EDITED BY **JEN-TSUNG CHEN**

GENOME AND EPIGENOME EDITING FOR STRESS-TOLERANT CROPS

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Edited by

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Preface

In recent decades, the world has faced serious crises mainly caused by the continuously expanding human population, the resulting environmental pollution, and the shortage of food and natural resources. Undoubtedly, these conditions can be worse under the scenario of climate change globally, such as a rising temperature that can lead to shortages of water resources and, consequently, increase the risk of agricultural and ecological droughts. To ensure sufficient agricultural nutrition and food production, scientists are expected to solve the problems by developing new crop breeding methods, particularly focusing on identifying the stress-tolerant traits and uncovering underlying in-depth machinery. Importantly, these approaches can advance agricultural biotechnology and crop breeding focusing on acquiring future crops with climate-resilient capacities, which might greatly ensure nutrition and food security under the challenge of upcoming global climate change. Therefore, nowadays, the demand to organize crop breeding programs for developing climate-resilient crops is inevitably increasing.

To support such demand, modern plant molecular biotechnology has been upgraded fast to achieve high-throughput, high-resolution, smart, and precision manners such as multiple omics-based functional genomics, CRISPR/Cas9-mediated genome editing, RNA technology, and so on. In the post-genomics era, the approaches of multiple omics continue to mine huge amounts of genetic resources and accumulate interesting key genes, and definitely need efficient ways of genetic engineering to benefit crop breeding and agricultural production. In the past decade, an emerging genetic engineering technology, that is, genome editing technology, has the power to release the potential of plant genomes through the precise delivery of tolerant genes for crop improvement. The system of genome editing has upgraded fast, and now, CRISPR/Cas9 has become the dominant technology. Thus, the integration and coordination of multiple omics and CRISPR/Cas9 for organizing omics-CRISPR breeding strategies inevitably become the next wave of important tasks in plant science. In addition, in recent years, RNA technology/epigenetic regulation has been introduced into diverse fields of plant science and has been proven to its great potential in mitigating plant stress responses as well as being a fine-tuning regulator in some ways to advance crop improvement and breeding. The role of noncoding RNAs (ncRNAs) in the management of CRISPR-edited crop production has become an important topic. In the future, achieving climate-resilient agriculture needs the coordination of some crucial technologies involving multiple omics, CRISPR/Cas9, and functional RNA technology/epigenetics.

In this book, the ways for vertical integration and horizontal coordination of the crucial technologies were comprehensively discussed to advance crop breeding programs toward stress-resilient agriculture.

This book presents the coordinated CRISPR-noncoding RNAs (ncRNAs) strategies for combating diverse stressors and complicated or multiple stress conditions. It covers both abiotic and biotic stress, including stressors of salinity, temperature, drought, heavy metals, pests, pathogens, and so on, and proposes strategies to develop stress-tolerant crops with high-yield and high-quality traits through the integration or coordination of the mainstream technologies, that is, multiple omics, CRISPR/Cas, and ncRNA-based epigenetics.

This book is an ideal reference to integrate the emerging field of multiple omics, CRISPR/Cas, and ncRNA-based epigenetics by sharing crucial aspects of methods, applications, and future directions. It opens doors for students and researchers to efficiently overview these critical subtopics of plant science and technology and thus realize the concept and, hopefully, inspire the ideas of future experiments and the exploration of the knowledge and, eventually, lead to better development of future crops by scientists, plant biologists, and crop breeders.

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Mitigating Heat Stress Response in CRISPR/Cas-Mediated Edited Crops by Altering the Expression Pattern of Noncoding DNA

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1.1 Introduction

Heat stress, a significant constraint in crop production, is becoming increasingly threatening with the onset of climate change and the increased frequency of extreme heat waves. Plants are highly vulnerable to temperature fluctuations beyond their optimum range for growth, development, and reproduction (Govindaraj et al., 2018). Previously, elevated temperatures, especially heat waves, have already substantially reduced the regional yields of rice, wheat, and corn in Asia, North America, India, and Europe (Hassan et al., 2021). The Intergovernmental Panel on Climate Change's Sixth Assessment Report (IPCC's AR6) indicated that the global surface temperature rose by 1.09 °C from 1850–1900 to 2011–2020, with an anticipated rise of at least 0.2 °C per decade without substantial mitigation (IPCC, 2021). This could potentially compromise our capacity to attain food security in the future; the current predictive models suggest that yield losses among staple crops could be as high as 8% per 1 °C increase in global temperature (Zhao et al., 2017; Lee et al., 2024). While adaptive agricultural practices such as altering planting dates, improving irrigation availability and efficiency, utilizing shade structures, growing in controlled environments, and applying osmoprotectants could alleviate the effects of heat stress on crop yield, these approaches are not always cheap and flexible, especially at higher production levels. The deployment of thermotolerant cultivars that can withstand elevated temperatures without yield or agronomic penalties is still seen as the most sustainable solution to feeding the ever-growing human population amidst rising global temperatures.

Breeding for thermotolerance is associated with complexities originating from this trait's polygenic nature and the complex genetic regulatory network that controls its manifestation (Yeh et al., 2012). Currently, we are progressing in explaining the mechanisms and regulatory control of thermotolerance in model species such as *Arabidopsis* (*Arabidopsis thaliana*), but given the diversity of thermotolerance phenotypes among different plant species, this may not be sufficient (Yeh et al., 2012). Elucidating species-specific thermotolerant mechanisms will greatly benefit the development of thermotolerant cultivars.

Gene-editing platforms, particularly clustered regularly interspaced palindromic repeats (CRISPRs)/CRISPR-associated protein 9 (Cas9) and their derivatives, have been beneficial in generating novel thermotolerant phenotypes and functional elucidation. In plant breeding, the most

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mainstream gene-editing approach is to mutate the coding region of a candidate protein-coding gene in a targeted manner to generate phenotypic variability to select upon. To a lesser extent, the mutation usually results in abolished gene function (knockout), gain-of-function, or neofunctionalization. Unfortunately, negative pleiotropic effects may also result when targeting genes involved in complex gene networks.

An emerging approach in genome editing for crop improvement is targeting noncoding DNA, particularly *cis*-regulatory elements (CREs). These noncoding DNA segments regulate gene expression when interacted with *trans*-regulatory elements. Contrary to the mainstream approach of modifying gene products, this approach aims to quantitatively modify the levels and/or the temporal–spatial gene expression to achieve phenotypic diversity while at the same time minimizing or avoiding pleiotropic effects (Swinnen et al., 2016). The transcriptional rewiring associated with the selection of CRE variants during the domestication of our staple crop species (Swinnen et al., 2016) and the diverse phenotypic changes induced when editing CREs (reviewed in Saeed et al. (2022)) greatly emphasize the phenotypic contributions of CREs in particular and noncoding DNA in general, making a strong case for the utility of this approach in accelerating crop improvement.

This chapter will review approaches for improving thermotolerance by modifying CREs and other functional, noncoding DNA elements using genome-editing tools, particularly those derived from CRISPR/Cas9. Since thermotolerance is a quantitative polygenic trait tightly regulated by a complex regulatory network, we believe this approach could greatly benefit its development.

1.2 Impact of Climate Change and Heat Stress on Crop Productivity

1.2.1 A Physiological Impact of Heat Stress on Plant Growth and Development

Heat stress is a significant environmental factor affecting crop yield and quality. The definition of heat stress depends on the natural habitat and is species-specific (Yeh et al., 2012). Mild temperature increase generally induces plant development and early flowering and alters immunity (Hua, 2013; Verhage et al., 2014; Capovilla et al., 2015; Gangappa et al., 2017). Plants can adapt to suboptimal temperatures through thermomorphogenesis – a range of morphological adaptations, including hypocotyl elongation, upward leaf movement (thermonasty), petiole elongation, reduced stomatal density, and formation of smaller and thinner leaves (Quint et al., 2016; Casal & Balasubramanian, 2019). Eventually, plants can cool themselves through open rosette structures and transpiration (Crawford et al., 2012; Park et al., 2019).

Extreme temperatures can cause irreversible plant damage, significantly affecting crop development and profitability and seriously threatening national and global food security (Lesk et al., 2022). Temperature stress disrupts photosynthesis, water metabolism, nutrient cycling, protein synthesis, reproduction, and the functionality of various enzymes, phytohormones, pollen development, and signaling molecules, resulting in sizeable reductions in yields (Crafts-Brandner & Salvucci, 2002; Zinn et al., 2010; Mishra et al., 2023). Molecular mechanisms of thermosensing and signaling have been primarily explored in *Arabidopsis* with the basic Helix-Loop-Helix (bHLH) transcription factor (TF) PHYTOCHROME INTERACTING FACTOR 4 (PIF4) considered as the core of temperature signaling pathways (Gangappa et al., 2017; Casal & Balasubramanian, 2019). One of the key downstream targets of the PIF4 pathway involved in promoting growth is *YUCCA8* (*YUC8*), which encodes a rate-limiting enzyme in auxin biosynthesis and is critical for thermomorphogenesis (Franklin et al., 2011; Lee et al., 2014).

At the same time, the PIF4-independent signaling pathways (PIF4 and PIF7 alike) were discovered to directly stimulate auxin biosynthesis and trigger thermomorphogenesis in a brassinosteroid-dependent manner (Chung et al., 2020; Fiorucci et al., 2020; Vu et al., 2021). The active form of the photoreceptor phytochrome B (phyB) senses elevated temperature and is converted from the active Pfr form to the inactive Pr form. The nuclear export of the phyB-Pr upon warmth releases PIF4 inhibition, initiating thermomorphogenesis (Legris et al., 2016; Qiu et al., 2019). Additionally, other mechanisms of temperature sensing were reported in *Arabidopsis*. The translation of *PIF7* mRNA is enhanced by elevated temperature through the relaxation of the *PIF7* mRNA hairpin structure, leading to the accumulation of PIF7 protein (Chung et al., 2020). Also, the transcriptional repressor EARLY FLOWERING 3 (ELF3) aggregates into inactive condensates during warming, contributing to sensing elevated temperatures (Jung et al., 2020).

Heat can damage photosynthetic machinery by inhibiting enzymes like RUBISCO and denaturing chloroplastic proteins. This leads to stomatal closure, limiting CO_2 uptake and resulting in the buildup of reactive oxygen species (ROS), which cause cellular damage, affecting lipids, proteins, and nucleic acids (Allakhverdiev et al., 2008; Wang et al., 2018). Heat stress significantly impacts nutrient cycling and plant nutrition. High temperatures can impair root function, reducing the plant's ability to uptake essential nutrients like nitrogen, phosphorus, and potassium. For instance, in tomato plants, heat stress has been found to decrease levels of nutrient-uptake and -assimilation proteins in roots, leading to reduced nutrient absorption (Giri et al., 2017). Additionally, soil warming can accelerate nutrient cycling, resulting in nutrient losses due to faster decomposition rates and reducing nutrient availability in the long term. High temperatures also interfere with plant nutrient translocation, further complicating metabolic processes essential for growth and productivity (Mishra et al., 2023). At the cellular level, heat stress can cause protein denaturation, membrane destabilization, and oxidative stress. Persistent heat stress may result in cell death and tissue necrosis, compromising the overall health and productivity of the plant (Haider et al., 2021). These changes lead to the accumulation of ROS, which can damage cellular components like lipids, proteins, and nucleic acids. Enzymes lose efficiency as temperatures exceed their optimal range; continued stress triggers phytohormonal imbalances, such as altered abscisic acid levels, which affect plant responses. These imbalances activate complex signaling cascades involving calcium, ROS, and other molecules, further challenging the plant's ability to cope with stress (Potters et al., 2007; Saidi et al., 2009; Devireddy et al., 2021).

1.2.2 Heat Shock Proteins (HSPs) and Their Roles

HSPs are a crucial component of a plant's response to heat stress, playing key roles in maintaining protein integrity and enabling cellular homeostasis under stressful conditions. These molecular chaperones assist in the folding, refolding, transport, and degradation of proteins, preventing their denaturation and aggregation caused by heat stress (Feder & Hofmann, 1999). They are often regulated by heat shock factors (HSFs), which are activated during extreme temperatures. In wheat, *HSFs* such as *HSFA2* and *HSFA6* are activated during heat stress, modulating downstream responses to improve heat tolerance (Xue et al., 2014). Once induced, HSPs bind to misfolded proteins, aid in refolding, and prevent the formation of toxic aggregates, playing a significant role in cellular survival under heat stress (Wu, 1995). Throughout the day, varying temperatures trigger the production and accumulation of different HSPs in plants. In the mid-morning, HSP20s are synthesized to prevent proteins from misfolding and aggregating before the temperature peaks by noon. Later in the day, as temperatures rise further, the production shifts to HSP60s, HSP70s, HSP90s, and HSP100s, which help resolubilize and reactivate proteins that may have become

inactive or misfolded due to heat stress, ensuring their proper functioning (Finka et al., 2016). For instance, overexpressing certain HSPs in some crops like rice (HSP18), barley (HSP70, HSP90, and HSP100), *Arabidopsis* (HSP101), and cotton (HSP70-26) improved resilience, making them valuable targets in breeding programs for heat tolerance (Queitsch et al., 2000; Kuang et al., 2017; Chaudhary et al., 2019; Zhiyong et al., 2021). Modifying the expression of HSFs and other directly related noncoding DNA regions that regulate HSPs could be a strategic approach to enhance the overexpression of key HSPs, ultimately developing heat-resilient plants.

1.2.3 Epigenetic Regulation of Stress Response Pathways (Histone Posttranslational Modifications, DNA Methylation, and ncRNA Expression)

Recent evidence suggests that epigenetic mechanisms of gene expression and regulation are actively involved in thermosensing and heat stress tolerance in plants. Epigenetics, in a broader sense, refers to "chromatin modifications" through chemical modifications of histone proteins or DNA wrapped around them that do not change the base sequence (Deichmann, 2016). The regulation of gene expression occurs through different pathways, including DNA methylation, small RNAs, ATP-dependent chromatin remodeling, histone variants, histone modifications, histone chaperones, and long noncoding RNAs (lncRNAs). Some of these pathways regulate the expression of high-temperature-responsive genes to prevent heat-related damage and promote subsequent adaptation (reviewed in Perrella et al. (2022)). For example, the expression of many thermoresponsive genes is regulated by the repressive histone variant H2A.Z deposited in nucleosomes of temperature-regulated loci primarily at the +1 site (Kumar & Wigge, 2010). The deposition and eviction of repressive H2A.Z in exchange for permissive H2A variant are performed by the Snf2 ATPase remodeling complexes, SWR1-C and INO80-EIN6 ENHANCER (EEN), respectively (Xue et al., 2021). A component of SWR1-C, ACTIN-RELATED PROTEIN 6 (ARP6), is a mediator of temperature responses in *Arabidopsis,* and *arp6* mutants demonstrate elongated hypocotyls already at low temperatures, indicative of a constitutive warm temperature phenotype (Kumar & Wigge, 2010). INO80 and EEN are directly associated with PIF4 in activating the transcription of auxin-related genes under elevated temperatures through the H2A.Z eviction. The contribution of H2A.Z to stress-induced gene activation was further supported by the fact that its eviction was compromised in both *pif4* and *ino80* mutants. Overall, the H2A.Z-containing nucleosomes are not temperature sensors per se, but rather their presence depends on transcriptional regulators allowing for environment-dependent chromatin reorganization and release of stress-responsive gene expression (Cortijo et al., 2017).

In addition to chromatin remodeling, histone methylation and histone acetylation marks have been implemented to regulate the gene expression of growth-promoting genes in response to heat stress. For example, histone H3K4me2 (an activating mark) is demethylated in response to the binding of FLOWERING CONTROL LOCUS A (FCA) to PIF4-activated growth-promoting genes, like *YUC8*, therefore eventually suppressing high-temperature-induced hypocotyl elongation (Lee et al., 2014). Therefore, *fca* mutants with a continuous expression of the *YUC8* gene display hyperelongated hypocotyls when exposed to 28 °C. At the same time, the H3K4me3 (considered an activating mark) demethylation of double mutant Jumonji C (JmjC) JMJ14 with its cofactor-producing enzyme cytosolic isocitrate dehydrogenases (cICDHs) showed suppression of several auxin-related genes, including *YUC8*, and resulted in reduced thermomorphogenesis capacity (Cui et al., 2021). These results suggest that distinctive histone demethylases can have either negative (FCA) or positive (JMJ14, JMJ15, and JMJ18) effects on the genes involved in thermomorphogenesis. Histone methylation can also affect alternative splicing (AS), and H3K36me3 enrichment was associated