

Research for Better Rice to Cope With Heat Stress

Dheeraj Mittal and Anil Grover

Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi-110021, India. Email: agrover@south.du.ac.in; anil.anilgrover@gmail.com

Abstract

Heat stress is detrimental to the optimal growth and productivity of rice plants. The looming threat of global warming warrants that ways to design rice which can withstand heat stress in both minimum temperature and maximum temperature cycle of the day are optimized. There is no definite success yet in breeding field-level tolerance to heat stress in rice, particularly at the time of flowering when rice plants are most sensitive to heat. Transgenic methods may provide means to breed heat tolerance in rice plants by employing genes from rice itself through their altered expression levels or from across the spectrum of living systems. To achieve this objective, it is important that the most basic and pertinent question as to which genes, proteins or processes contribute to heat tolerance, is addressed to. As rice is a model cereal system, research findings on rice would facilitate genetic engineering of heat tolerance in other cereal and non-cereal crops too.

Keywords: Heat stress, oxidative stress, rice, abiotic stresses, heat shock proteins, tolerant transgenic crops

Rice cultivation and heat stress

Rice (*Oryza sativa* L.) is staple food crop not just for the Indian masses but for the whole Asian community, and remains an important food crop for people of the entire world; estimates show that more than 3.5 billion people (out of estimated ~6.8 billion present population) depend on rice for more than 20% of their daily calories (<http://irri.org>). Rice crop is hence vital for the world food security.

Abiotic stresses (i.e. drought, salinity, flooding, extreme low and high temperatures and oxidative stresses) result in a huge loss in rice production (Grover and Pental 2003; Grover et al 2009). Plant abiotic stress responses are orchestrated by generation of concerned signals, perception of signals and subsequent transduction leading ultimately to activation of the transcriptional machinery such that plants are able to fight or succumb to stress (Grover et al. 2001). Emerging threats of global warming and climate change make it necessary that heat tolerant rice is designed to enhance its productivity as well as total production. Heat stress (HS) affects rice in several ways. Rice seed germination is reduced in response to HS. HS tends to lower the vigor of rice seedlings and cause abnormal branching patterns of roots. The growth processes are appreciably hastened with HS and as a result, the durations of various developmental phases are altered as temperature rises. In rice, number and height of tillers and tillering duration are severely reduced in response to HS. Rice is most susceptible to heat injury during flowering time

(Jagadish et al 2010a). Pollen viability is particularly sensitive to HS. Even high temperature at anthesis for 1-2 h may result in high spikelet sterility. However, understanding of physiological and molecular mechanisms determining the heat stress tolerance at the time of anthesis is limited (Jagadish et al 2007; Jagadish et al 2008; Jagadish et al 2010a). Both high day temperatures (Jagadish et al 2010a; Jagadish et al 2010b; Jagadish et al 2011) and high night temperatures (Welch et al 2010) negatively affect the rice spikelet fertility and yield. The duration of grain filling in rice is highly sensitive to elevated temperatures. The grain yield of rice has been estimated to have dropped by 10% for every 1°C increase in growing season minimum temperature in the dry season (Peng et al 2004).

Downstream proteins related to heat stress

Heat stress response (HSR) elicited in plant cells against HS can largely be considered as a defense mechanism. At the molecular level, HSR is characterized by rapid reprogramming of genetic machinery leading to expression of a large number of genes and the accumulation of heat shock proteins (Hsps) (Finka et al 2010). Most Hsps are molecular chaperones, which have a role in the stabilization and/or refolding of denatured proteins. In response to supra-optimal heat, cellular proteins tend to lose the biological activity due to aggregation and/or misfolding. The accumulation of stress-induced aggregated/misfolded proteins is deleterious to cells; in fact the abnormal state of proteins is considered to trigger the HSR (Vierling 1991;

Grover 2002; Wahid et al 2007). HS further leads to stimulated protein degradation, which is mediated by synthesis of specific proteases involved in the degradation of abnormal proteins. Overall, Hsps through their chaperoning activities provide a functional network in protecting cells against damage due to HS (Katiyar-Agarwal et al 2001; Singh and Grover 2008; Xu et al 2011). The increased levels of Hsps under HS may have a direct bearing on reducing the levels of denatured or aggregated proteins and thus are critical for the protein homeostasis.

Rice seedlings rapidly and predominantly synthesize and accumulate Hsp100 proteins in response to HS (Singla and Grover 1994). Rice Hsp100 is immunologically related to yeast Hsp104 (Singla and Grover 1993). Later studies showed that the expression of Hsp100-related proteins is a universal response in plants (Pareek et al 1995). Apart from stress, developmental cues are shown to regulate cellular levels of these proteins (Singla et al 1998). Rice Hsp100 protein allows survival of yeast *Ahsp104* mutant under HS through promoting disaggregation of stress-induced protein granules (Agarwal et al 2003). The levels of Hsp100 transcripts or proteins are noted to be differential in *indica* and *japonica* rice types. More recent work has shown that Hsp100 proteins belong to bacterial ClpB family of proteins; hence it is apt to consider Hsp100 proteins as ClpB-like proteins. Rice genome contains 3 Hsp100/ ClpB-like proteins namely ClpB-cytoplasmic (Os05g44340), ClpB-mitochondrial (Os02g08490) and ClpB-chloroplastic (Os03g31300) (Singh et al 2010). Singh and Grover (2010) have shown that all three rice Hsp100 isoforms are highly comparable to yeast Hsp104 protein.

Singh et al (2012) noted that both transcriptional and post-transcriptional regulation mechanisms govern the synthesis of ClpB-cytoplasmic protein in rice. sHsp genes are among the most predominantly expressed stress protein family in rice. Detailed work on the genomic organization and expression profiling of rice sHsp genes under stress and development has been reported (Sarkar et al 2009). Rice genome sequencing data and various other gene expression datasets have shown that the spectrum of Hsps in rice is highly complex as multiple members belonging to Hsp10, Hsp40, Hsp60, Hsp70 and Hsp90 and Hsp100 families are expressed when the plants are challenged by HS. Jagadish et al (2010b) showed that greater heat tolerance of rice cultivar N22 is linked to higher accumulation of stress responsive cold and heat shock protein during anthesis. It is suggested that if the heat tolerance trait from tolerant donor N22 could be transferred into hybrid rice, rice yield could become sustainable in future (Madan et al 2012).

Understandably, much needs to be done and validated to understand the wide range of functions and complexities associated with Hsp genes and proteins in rice.

Besides the expression of Hsps that form a ubiquitous component in the HSR, there are Hsp-independent proteins or pathways also which are involved in HSR. For instance, proteins like late embryogenesis-related proteins (Lea proteins), dehydrins, glycine-rich RNA binding proteins, FK506 binding proteins and elongation factor (EF-Tu) have been implicated in HSR (Wahid et al 2007; Huang and Xu 2008). Recently reported MBF1c regulon is another example in this category. Many of the details by which the non-Hsp proteins work during the HSR are yet to be revealed. Singh and Grover (2008) proposed that increased osmolytes might make the cell environment highly reductive due to higher energy status and thus reduce damage to proteins. There are also indications that osmolytes bind with the cellular proteins to protect them from denaturation or aggregation (Ou et al 2001; Ignatova and Gierasch 2006). Cell detoxification components may likewise be important in making a reductive cell environment and in minimizing the loss of active proteins. It has been suggested that reduction in the damage to cellular proteins is the key process in the development of heat tolerance (Singh and Grover 2008).

Upstream genetic components related to heat stress

HSR involves coalition of several pathways, culminating in activation of HSFs and accumulation of Hsps/ non-Hsps proteins (Saidi et al 2010; Hua 2009). Heat shock transcription factors (HSFs) are considered to be the master regulators of the HSR. The plant HSFs can potentially exist in homo- or hetero-oligomeric states. HSFs through this changed state may show altered cellular localization (cytoplasmic or nuclear), resulting eventually in enhanced or suppressed transcription (Miller and Mittler 2006). HSFs specifically bind to palindromic heat shock element (HSE), sequences located upstream of Hsp genes. Binding of HSFs to HSEs leads to transcriptional regulation of HS responsive genes. Detailed analysis has shown that HSFs and Hsps represent an interaction point amongst multiple stress response pathways in plants (Swindell et al 2007). HSF:Hsp regulon is hence an important transcriptional regulatory network during HS (Nakashima et al 2009). HSFs and Hsps orchestrate an HSF:Hsp circuitry which involves additional upstream and downstream proteins (Mittal et al 2011).

In rice, 26 genes constitute the HSF family. Rice HSF genes are noted to respond to a variety of stresses (Mittal et al 2009). Detailed genomic features of various

rice HSF genes have been reported. HSF transcripts are shown to respond to HS as well as to low temperature and oxidative stresses (Mittal et al 2009). Transcript expression profiling data showed that 22 *OsHSF* genes are induced by HS. Induction of 10 and 14 *OsHSF* genes was also noted against cold stress (CS) and oxidative stress (OS), respectively. All *OsHSF* genes induced by OS were also induced by HS. Apart from stress inducibility, various *OsHSF* genes are developmentally-regulated in diverse tissue types (Mittal et al 2009). The binding affinities and interactions among different HSE types and *OsHSFs* have recently been addressed (Mittal et al 2011). Based on the differential binding affinities and interaction among HSFs (homomeric as well as heteromeric interactions), it has been suggested that such differential patterns may have a bearing on cellular functioning of *OsHSFs* under a range of different physiological and environmental conditions that influence synthesis of different target proteins governed by HSE-HSF interactions (Mittal et al 2011). Recently, it was noted that HSFs and Hsps (Hsp100 in particular) form a HSF/Hsp circuitry in rice that involves several additional proteins (Singh et al 2012). It was shown that *OsHSFA2c* and *OsHSFB4b* are involved in the transcriptional regulation of *OsClpB*- cytoplasmic or *Hsp100* gene in rice.

Mittal et al (2011) analyzed genome-wide distribution of HSEs in rice. This study showed that genome-wide 2830 genes have HSEs in their 1-kb upstream region. It was shown that only ~16% of the HS induced genes as per the microarray data contain the canonical HSE types (Mittal et al 2011). It is possible that rice genes that are HS inducible and do not contain typical HSEs may harbor other cis-elements which are HS responsive. In addition, it has been noted that many genes that are not HS inducible also have HSEs in their promoter region. Put together, these observations highlight the inadequacies in the current understanding of the relevance of HSEs in HS response in rice (Mittal et al 2011).

Besides HSFs, several other heat responsive transcription factors as well as multiple Hsp independent pathways mediated by auxins, abscisic acid (ABA), salicylic acid (SA), hydrogen peroxide and ethylene are shown to play a role in heat tolerance (Larkindale and Huang 2005; Hua 2009).

Genome-wide transcript expression analysis in relation to heat stress

Transcriptome studies using microarray technology have significantly enhanced understanding of the stress

responses (Kilian et al 2011). Omics approaches such as transcriptomics, proteomics, and metabolomics have helped in detailed understanding of various molecular facets of HSR in rice (Yamakawa et al 2007; Endo et al 2009; Hu et al 2009; Narsai et al 2010; Yamakawa and Hakata 2010; Zou et al 2011; Mittal et al 2012). Endo et al (2009) investigated gene regulatory mechanisms in anther during HS by microarray analysis. Among various HS responsive genes in anthers, 13 genes were found down-regulated and all of these were specific to immature anther; mainly the tapetum. This group suggested that HS primarily affects the tapetal cells. Yamakawa et al (2007) reported comprehensive expression profiling of rice grain filling related genes during HS. In this study, down-regulation of starch synthesis related genes and prolamin genes and up-regulation of α -amylases and Hsp genes was noted. Besides these, several redox responsive genes were differentially regulated. Yamakawa and Hakata (2010) analyzed the levels of metabolites and transcripts in developing caryopses (milky stage) exposed to HS. It was noted that HS results in inhibition of starch accumulation and induction of sucrose, amino acids, GABA (gamma-aminobutyric acid) and glutathione accumulation.

Mittal et al (2012) have recently reported genome-wide transcriptional changes in response to cold, heat and oxidative stresses in rice seedlings. Their analysis showed that HS results in predominant changes in transcripts of Hsp and HSF genes, as well as genes associated with synthesis of scavengers of reactive oxygen species (ROS) and genes that control the level of sugars, metabolites and auxins. From this study, it appeared that ROS homeostasis plays a central role in response to temperature extremes. Further, a considerable overlap was noted at the level of transcription as well as in regulatory modules of differentially-expressed genes following temperature stresses and oxidative stress. This work highlighted the genes associated with early expression changes (genes which showed rapid changes in their expression scored by employing early time points of stress), expressed in-common amongst different stresses (which might be part of general stress response), specifically-regulated genes (constituting specific components for a respective stress condition) and late responsive genes (genes which are either induced/repressed at a late time point or the one which showed a sustained expression). The genes expressed in common amongst different stresses may function as integrators of multiple environmental signals. Such genes may function as co-regulators that respond to a variety of abiotic stresses and/or represent the modules (response networks) that might be involved in the cross talk. Mittal et al (2012) further constructed

composite promoter models (CPMs) for differentially-regulated genes during HS using ExplainTM Plant 3.0 software program. This analysis was aimed at analyzing the over-represented transcription factor (TF) binding sites (F-Match) to unveil, which all matrices or TF-binding sites occur in the gene sets. The promoters of the differentially-expressed genes during HS were enriched for the HSF matrices along with ABRE-binding bZIP factor family members like ABF2, ABF3 and ABF4 and TFs like BZ8 and GBF family protein. The frequency of MYB family TF matrices was high among the promoters of the down-regulated genes during HS. Similar pattern was noted in case of the differentially-expressed genes during OS. A clear pattern of over-represented HSF binding sites in the promoters of up-regulated genes during HS and OS treatment and not in CS up-regulated genes was noted. A high similarity was found between the promoter models of HS and OS responsive genes. It is possible that the modules noted by Mittal et al (2012) represent function-specific regulation of transcription.

Addressing complexity of field-level abiotic stresses in transcript expression

Abiotic stresses seldom occur in isolation under field-conditions. Mostly, it is a complex scenario of different abiotic stresses affecting the plants in combination, occurring concurrently or separated temporally resulting in a cross talk. Miller et al (2008) proposed that during the cross talk, there may be summation of various signals, which may (1) cause an additive effect, (2) result in generation of a new signal as of the integration of different signals or (3) lead one signal to become epistatic over the other. Importantly, these possibilities may co-exist as well. On the basis of 'stress-matrix' discussed by Mittler and Blumwald (2010), it seems that HS in combination with drought, salinity, UV, ozone, high light intensity and pathogen may result in more deleterious effects on crop productivity. Combination of stresses may also have beneficial effects on the plants (Mittler and Blumwald 2010). Modulation of environmental responses as of cross talk events and prior stress exposure need to be taken into account in designing stress tolerant crops in field conditions (Wee and Dinnyen 2010). However, most studies on analyzing the response of plants to abiotic stresses emphasize a given stress type in isolation to simplify interpretation and are routinely carried taking only single or a few variables under controlled laboratory conditions. As the studies wherein single stress type is applied do not reflect the actual conditions that occur in the field (i.e. stress combinations or complex stresses), the understanding gained has glaring gaps. Understandably, the plants response to abiotic stresses in the field may be different from the response reflected in laboratory conditions.

It has been noted that the molecular and metabolic responses of plants to combination of drought and heat are unique and cannot be predicted directly from the studies where different stresses were applied individually (Mittler 2006). Stress response involving temperature extremes results in induced ROS production leading to oxidative damage (Apel and Hirt 2004). Suzuki and Mittler (2006) suggested that ROS could play a key role in mediating signal transduction event. During HS, plants activate mechanisms and pathways to control the ROS and redox homeostasis (Locato et al 2008). Recent reports suggest that HS is accompanied by some degree of oxidative stress (Frank et al 2009; Banti et al 2010). The accumulation of intracellular ROS following environmental stress further results in accumulation of mis-folded and damaged proteins via inhibition of 26S proteasome (Nishizawa-Yokoi et al 2010). In rice, Mittal et al (2012) have also highlighted the central role of ROS homeostasis during temperature extremes. Combination of drought and HS (Rizhsky et al 2002, 2004), HS and high light intensity (Kousevitsky et al 2008) and HS and salinity (Hewezi et al 2008) result in a unique stress response that cannot be extrapolated from the individual stress responses. Szucs et al (2011) also noted that combination of stresses (water shortage combined with HS) results in enhanced differential gene expression in wheat. Pretreatment of plants with stress conditions that induce 'oxidative burst' can trigger a protective function or immunize the plants against environmental stresses and thus could play a role in acclimatizing stress tolerance (Goswami et al 2010).

From the above account, it emerges that there is a need to mimic complex stress conditions in laboratory studies (Mittler 2006). Mittler and Blumwald (2010) advocated that stress combinations should be handled as a new state of abiotic stress in plants, which elicits a different stress response. The huge damage caused to agriculture by combination of stresses (Mittler 2006) underscores the need to study this complexity of stress response and to develop crops and plants with enhanced tolerance to such stress combinations. The development of tolerance to combination of different abiotic stresses is a much-desired target in breeding projects. To increase tolerance of rice plants to different abiotic stresses and to enable field-level resistance, there is need to unravel the details of molecular mechanisms related to tolerance against the complex forms of stresses. In a quest whether combination of stresses results in a different (which may be unique or additive) response in rice, our group has been analyzing how CS and OS together as well as HS and OS together (OS given as pre-treatment as well as co-treatment with temperature extremes) affect the gene expression in rice seedlings (D Mittal and A Grover, manuscript in preparation).

Designing heat tolerant transgenic crops

The genetic enhancement of heat tolerance in crops is a much desired objective. Conventional breeding for heat tolerance in crop plants has not been much successful due to several reasons like lack of suitable source of genes in sexually compatible gene pools, complex nature of the HS tolerance trait, lack of understanding on the molecular mechanisms of the HS tolerance response etc. As transgenic methods can bring genes for heat tolerance from sexually incompatible sources as well, genetically engineered plants against HS has emerged as a powerful alternative (Wahid et al 2007; Singh and Grover 2008; Hua 2009; Saidi et al 2010; Zinn et al 2010; Zou et al 2011). Overall, heat tolerance in plants through transgenic approach has been addressed to by (1) elevating levels of osmolytes, (2) increasing levels of cell detoxification enzymes, (3) altering membrane fluidity and (4) using chaperone genes (Singh and Grover 2008; Zou et al 2011). There are also attempts to employ genes involved in various metabolic processes such as polyamine biosynthesis, photosynthesis and NO (nitric oxide) synthesis to achieve enhanced heat tolerance (Singh and Grover 2008; Zou et al 2011). Transcription factor (TF) based technologies are advocated in the next generation of engineered crops (Century et al 2008). There are various reports where over-expression of HSFs has been utilized as a strategy to enhance heat tolerance (Singh and Grover 2008; Zou et al 2011; Schmidt et al 2012). Recent reports suggest that WRKYs and DREBs may regulate the HSFs and thereby act upstream in the HS responsive cascade. Heat tolerance was significantly induced in plants over-expressing DREB genes (Sakuma et al 2006; Lim et al 2007; Hong et al 2009; Chen et al 2010; Matsukura et al 2010; Kang et al 2011). Further, it has been reported that WRKY genes respond to a combination of drought and heat shock (Rizhsky et al 2002, 2004; Wu et al 2009). Over-expression of WRKY11, WRKY25 and WRKY39 in *Arabidopsis* markedly enhanced heat tolerance (Wu et al 2009; Li et al 2009, 2010). The nuclear transcription factor X-box binding 1 gene has also been shown to promote acquired heat tolerance (Larkindale and Vierling 2008). Over-expression of AtSAP10 (Zn-finger domain containing protein) in *Arabidopsis* conferred strong tolerance to heavy metals and HS (Dixit and Dhankar 2011). Over-expression of ABP9 gene (encoding abscisic acid responsive element binding protein 9) in *Arabidopsis* plants increased the photosynthetic capacity, elevated the carbon use efficiency and enhanced tolerance to heat and water stress (Zhang et al 2008). Gao et al (2008) identified a membrane-tethered bZIP TF (bZIP28) and showed its role in heat tolerance in *Arabidopsis*. Recent studies have shown that MBF1c protein is an important component of HS response in *Arabidopsis* which

functions upstream of SA (salicylic acid), trehalose and ethylene during HS (Suzuki et al 2008). Further, MBF1c has been implicated in control of DREB2a, 2 HSFs and several zinc finger proteins at the transcriptional level during heat shock (Suzuki et al 2011). MBF1c regulon has been suggested to work independent of the HSF:Hsp regulon (Suzuki et al 2011).

While generation of transgenic rice with enhanced HS tolerance provides an attractive approach, production of heat tolerant rice that can withstand field-level stress remains elusive. Katiyar-Agarwal et al (2003) overexpressed AtHsp100 in rice and showed that transgenic plants have enhanced HS tolerance. In fact, various downstream (*Hsp17.7*, *Hsp70* and *Fad7*) and upstream (*HSFA4d*, *WRKY11*, *SBPase* and *GSK1*) genes have been transformed into rice to increase HS tolerance (Zou et al 2011). In addition, it has been shown that a large number of rice proteins (i.e. Hsp100, Hsp90, Hsp16.9, HSF7, HSFA2e, DREB2b, AREB1, ZFP177 and AKR1) have resulted in enhanced thermotolerance in bacterial and plant systems (Zou et al 2011).

To further pursue this goal, the physiological processes, molecular mechanisms, biochemical enzymes, proteins and genes that impart HS tolerance need to be analyzed.

Prospects and challenges

In spite of intense studies undertaken so far, the complete understanding of the molecular basis of heat stress response in plants remains elusive. To achieve heat tolerant transgenic rice plants for field cultivation, it is important that this gap is filled. The various mechanisms involved in heat tolerance need to be catalogued and the 'master regulators' of heat tolerance response need to be identified. Heat stress-related microarray studies have shown that large number of genes implicated in heat response remains un-annotated. To achieve the task of completing the functional genomics of heat response, global co-operations are required to bring all relevant pieces of information at one place. It is equally important that the genetic resources pertaining to variations observed in heat stress response in diverse rice varieties and cultivars are globally shared. It is also important that the differences in gene expression profiles in such contrasting rice genotypes should be accounted for and their biological relevance should be delineated to understand the functional diversity of various alleles.

Transgenic crops have the potential to promote a revolutionary change in agriculture. For raising field-level HS tolerant transgenic rice plants, 'gene discovery' programs relevant to heat tolerance need to be supported. There may not be single gene-based solutions for efficient HS tolerance; however, the single gene

transformations open up avenues for possible pyramiding of various HS related genes at a later stage. Importantly, every gene that shows inducibility upon HS treatment may not have a primary role in stress adaptation. There is a need for devising suitable experimental approaches to delineate genes responsible for primary effects from those that have secondary or tertiary effects. Novel sources of transgenes from diverse stress-adapted species should be employed to provide rice plant the necessary adaptive advantage. There is need to apply gain and loss of function approach to understand the role of novel genes in rice as is being vigorously followed in *Arabidopsis*. Research efforts should in particular be enhanced to understand how rice plants cope with high temperature stress during the reproductive stages.

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