Effective Strategies for Enhancing Tolerance to High-Temperature Stress in Rice during the Reproductive and Ripening Stages

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ABSTRACT Temperatures that extend beyond normal levels of tolerance cause severe stress to plants, especially during the reproductive and grain filling/ripening stages. Heat stress leads to serious yield losses in many crop plants, including rice (*Oryza sativa*). In view of the current scenario of global climate change, frequent fluctuations and a significant increase in average temperatures will pose challenges to protecting those yields. Therefore, elucidating the molecular mechanisms that make crop plants more tolerant of heat, particularly in organs at the reproductive stage, is of utmost importance. Precise molecular information will be helpful for the manipulation and exploration of relevant genes for use in crop improvement programs. In this review, we highlight recent progress in research on the molecular responses to high temperatures in pollen and seed and provide a perspective on the development of heat tolerance in rice cultivars. The responsible mechanism is a very complex phenomenon that involves several biochemical and physiological changes, molecular responses, and a series of signal transductions. Improving our understanding requires detailed knowledge at various omics levels. Recent technological advancements have accelerated genomics, transcriptomics, and proteomics studies in rice, a model crop plant. Here, we discuss those technological and omics approaches being taken to investigate the heat tolerance mechanism, particularly in rice. In addition, we address the tools being used to identify key genes and QTLs that can then be utilized for molecular breeding and biotechnology.

Keywords Heat stress tolerance, Pollen development, Rice, Seed development, Spikelet fertility

INTRODUCTION

Rice (*Oryza sativa*) is a staple food crop worldwide, upon which almost half of the population depends. To meet growing demands by consumers there as well as in other parts of the world, annual grain yields must increase by 0.6 to 0.9% until 2050 (Carriger and Vallee 2007). However, it is difficult to combat the challenges associated with situations such as an ever-changing climate, decreases in

the amount of agricultural land available per capita, and greater frequencies of biotic and abiotic stresses. The primary concern is global warming and unpredictable temperature fluctuations. Conventional rice-growing areas feature relatively higher day and night temperatures (28/22°C) that are more strongly influenced by climate change (Prasad *et al.* 2006). A mathematical model has projected a 2.0 to 4.5°C increase in the global temperature by the end of the century (IPCC 2007). Those predictions

Received November 27, 2017; Revised November 27, 2017; Accepted November 27, 2017; Published March 1, 2018

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are even more intimidating for rice-growing regions such as Asia, the Middle East, and Africa. If the average temperature were to rise by 3 to 4°C in those areas that would lead to a 15 to 35% reduction in crop yields (Ortiz *et al.* 2008).

High-temperature stress (HTS) alters several physiological and metabolic processes in cells. The vulnerability of plants to HTS differs according to developmental stage, but it affects both vegetative and reproductive phases to some extent. Those effects depend upon species and genotype, and an ample amount of inter- or intraspecific variations can occur (Sakata and Higashitani 2008). Various morphological responses have been observed, e.g., wounding and aging of leaves; inhibition of leaf, stem, root, and shoot growth; a reduction in seed germination rates; and unevenness in seed maturation. Many species undergo structural changes to their hypocotyls and floral organs to avoid the consequences of high temperatures (Tian *et al.* 2009). Likewise, HTS affects the total assimilation rate in the shoot, which then contributes to a decrease in whole-plant dry weight (Wahid *et al.* 2007). Other physiological adjustments include the production of reactive oxygen species, increased water loss, greater membrane permeability, and decreased photosynthesis. Although these physiological effects have been evaluated in some plants (Wahid *et al.* 2007; Bokszczanin *et al.* 2013; Mathur *et al.* 2014), HTS responses in rice have not received much focus, and only a few studies have examined these responses at the flowering stage or in reproductive organs (Jagadish *et al.* 2010b; Rang *et al.* 2010).

The mechanism for heat tolerance has been elucidated in various crops (Iba *et al.* 2002; Chinnusamy *et al.* 2007; Kotak *et al.* 2007; Wahid *et al.* 2007; Zinn *et al.* 2010; Giorno *et al.* 2013; De Storme and Geelen 2014). Rice plants are most sensitive to high temperatures at the reproductive (anthesis) stage (Yoshida *et al.* 1981), and frequent increases in temperature above the critical level of 33°C result in spikelet sterility and serious losses in grain yields (Nakagawa *et al.* 2002). Therefore, the rice reproductive organs can be severely affected by HTS depending upon the duration and the extent of the stress (Wahid *et al.* 2007; Jagadish *et al.* 2013).

Here, we describe the recent progress made in research

that addresses these high-temperature influences during the reproductive and grain-filling/ripening stages in rice. We summarize the current understanding about the molecular and genetic events involved in male and female organ development under HTS, while presenting omics-scale studies and advanced breeding strategies being used to improve HTS tolerance. Key candidate genes, including transcription factors (TFs), and the molecular pathways involved in this mechanism, particularly during the reproductive stages, are also discussed, along with genetic engineering and molecular breeding approaches that are being applied to produce rice that is tolerant of high temperatures.

Effect of high-temperature stress on pollen and anther development

In flowering plants, sexual reproduction usually requires flowers that produce male and female gametophytes. This process can be restricted because formation of the male gametophyte (pollen) is more sensitive to the growing environment (Ahmed *et al.* 1992; Peet *et al.* 1998; Kim *et al.* 2001; Rieu *et al.* 2017). Pollen develops inside locules from diploid pollen mother cells that undergo cell division (meiosis) to generate four haploid microspores in the locular fluid. Each microspore then asymmetrically divides to form a vegetative cell (VC) and generative cell (GC) during pollen mitosis I. Small GCs are subsequently engulfed within the cytoplasm of the larger VC and further cell division occurs, i.e., mitosis (pollen mitosis II), to form two sperm cells (McCormick 2004). During this process, the tapetum is generated from the innermost anther wall (Goldberg *et al.* 1993). The tapetum plays an important role at the early microspore stage, providing the nutrients, carbohydrates, lipids, and enzymes necessary for synthesis of the outer pollen wall. This process is closely associated with microspore development, and the tapetum degenerates soon after the microspore is released from the tetrad. Therefore, proper tapetal functioning and its timely degradation are crucial components in pollen development (Rieu *et al.* 2017).

Abiotic-stress conditions such as HTS have a severe impact on pollen and anther development. Various reproduction-related events affected by heat stress are

shown in Fig. 1. Analysis of tapetum sensitivity, based on transcriptome studies of rice, has shown how the pattern of expression by heat-repressed genes can vary in tapetal tissue (Endo *et al.* 2009). Pollen tends to be more sensitive than the female gametophyte to HTS across plant species, including chickpea (*Cicer arietinum*) (Clarke and Siddique 2004; Devasirvatham *et al.* 2012, 2013; Kaushal *et al.* 2013), rice (Sakata and Higashitani 2008; Wassmann *et al.* 2009), tomato (*Lycopersicon esculentum*) (Giorno *et al.* 2013), maize (*Zea mays*) (Herrero and Johnson 1980), and *Vigna unguiculata* (Ahmed *et al.* 1992). When compared with other cereal crops such as maize and wheat (*Triticum aestivum*), rice is relatively more sensitive to HTS ($> 35^{\circ}\text{C}$), mainly during the stages of flowering (Prasad *et al.* 2006; Jagadish *et al.* 2007, 2008, 2010b) and gametogenesis (Jagadish *et al.* 2013). Under such high temperatures, microspores are aborted and pollen viability is reduced during anthesis, subsequently causing spikelet sterility (Cheng *et al.* 2009; Mohammed and Tarpley 2009a, 2009b, 2010). The relationship between heat stress and spikelet sterility has been reported in rice (Nguyen *et al.* 2013; Oort *et al.* 2014) and other crop species (Oshino 2007; Sakata *et*

al. 2008). As demonstrated in several investigations, HTS can severely alter the morphology, structure, and functions of male gametophytes. Because pollen quality is the most crucial factor in determining crop yields during periods of heat stress (Firon *et al.* 2006; Rang *et al.* 2010), the results of analyses on pollen viability and spikelet fertility under elevated temperatures can help improve our understanding of how heat tolerance is conferred in rice.

Effect of high-temperature stress on pistil development

As part of the reproductive process, pistils, the maternal organ, form in the flower, providing nutrition and protection to the female gametophyte while also supporting the male gametophyte. Although this organ is considered more tolerant of HTS than the male gametophyte (Hedhly *et al.* 2011), the effect of such stress on the sporophytic tissue of the pistil has not been widely studied. Nevertheless, high temperatures influence the pistil both pre-and post-pollination as well as during the stages of embryo development, silique formation, and seed set. Species can vary in their sensitivity to heat, based on genotype and the

Fig. 1. Effect of high temperature on flowering and reproductive phases.

temperatures to which they are exposed. In crops such as *Prunus armeniaca* (Rodrigo and Herrero 2002), HTS causes shorter styles and abnormal ovary growth. Similarly, post-pollination defects in the female gametophyte have been reported in chickpea (Srinivasan *et al.* 1999), and in rapeseed (*Brassica napus* L.) (Young *et al.* 2004). In many species, the impact of HTS can be more detrimental on microsporogenesis than on macrosporogenesis, and the receptivity of pollen on stigmas can be reduced, leading to failed fertilization (Srinivasan *et al.* 1999). For *Prunus persica* L., elevated temperatures mean that pollen germination and pollen tube growth cannot be supported (Hedhly *et al.* 2005). Reports have been made about ovary defects in wheat (Saini *et al.* 1983) and in Arabidopsis, where HTS is associated with a reduction in the number of ovules as well as more abortions (Whittle *et al.* 2009). In rapeseed, increasing the growing temperature from 26°C to 32°C is linked with defects of the embryo sac (Polowick and Sawhney 1988). Elevated temperatures cause pistil hyperplasia in rice, which leads to spikelet sterility and stamen hypoplasia (Takeoka *et al.* 1991).

Effect of high-temperature stress on developing seeds

Both monocotyledonous and dicotyledonous plants are affected by HTS (Herrero and Johnson 1980; Saini *et al.* 1983; Carlson 1990; Nuttal *et al.* 1992; Morrison 1993; Peet *et al.* 1998; Angadi *et al.* 2000; Sato *et al.* 2002). Heat stress that occurs during the period of seed development can decrease grain size for many cereal crops (Nagato and Ebata 1960; Hunter *et al.* 1977; Savin *et al.* 1996). In rice, the intricate process of reproductive differentiation and degeneration has three genetic components: diploid maternal tissues (e.g., pericarp, testa, and nucellus), triploid endosperm, and diploid embryos (Wu *et al.* 2016a, 2016b). There, the early stage of seed development is highly sensitive to heat stress (Folsom *et al.* 2014). Because the endosperm accounts for most of the rice caryopsis, any defect in it can affect the final seed size. Formation of the rice endosperm requires distinct developmental processes, i.e., syncytial nuclear division, cellularization, differentiation, and maturation (Olsen 2001; Sabelli and Larkins 2009; Wu *et al.* 2016a). Shrunken endosperm results from a noticeable reduction in starch synthesis (Lee *et al.* 2007).

The duration of the syncytial phase and the timing of cellularization are critical because they are correlated with the extent of nuclear proliferation and may influence grain filling and grain weight (Brown *et al.* 1996; Mitzutani *et al.* 2010). Heat stress is linked with diverse types of chalky rice grains, including white-black, white-belly basal white, and milky-white (Wada *et al.* 2015). Folsom *et al.* (2014) have demonstrated that initiation of endosperm cellularization is controlled by a rice Polycomb Repressive Complex2 member, Fertilization-Independent Endosperm 1 (*OsFIE1*), and can be influenced by HTS. This gene is maternally expressed and endosperm-imprinted (Zhang *et al.* 2012). Its expression increases after fertilization due to demethylation, which is associated with the initiation of endosperm cellularization (Folsom *et al.* 2014; Chen *et al.* 2016). When developing seeds are exposed to elevated temperatures, changes occur in both the methylation level of the 5' coding region of *OsFIE1* and its H3K9me methylation mark (Folsom *et al.* 2014). These reports suggest that *OsFIE1* is a potential component involved in regulating seed enlargement under heat stress. Other genes, such as rice *AGL36* and the orthologs of *Pheres1*, *OsMADS82*, and *OsMADS87*, are epigenetically repressed by *OsFIE1*. Chen *et al.* (2016) have identified putative imprinted genes during the endosperm transition from syncytium to the cellularization stage in young heat-stressed seeds. Such conditions promote a mis-timed release of imprinting from a subset of seed-specific genes in rice, thereby leading to the thermal resilience of rice during reproductive development. Indeed, one heat-sensitive imprinted gene, *OsMADS87*, a rice homolog of the Arabidopsis endosperm-specific *PHE1* (Köhler *et al.* 2005), directly regulates rice seed size (Chen *et al.* 2016).

When regions in diverse climates experience ever-changing temperatures during the reproductive stage, seed development and fruit set are altered. Although HTS can reduce seed production in various species, it remains unclear whether this is an outcome of decreased flowering, fruit abortion, or disruptions in fertilization or post-fertilization events. These impacts of HTS can be examined through approaches that utilize microarrays, RNA sequencing (RNA-Seq), or screening of genes that are expressed during the post-fertilization process. The results

of such research can be used to improve our understanding of the heat-stress response in developing rice seeds and the impact of HTS on crop yields.

Functional characterization of rice genes that confer tolerance to high-temperature stress

Because the rice genome is well-annotated, it provides a

Table 1. Summary of recent researches in rice for increased high temperature stress tolerance.

Gene	Gene symbol	Plant species	Methods	Known functions	Reference
Glycogen synthase kinase3-like gene 1	OsGSK1	Rice	Mutant	Cold, drought, salinity and heat tolerance	Koh <i>et al.</i> 2007
Oswrky11	OsWRKY11	Rice	Overexpression	Drought and heat tolerance	Wu <i>et al.</i> 2009
Sedoheptulose-1,7-bisphosphatase	SBPase	Rice	Overexpression	Photosynthetic ability under high temperature condition	Feng <i>et al.</i> 2007
Mitochondrial heat shock protein 70	mtHsp70	Rice	Overexpression	Heat and redox induced programmed cell death in protoplast	Qi <i>et al.</i> 2010
Small heat-shock protein17.7	sHSP17.7	Rice	Overexpression	Heat and UV tolerance	Murakami <i>et al.</i> 2004
Hsf binding protein 2	OsHSBP2	Rice	Knockdown Overexpression	Heat shock tolerance	Rana <i>et al.</i> 2012
Hsf binding protein 1	OsHSBP1	Rice	Knockdown Overexpression	Heat shock tolerance.	Rana <i>et al.</i> 2012
Osmyb55	OsMYB55	Rice	Overexpression	Growth under high temperature	El-kereamy <i>et al.</i> 2012
Rice carbon catabolite repressor 4(ccr4)-associated factor 1g	OsCAF1G	Rice	Others	ABA, heat stress tolerance	Chou <i>et al.</i> 2014
Rice carbon catabolite repressor 4(ccr4)-associated factor 1h	OsCAF1H	Rice	Others	Heat stress tolerance	Chou <i>et al.</i> 2014
Small heat shock proteins (shsps) oshsp16.9	HSP 16.9	Rice	Overexpression	Drought and cold, salt and heat tolerance	Jung <i>et al.</i> 2014
A 2 subunit 26s proteasome	TT1 (Os03g0387100)	Rice	Overexpression	Heat stress tolerance	Li <i>et al.</i> 2015b
Manganese superoxide dismutase	SOD 1 (MSD1)	Rice	Overexpression	Heat stress tolerance	Shiraya <i>et al.</i> 2015
Spl7	HSFA4d	Rice	Overexpression	Heat stress tolerance	Yamanouchi <i>et al.</i> 2002
Receptor-like kinase erecta	ERECTA	Rice	Overexpression	Heat stress tolerance	Shen <i>et al.</i> 2015
Hsp101	AtHsp101	<i>A. thaliana</i>	Overexpression	Heat stress tolerance	Katiyar-Agarwal <i>et al.</i> 2003
Fatty acid desaturase	FAD7	<i>A. thaliana</i>	Overexpression	Heat stress tolerance	Sohn <i>et al.</i> 2007
Polycomb group protein	FIE1	<i>A. thaliana</i>	Overexpression	Heat stress tolerance	Folsom <i>et al.</i> 2014
Stress-responsive nac gene	SNAC3	Rice	Overexpression	Heat stress tolerance	Fang <i>et al.</i> 2015

basis for comparative genomics among crop species. Many genes have been identified in rice through multiple approaches, including microarrays and RNA-Seq, proteomics data, protein-protein interactions, and genome-wide gene-indexed mutant populations (Chandran and Jung 2014). These data are helpful for predicting gene functions. The information made publicly available from genetic analyses, gene-indexed mutants, or transgenic plants have enabled us to learn the functions of more than 1,000 genes in rice (Yamamoto *et al.* 2012). Further progress can be made based on high-throughput omics data. For example, one important resource for functional genomics is provided by the International Rice Functional Genomics Consortium (IRFGC), which has generated gene-indexed mutants that can facilitate functional annotations for most rice genes. All of the information about functionally characterized genes can be accessed using the Overview of functionally characterized Genes in Rice Online database, or OGRO (Yamamoto *et al.* 2012; <http://qtaro.abr.affrc.go.jp/ogro>). Table 1 presents the genes that have been identified as responsible for HTS tolerance, based on functional characterization and validation through overexpression analysis, mutant screening, and transgenic approaches.

Newly identified rice genes for heat-stress tolerance that are expressed in the reproductive and grain filling stages

Several studies involving expression profiling have identified genes that are induced by environmental stresses (Rabbani *et al.* 2003; Bray 2004; Yamaguchi-Shinozaki and Shinozaki 2006; Zhang *et al.* 2012). Using microarrays, gene expression at the transcriptome level has been monitored in the reproductive and ripening tissues of heat-sensitive ‘Nipponbare’ rice and the heat-tolerant ‘996’ (Endo *et al.* 2009; Zhang *et al.* 2012). Furthermore, RNA-Seq has been conducted to examine the reproductive tissues during anthesis for heat-tolerant ‘N22’ rice and the sensitive cultivars ‘IR64’ and ‘6264’ (González-Schain *et al.* 2016). Endo *et al.* (2009) have found that, under HTS conditions, the expression of more than 1,400 genes is significantly changed in the anthers from ‘Nipponbare’ plants. Similarly, Zhang *et al.* (2012) have described

almost 2,500 heat-responsive genes in young florets during meiosis. González-Schain *et al.* (2016) have reported that, among 630 heat-responsive genes, a relatively small number (37) are core heat-responsive. Under combined drought and heat stress, 20 genes are up-regulated in pollinated rice pistils (Li *et al.* 2015a). Table 2 shows the heat-responsive genes expressed in rice reproductive and ripening tissues that can potentially be used to improve HTS tolerance in that crop. The large number of differentially expressed genes (DEGs) suggested by expression profiling will be useful in future studies that can expand our understanding of the overall molecular responses and probable pathways involved in the stress response. For example, Endo *et al.* (2009), Zhang *et al.* (2012), and González-Schain *et al.* (2016) have determined that TFs are over-represented among the DEGs that respond to heat stress during the reproductive stage in rice. Genes that regulate the pathways for sugar and amino acid metabolism in rice also have important roles in heat-stress tolerance (Li *et al.* 2015a). Therefore, these recent reports of numerous DEGs in rice can help us interpret the interactions and possible involvement of these key reproduction-related genes that might also confer HTS tolerance.

Chalkiness and smaller-than-normal grains are typical of rice exposed to heat stress during the stage of caryopsis maturation. Such symptoms have been reported in transgenic and mutant rice with decreased or deficient expression of various starch synthesis-related genes, including those for a sucrose transporter (*SUT1*), invertase (*CIN2*), UDP-glucose pyrophosphorylase (*UGPase*), ADP-glucose pyrophosphorylase (*AGPS*), soluble starch synthase (*SSIIIa*), and branching enzyme (*BEIIb*) (Nishi *et al.* 2001; Kawagoe *et al.* 2005; Fujita *et al.* 2007; Wang *et al.* 2008; Woo *et al.* 2008; Ito *et al.* 2009). Transcriptomic profiling has revealed that high temperatures down-regulate the expression of those genes as well as various others involved in sucrose import/degradation and starch biosynthesis while up-regulating genes for starch degradation (Yamakawa *et al.* 2007; Yamakawa and Hakata 2010). Transgenic rice plants that over-express amylase genes also display the chalky endosperm phenotype, suggesting that premature autolysis of starch

Table 2. Summary of heat stress-responsive core genes in reproductive tissues of rice.

MSU-LOCUS ID	RAPDB-LOCUS ID	Description	References
Transcription factors			
LOC_Os02g52190	Os02g0759000	Helix-loop-helix DNA binding Protein	Mittal <i>et al.</i> 2012
LOC_Os09g35790 ²⁾	Os09g0526600	Hsfb2c	Mittal <i>et al.</i> 2012
LOC_Os07g48550	Os07g0684800	No apical meristem protein	Gonzalez-Schain <i>et al.</i> 2016
Chaperones			
LOC_Os01g08860	Os01g0184100	Hsp20/alpha family protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g16860	Os03g0276500	Dnak family protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os01g42190 ²⁾	Os01g0606900	Heat shock protein dnaj	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os02g52150 ²⁾	Os02g0758000	Heat shock 22 kda protein, mitochondrial precursor	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os02g54140 ²⁾	Os02g0782500	Hsp20/alpha crystallin family protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g14180 ²⁾	Os03g0245800	Hsp20/alpha crystallin family protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g16040 ²⁾	Os03g0267200	Hsp20/alpha crystallin family protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os04g01740 ²⁾	Os04g0107900	Heat shock protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os04g36750 ²⁾	Os04g0445100	Sp20/alpha crystallin family protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os04g45480 ²⁾	Os04g0538000	Heat shock protein STI	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os05g44340 ²⁾	Os05g0519700	Oscplp-cyt	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os06g09560 ²⁾	Os06g0195800	Heat shock protein dnaj	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os11g13980 ²⁾	Os11g0244200	Hsp20/alpha crystallin family protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
Transporters			
LOC_Os01g17214	Os01g0279400	Major facilitator superfamily antiporter	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os02g50680 ²⁾	Os02g0740300	AAA-type atpase family protein	Mittal <i>et al.</i> 2012
LOC_Os03g24870	Os03g0363600	Transporter family protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g46440	Os03g0667100	BTBA4 - Bric-a-Brac, Tramtrack, Broad Complex BTB domain with Ankyrin repeat region	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os06g39260 ²⁾	Os06g0593100	Solute carrier family 35 member B1	Mittal <i>et al.</i> 2012
Cell Wall Modification			
LOC_Os01g24710	Os01g0348900	Jacalin-like lectin domain containing protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os02g44108	Os02g0658800	Expansin precursor	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os09g36060	Os09g0530200	Endoglucanase	Gonzalez-Schain <i>et al.</i> 2016
None	Os11g0702100	Similar to Class III chitinase homologue (oschib3hh) (Fragment)	Gonzalez-Schain <i>et al.</i> 2016
Metabolism			
LOC_Os01g07530 ²⁾	Os01g0170000	Uncharacterized glycosyltransferase	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os01g27360 ²⁾	Os01g0371200	Glutathione S-transferase	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016

Table 2. Continued.

MSU-LOCUS ID	RAPDB-LOCUS ID	Description	References
LOC_Os03g59430 ²⁾	Os03g0808900	Uncharacterized glycosyltransferase	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os06g10510	Os06g0206900	Oxidoreductase transition metal ion binding protein	Gonzalez-Schain <i>et al.</i> 2016
Calcium			
LOC_Os01g55270 ²⁾	Os01g0757500	SGS domain containing protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os01g59530	Os01g0810300	Oscml1 - Calmodulin-related calcium sensor protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os02g15930 ²⁾	Os02g0259900	BAG6-like	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
RNA			
LOC_Os02g40900	Os02g0622500	RNA recognition motif containing protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g21160	Os03g0329200	RNA-binding zinc finger protein	Gonzalez-Schain <i>et al.</i> 2016
Others			
LOC_Os03g16460 ²⁾	Os03g0271400	Expressed protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016
LOC_Os03g17790	Os03g0286900	OsRCI2-5 - Putative low temperature and salt responsive protein	Gonzalez-Schain <i>et al.</i> 2016
LOC_Os11g31740 ²⁾	Os11g0518600	Expressed protein	Mittal <i>et al.</i> 2012; Gonzalez-Schain <i>et al.</i> 2016

²⁾Heat-responsive genes from seedlings (Mittal *et al.* 2012) and also found during anthesis (Gonzalez-Schain *et al.* 2016).

granules is one reason that this phenotype occurs for rice grains that ripen under high temperatures (Asatsuma *et al.* 2006). Examination of the rice grain proteome under HTS has revealed differential expression (up- or downregulation by more than 2-fold) for 61 genes implicated in diverse metabolic and physiological functions, such as protein synthesis, redox homeostasis, lipid metabolism, and starch biosynthesis/degradation (Kaneko *et al.* 2016). In addition to proteins related to starch metabolism, storage proteins also accumulate, accounting for up to 10% of the total dry matter and affecting the physicochemical characteristics of rice flour and brewing quality that are also differentially affected by heat stress. Meanwhile, accumulations of prolamin and globulin, but not of glutelin and albumin, are reduced under high temperatures (Yamakawa *et al.* 2007; Lin *et al.* 2010). Enhanced expression of small heat shock proteins or redox homeostasis-related genes under high temperatures is strongly correlated with the occurrence of chalky grains, as demonstrated in studies of various conventional cultivars and transgenic lines of rice (Lin *et*

al. 2005, 2014; Liu *et al.* 2010, 2011). Metabolomic analysis of the developing caryopsis has indicated that high temperatures decrease the levels of glucose, fructose, most sugar phosphates, and organic acids involved in glycolysis/gluconeogenesis and the TCA cycle while increasing the amounts of sucrose, UDP-glucose, and ADP-glucose (Yamakawa and Hakata 2010). Such metabolomic changes also closely parallel the inhibition of starch accumulations and transcriptomic alterations in the developing caryopsis under high temperature (Yamakawa *et al.* 2007; Yamakawa and Hakata 2010).

Research with genetic and transcriptome profiling has shown that the high temperature-induced decline in grain weight and quality is a very complex phenomenon that requires the interactions of various metabolic pathways, including those for sugar import/degradation, starch synthesis/degradation, storage protein synthesis, and maintenance of homeostasis. Several candidate genes involved in the mechanism for heat-stress tolerance have already been identified in many plant species. To elucidate

these multifaceted effects of high temperature on the various metabolic and physiological processes of filling grains, researchers must still investigate how such stress can crosstalk with the developmental program of seed ripening or metabolic regulation when the caryopsis is maturing.

Breeding strategy for introducing tolerance to high temperatures

Breeding approaches involve the improvement of wild species to produce elite or domesticated breeding lines that introduce novel alleles into desired crops. High-temperature tolerance is a polygenic and complex trait highly influenced by environmental variations, but the genetics behind such tolerance is less understood (Wahid *et al.* 2007; Collins *et al.* 2008; Ainsworth and Ort 2010). Due to uncontrolled environmental factors and the influence of additional biotic stresses, it can be difficult to select for high-temperature tolerance through conventional breeding. Therefore, better methods are needed for conducting more accurate greenhouse experiments. To gain further understanding, rice researchers in the past decade have looked to other tools for identifying the genes and quantitative trait

loci (QTLs) associated with HTS tolerance. The availability of the well-annotated genome sequence (IRGSP 2005) has enabled significant advances to be made in this relatively new genomics field. Marker assisted recurrent selection (MARS) and genomic selection (GS) are proving efficient for the development of novel cultivars in many crops (Varshney *et al.* 2009). Each tool has a potential advantage over marker-assisted back-crossing (MABC), which requires prior knowledge about the genetic markers associated with genes, QTLs, or alleles that affect whole-plant stress tolerance or contributing mechanisms. Both MARS and GS can also handle the problem of stacking minor alleles and QTLs. Although MARS breeding is widely used in the private sector (Ribaut *et al.* 2010), its application has not been reported in the public sector. The second most comprehensive approach for predicting the breeding value of an individual within a breeding population has been the GS method (Meuwissen *et al.* 2001). However, Collins *et al.* (2008) have demonstrated that relying upon complex traits, e.g., heat-stress tolerance, for crop improvement is not effective mainly due to genotype $\times$ environment and gene-gene (i.e., epistasis) interactions, which frequently result in low breeding

Fig. 2. General strategies used to breed HTS-tolerant plants.

efficiency. In general, monogenic or less complex traits are easier to predict with GS tools while traits such as stress responses are more complex and harder to estimate by the genomic estimated breeding value, or GEBV. When heat-stress tolerance, which has a quantitative hereditary characteristic, is the focus, then MARS seems to be one of the most efficient methods for plant breeding. After using MARS, Jain *et al.* (2014) have described improvements in drought and heat tolerance in wheat, while International Maize and Wheat Improvement Center (<http://www.cimmyt.org>) have started utilizing this approach in maize, and Spindel *et al.* (2015) have made strong advancements via MARS in rice.

Efforts to achieve HTS tolerance are still in the early phases. Fig. 2 presents several general strategies that are being explored. The complexity of this trait makes it difficult to obtain suitable breeding material using inbred lines and commercial hybrids. Progress might be accelerated by identifying and characterizing the genetic resources for HTS and screening a large population that covers various developmental stages (Wahid *et al.* 2007). An advanced molecular breeding approach would also be advantageous because it could provide data that would contribute significantly to the development of HTS-tolerant rice plants.

Future directions in studying the mechanism for high-temperature tolerance in rice

Abundant information is available regarding the physiological and metabolic traits that define HTS tolerance. However, little is known about the responsible mechanism(s). With the goal of improving crop yields under various stress conditions, two standard biotechnological approaches have been taken: development of transgenic plants and advanced molecular breeding.

Transgenics tools have become more efficient for introducing novel traits for biotic and abiotic stresses while also annotating candidate genes for molecular functions and regulation. Tables 1-3 show the genes, TFs, and QTLs already identified in rice through various means. Genetic engineering can also be applied to manipulate genes from other species that might be transferred to an heterologous system. Katiyar-Agarwal *et al.* (2003) have demonstrated

enhanced heat tolerance in a transgenic line of 'Pusa Basmati 1' rice by over-expressing an Athsp101 protein. Their transgenic plants are able to survive being exposed to severe heat stress, i.e., 45 to 50°C, and they show vigorous growth during subsequent recovery at 28°C. Shen *et al.* (2015) have reported that overexpression of the *Arabidopsis thaliana* receptor-like kinase *ERECTA* (*ER*) improves thermotolerance in *Arabidopsis*, rice, and tomato. A transgenic rice line that over-expresses *ER* has 55 to 70% higher seed set when compared with the non-transformed control line. Similarly, Li *et al.* (2015b) have indicated that a natural allele of a proteasome alpha 2 subunit gene can contribute to thermotolerance and adaptation by African rice species. Fang *et al.* (2015) have described a stress-responsive NAC TF, *SNAC3*, that confers heat and drought tolerance in rice. Furthermore, when that gene is knocked out, the transgenics become susceptible to HTS. However, despite these reports of transgenic heat-tolerant rice, less effort has been applied in that research area when compared with the efforts made to develop lines that are salt-, cold-, or drought-tolerant.

Heterologous expression of genes in transgenic plants is highly influenced by the choice of promoter used to regulate the transgene. Several well-characterized promoters that provide ubiquitous or tissue-specific gene expression are available for rice. Jeong and Jung (2015) have reviewed techniques that utilize a tissue-specific promoter rather than constitutive and spatio-temporal expression to exploit an effect of the transgene on transgenic plants. However, such approaches have not been widely applied in crop biotechnology and require further evaluation if they are to be successful in crop improvement. Genetic engineering provides great promise for enhancing plant tolerance to emerging abiotic stresses, and it can have a significant impact on the manipulation of innate genetic potential. In rice, this technology is still in the early phase but might possibly be used to transfer multiple genes for abiotic-stress tolerance. Additional knowledge about tolerance modules, including the identification of tissue-specific promoters and responsive genes, might enable the transformation of rice plants with multiple genes. Only a few field tests with transgenic rice have been reported. Before conducting such studies under field conditions,

Table 3. Compilation of mapped QTLs and major genes tested for high temperature stress resistance.

Trait	Plant species	QTL population	Chromosome	Reference
Photosynthetic rate and heat tolerance tillering and heading	Rice	IR64 × Azucena	6	Cao <i>et al.</i> 2003
Heat-tolerance at grain filling	Rice	Nipponbare/Kasalath// Nipponbare	3	Zhu <i>et al.</i> 2005
Thermo-tolerance of amylose content and gel consistency	Rice	Nipponbare/Kasalath// Nipponbare	9	Zhu <i>et al.</i> 2006
High temperatures associated with white-back kernels during ripening	Rice	Hana-echizen × Niigata-wase	4	Tabata <i>et al.</i> 2007
White-back and basal-white kernels under high temperature stress	Rice	Hana-echizen × Niigata-wase	2	Kobayashi <i>et al.</i> 2007
Heat tolerance of the tassel period	Rice	RIL (Zhongyouzao No. 8 × Fengjin)	3	Zhang <i>et al.</i> 2008
Filled grains per panicle and grain yield	Rice	F2 (996 × 4628)	2	Zhang <i>et al.</i> 2009
High temperature Tolerance at seeding stage	Rice	F1 and F2 (HT54 × HT13)	1	Wei <i>et al.</i> 2013
Heat response in seedling stage.	Rice	Introgressed line YIL106 (Teqing × <i>O. rufipogon</i>)	5	Lei <i>et al.</i> 2013
Filled grains per panicle, unfilled grain percentage	Rice	BC2F2 (OM5930 × N22)	4+2+1+1+1	Buu <i>et al.</i> 2014
Heat tolerance in spikelet fertility	Rice	Bala × Azucena	1+2+8	Jagadish <i>et al.</i> 2010a
Heat tolerance in spikelet fertility	Rice	996 × 4628	2	Xiao <i>et al.</i> 2011
Heat tolerance in spikelet fertility	Rice	IR64 × N22	2	Ye <i>et al.</i> 2012
Heat tolerance during grain development	Rice	Tohoku-168 × Kokoromachi	1	Shirasawa <i>et al.</i> 2013
Anthers heat tolerance	Rice	(Nipponbare × Kasalath) × Nipponbare	4	Tazib <i>et al.</i> 2015
Spikelet sterility escape from heat stress	Rice	<i>O. officinalis</i> introgression lines	1	Hirabayashi <i>et al.</i> 2015
Heat tolerance in spikelet fertility	Rice	IR64 × N22	1	Ye <i>et al.</i> 2015a,b
Heat tolerance in grain ripening	Rice	Tsukushiroman' × 'Chikushi 52'	6	Wada <i>et al.</i> 2015

researchers must have a clear understanding of the genetic, biochemical, metabolic, and physiological mechanisms by which plants achieve HTS tolerance. Moreover, an advanced molecular breeding approach has a great advantage over the transgenic approach because it is universally accepted as a technology that can contribute significantly to the development of HTS-tolerant rice plants.

Because of predicted global climate changes, an appropriate strategy is urgently needed to combat the losses that will occur due to high-temperature stress. Rice is a very

important crop for supporting food security. Plants are much more sensitive to high temperatures when exposed during the reproductive stage. Production of functional and viable pollen that complete the pollination process is a crucial aspect to ensuring natural fertility and stable crop yields. Understanding the complexity of regulatory molecular mechanisms involved in these processes can be eased through omics-based studies that lead to the identification of many key genes, TFs, and QTLs. Genome-wide analyses that rely upon microarrays or next generation technologies such as RNA-Seq have started to

provide molecular information on candidate genes involved in heat tolerance, which can then be further characterized in depth. Additional efforts should be made to generate integrated data from omics, metabolic, and proteomics analyses. Heat stress is a polygenic characteristic that is often defined based upon complex traits such as yield under stress. Therefore, introgression of genes, TFs, and QTLs through conventional breeding is not feasible when developing an HTS-tolerant cultivar. Instead, alternative strategies such as advanced molecular breeding or genetic engineering are necessary if we are to develop rice plants that are tolerant to high temperatures.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Next-Generation BioGreen 21 Program (Agricultural Biotechnology Research Center. Project No. 01369001 to SKP) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2016R1D1A1A09919568 to KHJ).

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