different abiotic stress conditions. Expression of miR408 is significantly induced by abiotic stresses including heat (also cold, salt, oxidative, osmotic drought) in *Arabidopsis* and important crop plants. miR408 targets are the *PHYTOCYANIN* family genes that function as electron-transfer factors to regulate redox state (Hivrale et al. 2016; Kumar et al. 2015; Ma et al. 2015; Mangrauthia et al. 2017; Zhou et al. 2016). miR398 and miR408, therefore, may provide abiotic stress tolerance through regulation of redox status during heat or combined stress conditions. Manipulation of redox state through alteration of miR398, miR408 or their targets, therefore, may be a useful strategy to breed thermotolerant crops.

9.10 Breeding for Thermotolerance

In order to enhance HS tolerance in crop plants, it is necessary to better understand the molecular mechanisms that regulate the HSR and to develop new breeding strategies. In a large number of studies, the biological relevance of sRNA and target changes in

HSR has been demonstrated using sRNA or target mutants, miRNA-overexpressors, or target-mimicry lines. These findings spurred the idea of developing crops having altered miRNA or target level leading to improved agronomical performance, yield or nutritional value amongst abiotic (e.g. heat stress) conditions. This may be achieved through classical or molecular breeding. Artificial miRNAs (amiRNA) have been successfully employed to silence endogenous targets in model plants, crop plants and unicellular organisms (Alvarez et al. 2006; Khraiwesh et al. 2008; Molnar et al. 2009; Schwab et al. 2006; Warthmann et al. 2008). amiRNAs may be designed to have high specificity (avoid off-target effects). Oppositely to amiRNA technology that suppresses target levels, target-mimicry constructs lead to the abolishment of miRNA activity and consequently target-mRNA accumulation (Franco-Zorrilla et al. 2007). amiRNAs and target-mimicry constructs provide a flexible tool for downregulation of related genes or gene families. In contrast to miRNA-based methods, siRNA-based RNA silencing technology (RNA interference or RNAi) cannot be successfully employed for heat-tolerant plant breeding since these depend on the activity of RDR6/SGS3 complex (SGS3 protein is degraded under high-temperature conditions) (Liu et al. 2019) for amplification of gene silencing response. At the same time, it is important to consider the possible side-effect of the improved abiotic tolerance: improved heat-tolerance weakens immunity and might cause poor development under non-stress conditions (Liu et al. 2019).

9.11 Conclusions

There is an increased risk of crop yield reduction worldwide caused by global warming and extreme temperature fluctuations. Elucidation of temperature response, therefore, is becoming more important. Present evidence suggests that epigenetic, transcriptional, sRNA-mediated post-transcriptional and protein-level regulatory mechanisms are all intercalated to precisely and flexibly adjust the trade-off between development and stress response within and trans-generationally. Future research is needed for functional confirmation of HS-responsive sRNA/target changes and for the elucidation of the relationship of sRNA-mediated HSR and other regulatory networks. Complete understanding of the mechanism of sRNA action during HS, combined abiotic stresses or cross-protection between stresses may provide tools for breeding resilient crops.

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