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Genome-Wide Transcriptome Analysis During Anthesis Reveals New Insights into the Molecular Basis of Heat Stress Responses in Tolerant and Sensitive Rice Varieties

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Rice is one of the main food crops in the world. In the near future, yield is expected to be under pressure due to unfavorable climatic conditions, such as increasing temperatures. Therefore, improving rice germplasm in order to guarantee rice production under harsh environmental conditions is of top priority. Although many physiological studies have contributed to understanding heat responses during anthesis, the most heat-sensitive stage, molecular data are still largely lacking. In this study, an RNA-sequencing approach of heat- and control-treated reproductive tissues during anthesis was carried out using N22, one of the most heat-tolerant rice cultivars known to date. This analysis revealed that expression of genes encoding a number of transcription factor families, together with signal transduction and metabolic pathway genes, is repressed. On the other hand, expression of genes encoding heat shock factors and heat shock proteins was highly activated. Many of these genes are predominantly expressed at late stages of anther development. Further physiological experiments using heat-tolerant N22 and two sensitive cultivars suggest that reduced yield in heat-sensitive plants may be associated with poor pollen development or production in anthers prior to anthesis. In parallel, induction levels of a set of heat-responsive genes in these tissues correlated well with heat tolerance. Altogether, these findings suggest that proper expression of protective chaperones in anthers is needed before anthesis to overcome stress damage and to ensure fertilization. Genes putatively controlling this process were identified and are valuable candidates to consider for molecular breeding of highly productive heat-tolerant cultivars.

Keywords: Anthesis • Heat stress • Pollen • Rice • RNA-seq • Spikelet fertility.

Abbreviations: DEG, differentially expressed gene; FC, fold change; FDR, false discovery rate; GO, Gene Ontology; HS, heat stress; HSF, heat shock factor; HSP, heat shock protein; RNA-Seq, next-generation RNA-sequencing; RPKM, reads per kilobase of coding sequence per million reads; RT–qPCR; reverse transcription-quantitative real-time PCR; SA, salicylic acid; TF, transcription factor.

Introduction

Rice is one of the major staple cereals in the world, providing essential caloric requirement for billions of people (Khush 2005). Production of rice will need to be increased by 40% in 2030 to satisfy a steadily increasing demand from a fast growing world population (Anderson et al. 2004). The challenge, however, will have to be met with less land (urbanization), less water (human and industrial needs, climate change) and increasing pest and disease pressure. Predicted global increases in temperatures, during the coming decades due to climate change, will pose a serious threat to crop productivity and to sustain global food security (Wheeler and von Braun 2013). A sustainable increase in productivity requires intensified efforts to develop cultivars with improved yield potential, having greater stress tolerance and superior resource use efficiency.

Among the key climate change drivers, high temperatures influence all growth stages during the rice life cycle (Shah et al. 2011). However, it has been well documented that anthesis in rice is the stage most sensitive to high temperatures (Yoshida et al. 1981, Prasad et al. 2006). A spikelet tissue temperature of $\geq$33.7°C for an hour coinciding with anthesis is documented to be sufficient to induce spikelet sterility (Jagadish et al. 2007), while exposure to temperatures even at 38 or 41°C an hour after anthesis did not induce sterility (Yoshida et al. 1981). During anthesis, many physiological processes occur in a short period of about 45 min to 1 h, including anther dehiscence, pollination and pollen germination on the stigmatic surface and pollen tube growth to reach the ovule (Cho 1956, Jagadish et al. 2010). All these processes are negatively influenced by heat stress (HS) (Jagadish et al. 2014).

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N22 is one of the most heat-tolerant rice cultivars known to date, but its agronomic performance is poor (Bahuguna et al. 2014). Nevertheless it is used routinely in breeding programs as a source of tolerance not only for heat but also for drought stress (Vikram et al. 2011, Ye et al. 2012). The negative influence of HS on pollen production, shedding and viability in N22 is significantly lower compared with many other cultivars (Prasad et al. 2006). Very few cultivars behave similarly to N22 with respect to the physiological responses to high temperatures affecting overall fertility; N22 stands out, with less yield penalty when exposed to HS (Jagadish et al. 2008, Jagadish et al. 2010). This suggests that there should be molecular or biochemical superiority, in addition to physiological/anatomical differences in its background, that has adapted to better tolerate high temperatures. Proteomic studies have identified cold and heat shock proteins that may be involved in conferring tolerance to heat in N22 (Jagadish et al. 2010). Two other studies have evaluated transcriptomic changes accompanying HS in reproductive tissues from the heat-tolerant cultivar 996 (Zhang et al. 2012) and the heat-sensitive variety Nipponbare (Endo et al. 2009) by microarray analysis. However, both studies focused their analysis on earlier stages of reproductive development (pre- and during meiosis). This study depicts for the first time the global transcriptional response to HS of the reference heat-tolerant cultivar N22 in reproductive tissues during anthesis by RNA sequencing (RNA-Seq) analysis. In addition, we tested whether the responses are also affected at the physiological and molecular levels in two heat-sensitive cultivars: a different accession of N22 that is susceptible to HS and a widely grown popular variety IR64. Overall and specific HS responses as well as promising candidate genes for future breeding programs were identified and discussed.

Results

HS-induced transcriptional changes in heat-tolerant N22 during anthesis

In order to study the early molecular response to HS in the tolerant rice cultivar N22, IRGC accession 19379, pollinated pistils were isolated from 30 min heat-treated (38°C) spikelets specifically coinciding with anthesis. The same type of tissues was collected from plants subjected to a normal growing temperature (29°C) and used as controls (C). Three biological replicates from each treatment were obtained. Total RNA was extracted from these tissues and used for Illumina RNA-Seq experiments. More than 20 million reads were obtained from each of the six samples (3HS, 3C) and mapped to the *Oryza* MSU7.0 database using the commercially available CLC Genomics Workbench (CLC bio). Gene expression was quantified as reads per kilobase of coding sequence per million reads (RPKM). After normalization using total reads, statistical analyses were carried out (Baggerley's test), and a list of 630 differentially expressed genes (DEGs) was obtained, 259 up- and 371 down-regulated by HS, setting the false discovery rate (FDR) at <0.05 and fold change (FC) at >2 as cut-offs (**Supplementary Table S1**). In order to verify the validity of these results, five genes from each list, with altered expression levels and FCs, were chosen as representatives to quantify their expression by RT-qPCR. Results shown in **Fig. 1A** confirm the robustness of global expression data obtained for both up- and down-regulated genes (upper and lower panel, respectively).

Based on the putative functions assigned by the Rice Genome Annotation Project to the 630 identified DEGs, we grouped them into functional categories. **Fig. 1B** shows that the main categories affected by heat stress comprised genes encoding chaperones, transcription factors (TFs), and those involved in metabolic processes, transporters and signaling-related kinases and phosphatases. As expected, chaperone-encoding genes were massively induced by heat. Remarkably, a rapid response to heat triggered the down-regulation of a substantial number of TF-encoding genes and only a few of them were induced, of which those belonging to the heat shock factor (HSF) gene family are the most representative. In contrast, expression of TF genes that are members of families such as *WRKY*, *MYB*, *AP2/ERF*, *bHLH*, etc. were repressed by HS (**Supplementary Table S2**). Notice that while some of these TF genes seem to be part of a general response due to their identification in other global expression profiling experiments during HS (see below and **Supplementary Table S2**), many others have not been described before and their change in expression may be important for the responses to heat during anthesis.

As reproductive tissues in different developmental stages can be affected by increasing temperatures, we tested whether there is tissue-specific and developmental regulation of the expression of DEGs identified by the RNA-Seq analysis of pollinated pistils. Thus, we carried out an in silico gene expression analysis from the whole inflorescence, anthers, pistil, and lemma and palea during reproductive organ/tissue development using RiceXPro (http://ricexpro.dna.affrc.go.jp/). As the maximum number of genes that can be processed at the same time using this program is limited to 100, we selected the most up- or down-regulated genes from the RNA-Seq list. **Supplementary Fig. S1** shows that most of the top 100 up-regulated genes (31 genes; **Supplementary Table S3**) were preferentially expressed in anthers, many of them at a later stage of anther development when pollen grains are formed. Almost 30% of those 100 most up-regulated genes were also found to be pollen/sperm cell-expressed genes in another transcriptomic analysis from rice tissues (Russell et al. 2012; **Supplementary Table S3**). Thus, some of these genes might be expressed as a protective reservoir during pollen maturation while others might be specifically expressed after heat shock in reproductive tissues during anthesis. On the other hand, down-regulated genes did not appear to be preferentially expressed in any of the tissues during reproductive development (**Supplementary Fig. S2**).

Enriched gene categories related to heat stress

In order to understand which categories are over-represented in the list of DEGs in comparison with the whole rice genome, all 630 genes were further analyzed for Gene Ontology (GO) functional annotations with agriGO analysis tools (http://bioinfo.cau.

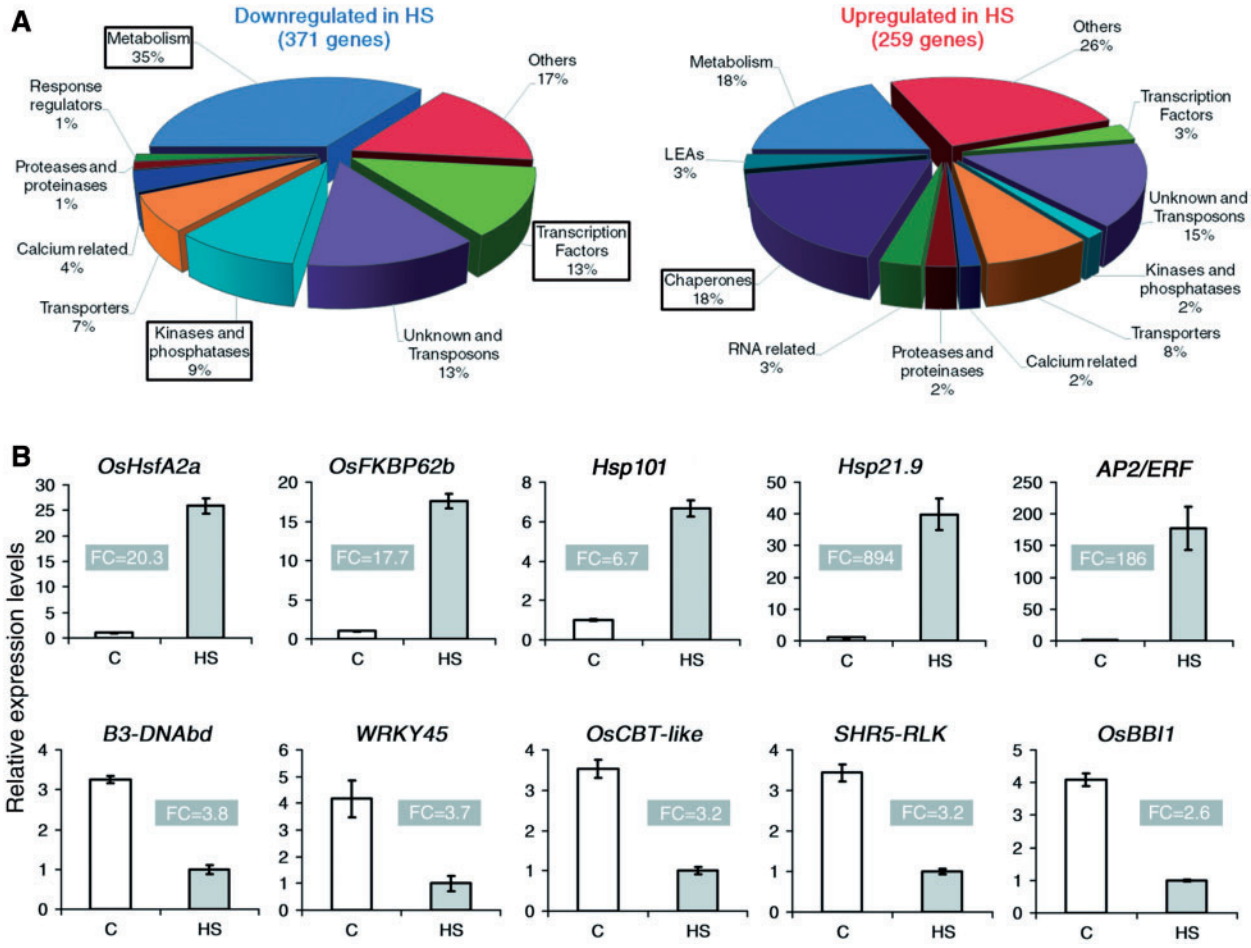

Fig. 1 Classification of differentially expressed genes during heat stress in N22 and validation by RT-qPCR. (A) Genes were grouped based on their putative functions assigned by the Rice Genome Annotation Project. The percentage of genes for each data set is also shown and more significant classes are boxed. (B) Expression levels of 10 selected genes were quantified by RT-qPCR. Relative expression levels shown were calculated as the ratio of gene/OsEF1 and NABP expression levels and normalized to 1 in controls (for HS-up-regulated genes) and in heat-stressed samples (for HS-down-regulated genes). Fold change (FC) from RNA-Seq analyses is also shown in boxes. HS, heat-stress; C,controls. Bars represent the SEM.

edu.cn/agriGO/analysis.php) and Mapman Software (Thimm et al. 2004). The up-regulated DEGs are enriched with genes involved in stress and stimulus responses belonging to the 'Biological Process' category (**Fig. 2**). Down-regulated genes are enriched in signal transduction, biosynthetic and metabolic processes (**Fig. 2B**, right panel) that can account for the high percentage of repressed genes associated with metabolism and kinases and phosphatases (**Fig. 1B**). Similarly, GO analysis confirmed the over-representation of TF-encoding genes in the group of down-regulated genes which fall into the 'Molecular function' category, together with genes belonging to catalytic activity. Concerning the latter, most of them encode enzymes linked to metabolism (**Supplementary Figs. S3, S4**) and receptor-like kinases belonging to three groups: cytoplasmic, leucine-rich repeat (LRR) and S-locus type (**Supplementary Fig. S5**). Further, significant (FDR < 0.05, **Supplementary Table S4**) enrichment of mitochondrial- and vacuolar-associated genes induced by heat stress was recorded (**Fig. 2A**, right panel), many of them belonging to both GO accessions. Details of the GO accessions enriched and the associated FDRs of DEGs in HS are provided in **Supplementary Table S4**.

Common and specific heat-responsive genes in reproductive tissues in rice

In rice, male reproductive organs are most sensitive to HS, thereby affecting spikelet fertility and grain yield (Jagadish et al. 2010). Two previous studies have identified sets of heat-responsive genes during reproductive development. Endo and colleagues found >1,400 genes whose expression changed significantly in anthers in the heat-sensitive reference variety Nipponbare (Endo et al. 2009). A second publication describes almost 2,500 heat-responsive genes in young florets during meiosis from the heat-tolerant cultivar 996 (Zhang et al. 2012). Comparison of the data sets between the above-mentioned studies and our analysis shows a higher number of common deregulated genes between our work and Zhang's data sets (216 genes, 34% of all 630 deregulated genes in our data sets) than with Endo's data sets (71 genes, 11% of total genes in our data sets) (**Fig. 3**). We noticed that many of the genes were common between Zhang and our work but absent in the data set of Endo et al. indicating that the common genes may be associated with heat

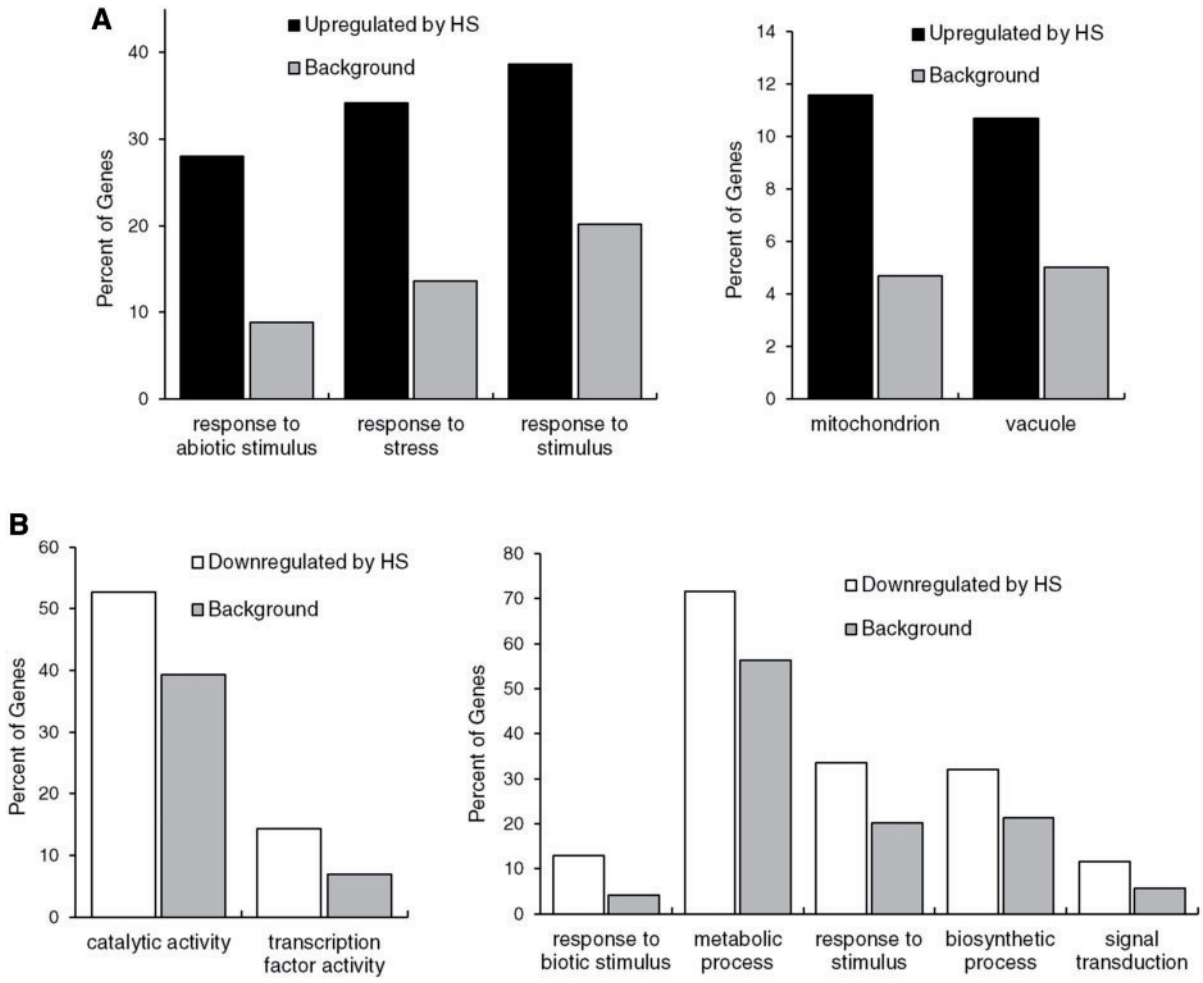

Fig. 2 Gene Ontology enrichment analyses of data sets obtained by RNA-Seq. The most representative (lowest FDR) GO accessions are shown for HS-up-regulated (A) and HS-down-regulated (B) genes. Backgrounds correspond to the percentage of genes from the whole genome belonging to each GO accession. The full list of statistically significant (FDR < 0.05) GO accessions enriched is given in **Supplementary Table S3**.

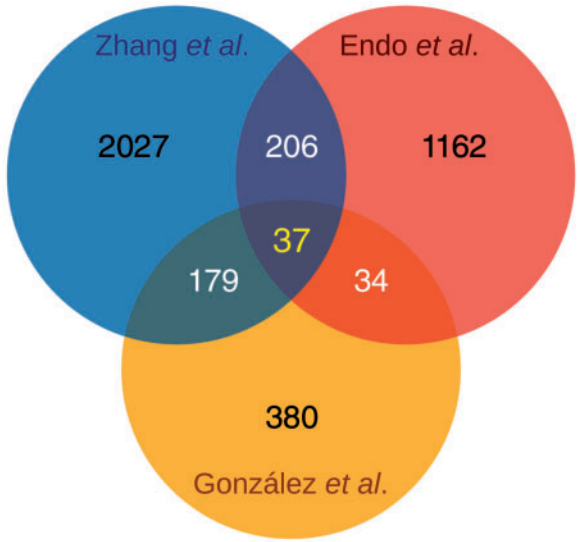

Fig. 3 Venn diagram showing heat-responsive genes identified in three independent rice heat stress data sets. Lists of heat-responsive genes from Endo et al. (2009) and Zhang et al. (2012) were compared with the list obtained by RNA-Seq analyses in this study.

tolerance. In addition, there are a number of common heat-responsive genes between the work of Zhang et al. and Endo et al. that are not identified in our studies, suggesting specificity of expression patterns during earlier anther developmental stages compared with the fully mature stage. Remarkably, **Fig. 3** shows a relatively small number of heat-responsive genes common to all three studies (see also **Supplementary Table S5**). Some of them, such as small heat shock protein (*HSP*) genes, *HSP101* or heat shock factor (HSF) genes (*HSFB2c*), are well-known members of the canonical response to HS in plants. However, this list also includes other as yet uncharacterized genes such as two TFs, cell wall modification and sugar partitioning enzymes, transporters and two RNA-binding proteins. Some of these 37 heat-responsive genes in reproductive tissues in rice might be part of the basal response to HS. To investigate this possibility further, we compared these data sets with another study carried out using PUSA BASMATI heat-stressed seedlings (Mittal et al. 2012). Twenty-two out of those 37 genes (marked by asterisks in **Supplementary Table S5**) were also identified by Mittal et al. thus establishing a core heat-responsive gene set in

rice, while the other 15 genes seem to be part of the basal response specific to reproductive tissues. All these 37 genes have canonical or non-canonical heat shock elements in their promoters (data not shown). Common genes between this work and all three data sets mentioned above are provided in **Supplementary Table S6**.

Pollen development within anthers is compromised by heat in heat-sensitive cultivars

Previously, Jagadish et al. (2010) described the physiological response to heat during anthesis in different cultivars. We wanted to address the molecular response in heat-sensitive cultivars, compared with the tolerant N22 (IRGC accession 19379). Therefore, we selected a widely grown popular rice variety IR64 already described as moderately heat sensitive and another accession of N22, IRGC accession 6264, identified to be sensitive to heat (K. Jagadish, unpublished results). There are in total eight different accessions of N22 in the IRRI genebank with different levels of tolerance to HS during flowering, among which N22 (19379) has been documented to be highly tolerant (Rang et al. 2011). We performed identical experiments to the one carried out with N22 19379 (see above), treating plants during anthesis with heat or control temperatures for all three cultivars (N22 19379, N22 6264 and IR64) in triplicate. Fertility analyses clearly showed the tolerant behavior of N22 19379 (around 70% fertility among HS-treated spikelets) while N22 6264 and IR64 displayed higher spikelet sterility after exposure to HS (fertility around 30%) (**Fig. 4**, top panel). The degree of fertility can be affected by poor pollen development, number of pollen grains deposited on the stigma, their germination, and pollen tube growth rate to reach the ovaries for successful fertilization. The number of pollen grains on stigmas was affected by heat treatments in the susceptible N22 6264 (18.85 ± 4.64 in HS, 59.33 ± 5.21 in C; mean ± ?SEM, $P < 0.05$) (**Fig. 4**, middle panel), with IR64 behaving similarly while the tolerant N22 19379 had no significant decline in pollen count. Both N22 6264 and IR64 display limited pollen germination (<20 germinated pollen grains/spikelet, **Fig. 4**, bottom panel). Furthermore, we found only a few spikelets with pollen tubes reaching the ovary in heat-stressed IR64 (15%) and also for N22 6264 (45%) compared with 68% spikelets with at least one pollen tube reaching the heat-stressed ovary (**Supplementary Table S7**). In summary, these results suggest that pollen development in anthers prior to anthesis may be the most heat-sensitive process and explains the low fertility rates in these cultivars.

Heat tolerance during anthesis correlates with the extent of heat-induced molecular responses in anthers

Considering the physiological data obtained from the heat-tolerant and -sensitive cultivars (**Fig. 4**), and that the genes up-regulated by HS in N22 19379 are preferentially expressed in late developmental stages of the anthers (**Supplementary Fig. S1**), we then focused on the molecular analyses of these tissues. From the same experiment that was conducted to analyze the fertility, pollen count and germination, we collected

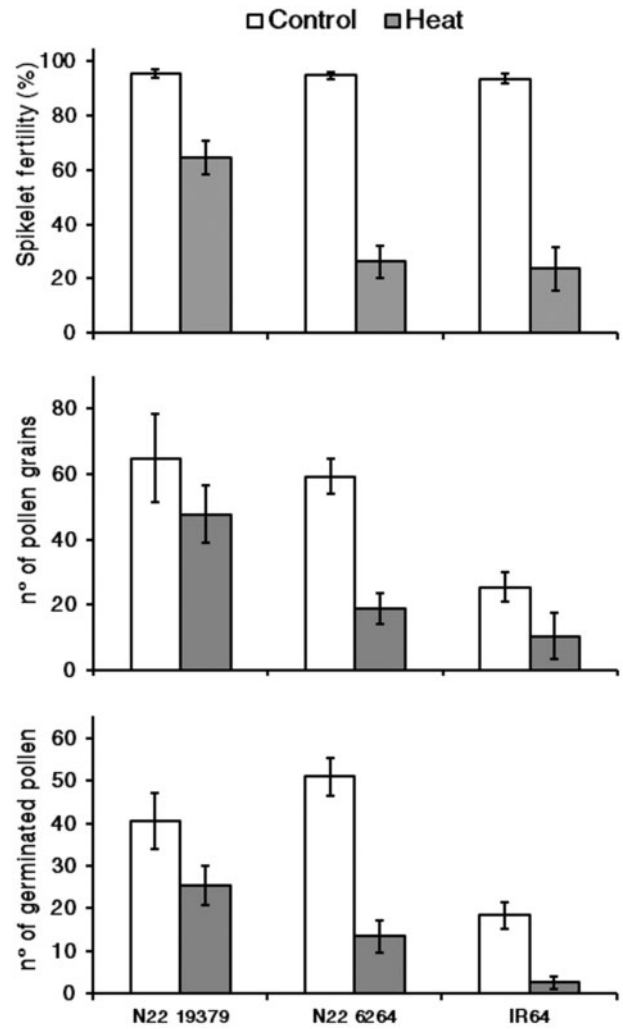

Fig. 4 Physiological response to heat stress in three contrasting varieties. Heat-tolerant N22 (19379), heat-sensitive N22 (6264) and IR64 cultivars were evaluated. Spikelet fertility as the percentage of filled/total spikelets after grain maturation (top panel), number of pollen grains counted on stigmas (middle panel) and number of germinated pollen grains on stigmas (bottom panel) are shown. From 10 to 45 spikelets from different tillers and at least three replicas were used. Bars represent the SEM.

anthers just prior to anthesis under heat and control temperatures from all three cultivars in triplicate. After RNA extraction and cDNA synthesis, we carried out an expression analyses of 19 selected genes by large-scale quantitative real-time PCR with Fluidigm technology (Fluidigm Corp.). All genes that we selected were previously shown to be up-regulated by HS in N22 19379 (**Supplementary Table S1**). Some of them are part of the basal heat-responsive core described above (*BAG6-like*, *Hsp101*, *Calcyclin-BP* and *OsSTI1*), TF-encoding genes previously described in other works (*OsHSFA2a* and *AP2/ERF*, see **Supplementary Table S2**) or were also heat-responsive genes in common with other studies (**Supplementary Table S6**) (*OsHSP16.9A* and *17.9A*, *OsFKBP62b*, *OsBI1-like*, *AWPM-19 like*, LOC_Os04g31710, *OsDMC1B*, *OsCBSX5*, CS domain, *RNApolII-AP3*, *OsADF3* and *SR33*). After calculating

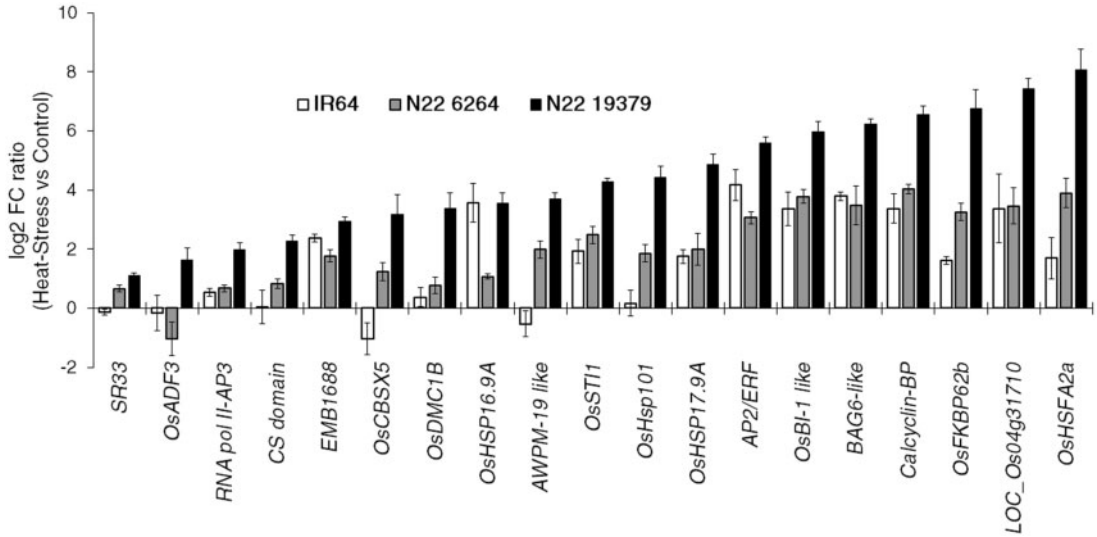

Fig. 5 Molecular response to heat stress in anthers of three contrasting cultivars. Expression of 19 heat-responsive genes was analyzed by large-scale RT-qPCR. Heat stress/control values of gene/OsUBQ expression levels were $\log_2$ tranformed and are presented in ascending order from N22 19379 values. Bars represent the SEM.

the expression ratio (FC) between HS and control for each gene and cultivar, we transformed these values to $\log_2$FC and represent them as a graph in ascending order of induction levels from N22 19379. **Fig. 5** shows that, except for *HSP16.9A* in IR64, all genes analyzed were induced by HS at higher levels in N22 19379 than in the sensitive cultivars ($P < 0.05$). Also, the expression of some genes such as *OsADF3*, *OsCBSX5* and *AWPM-19 like* was repressed in at least one sensitive cultivar. Note that log2 transformation of FC values tends to decrease the differences; for example, the level of induction of *OsHSFA2a* in N22 19379 was 268.1-fold while in IR64 and N22 6264 it was 3.2- and 14.9-fold, respectively. These results, together with the fertility analyses, strongly suggest that the strength of the molecular heat response in anthers is crucial to ensure fertility during reproductive stages.

The ability to display a successful response to HS in the tolerant line N22 19379 may be due to an inherent capability of one or more key factors involved in this response (e.g. faster sensing, quicker signaling or improved activity), and/or differences in background expression levels of these factors. To test this last hypothesis, we compared the relative expression levels of the same set of 19 genes between anthers collected under control conditions from N22 19379, N22 6264 and IR64 plants. Expression levels of many of the 19 genes shown in **Fig. 6** were significantly different ($P < 0.05$) between sensitive and tolerant cultivars, such as *OsADF3*, *OsDMC1b* or *Hsp101*. Strikingly, background levels of *OsFKBP62b* in IR64 and N22 6264 are 325- and 7-fold higher, respectively, compared with N22 19379. Taking advantage of the 3,000 rice genomes recently sequenced and published (The 3,000 rice genomes project 2014), we used the genome sequences of IR64 (ID: CAAS_CX403) and two different accessions of N22 (IDs: IRIS_313-10150; CAAS_CX368) to perform a promoter analysis of this gene by CLC software analysis. For this, 2 kb upstream sequences of all annotated rice genes from Nipponbare (http://rapdb.dna.affrc.go.jp/download/irgsp1.html) were used as a reference to perform a de novo assembly of all three genome sequences from IR64 and the two accessions of N22. This analysis showed that only 192 bp upstream of the *OsFKBP62b* 5′-untranslated region (UTR) from N22 (both accessions have identical sequences in this region) were assembled into the Japonica's reference genome sequence. No further reads were reliably assembled upstream of this small promoter region. On the other hand, a 2 kb sequence was fully assembled in IR64 and no mismatches were detected compared with the reference sequence. Thus, promoter regions might be very different in N22, which might account for the contrasting expression levels of *OsFKBP62b* in the cultivars analyzed. Furthermore, sequence alignment of the 192 bp shared upstream regions showed only a few polymorphisms (**Supplementary Fig. S6**); two of them (positions −137 and −66) create in N22 new pentanucleotides nTTCn, which is the basic unit of heat-responsive *cis*-elements. It would be interesting to know whether these polymorphisms may affect the expression of *OsFKBP62b* in response to HS.

Discussion

Heat-induced transcriptional reprogramming in tolerant rice cultivar N22 during anthesis

To our knowledge, this is the first report that describes the genome-wide transcriptional changes underlying HS responses in N22, which over the last few years has become a model cultivar for genetic improvement for tolerance of heat and other abiotic stresses in rice. The results presented here show that reproductive tissues respond quickly (<1 h) to adjust their transcriptome in order to prevent damage produced by high temperature (38°C). The overall strategy is to activate the chaperone network on a significantly large scale in order to avoid damage of proteins and other macromolecules caused by misfolding, and to repress several TF-, signaling- and metabolic-related genes. Changes in expression of some of these

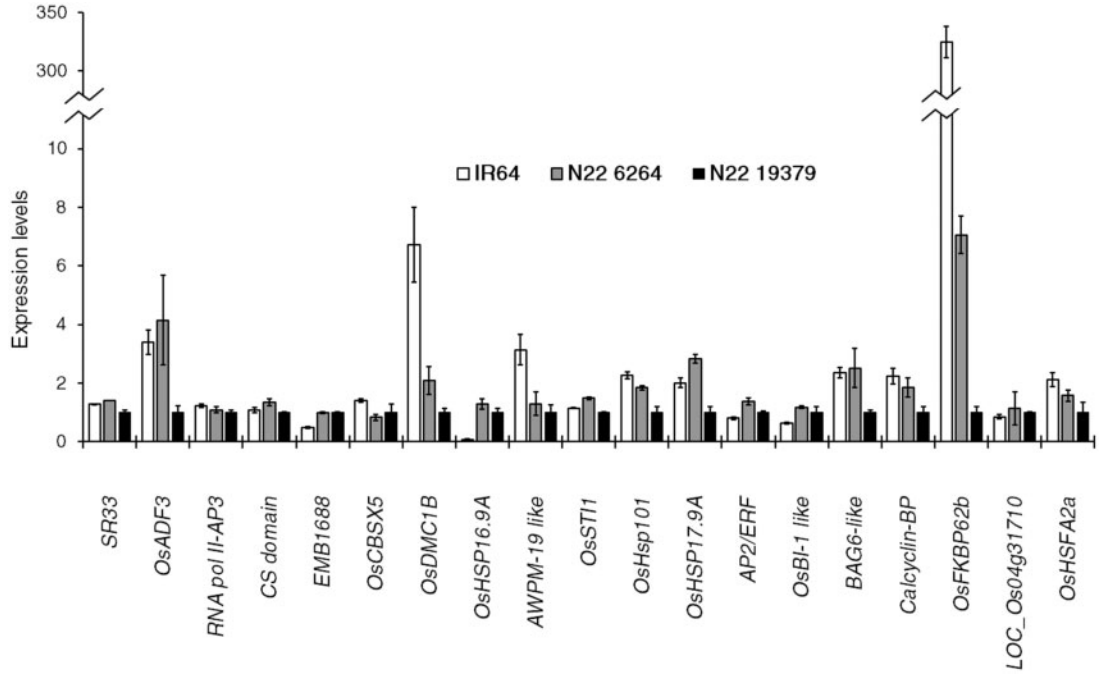

Fig. 6 Background expression levels of 19 heat-responsive genes in anthers of three contrasting cultivars. Expression of 19 selected genes was analyzed by large-scale RT-qPCR. Gene/OsUBQ expression levels and normalization to N22 19379 (value = 1) from anthers collected under control conditions from N22 19379, N22 6264 and IR64 are shown. Bars represent the SEM.

genes were validated by qPCR, and the overall results showed a good correlation with FCs obtained from the RNA-Seq analysis.

Induction of chaperone network

The rice genome encodes 25 HSFs and 74 HSP (sHSP, HSP70, HSP90, HSP100) families (Hu et al. 2009). Additionally, there are many co-chaperones, such as HSP40, or FK506-binding protein (FKBP) families considered to be core components of the chaperone machinery (Blatch 2010). In our study, a set of 46 genes representing HSFs, molecular chaperone and co-chaperone families were up-regulated during heat treatment in reproductive tissues during anthesis (**Fig. 1**; **Supplementary Table S1**). In a previous study, Jagadish and colleagues (2010) found two proteins highly induced in heat-stressed N22 during anthesis using a proteomic approach. One of these proteins is a small HSP (gene ID: LOC_Os02g52150) and the encoding gene is also highly up-regulated in our transcriptomic data set.

OsHSFA2a is one of the most HS-responsive *HSF* genes in various tissues (Chauhan et al. 2011, Jin et al. 2013). The present work also shows the predominance of this HSF and two additional *OsHSF* genes (*OsHSFA2f* and *HSFB2c*) significantly induced in these tissues.

Repression of transcription factors

With the exception of *OsHSF* genes and a few other TF genes whose expression was induced by HS, such as *AP2/ERF* (LOC_Os02g34260), most TF-encoding genes from different families were repressed after heat treatment. It is worth mentioning that Arabidopsis *RAP2.6L*, which is induced by several environmental stresses and is specifically expressed in developing pollen (Krishnaswamy et al. 2011), is the Arabidopsis gene most homologous to the heat-induced *AP2/ERF* factor. Functional analysis of this gene in rice will be interesting to understand its role in HS responses during pollen development prior to anthesis.

WRKY TFs can act as transcriptional activators or repressors, and many components of this large family in plants respond to biotic and abiotic stresses (Bakshi and Oelmuller 2014). We found nine *OsWRKY* genes that were repressed by heat; some of them have also been found to be repressed in other expression profiling studies (see **Supplementary Table S2**). *OsWRKY45* is linked to defense responses and tolerance to salt and drought (Shimono et al. 2007, Qiu and Yu 2009, Tao et al. 2011); however, the functional role of all the remaining *WRKY* genes found to be down-regulated in this study remains unknown. There are also nine *MYB* TF genes down-regulated by heat stress, and none of them has been characterized yet. Only a few *MYB* genes have been characterized as components of abiotic stress responses (El-kereamy et al. 2012, Yang et al. 2012, Xiong et al. 2014). Components of other TF family genes, such as *AP2/ERF*, *bHLH*, *NAC/NAM*, etc., were also down-regulated by heat during anthesis, raising the question of whether they may act as global repressors of heat responses. On the other hand, these genes could also be involved in the regulation of processes that have to be silenced upon HS.

Reorganization of metabolic and signaling pathways

Heat-induced transcriptional changes in the tolerant N22 variety include a massive down-regulation of genes related to metabolic processes and signal transduction pathways (**Figs. 1B**, 2B). The former implies a reorganization of lipid metabolism (**Supplementary Fig. S3**) altering the expression of several *UDP glycosyl transferase* genes, *GDSL-lipase* genes, etc.

(Supplementary Fig. S4) possibly to overcome the effects of changes in temperature on cellular membranes. Also, synthesis of aromatic compounds such as terpenes, flavonoids and phenylpropanoids was affected, which may be metabolically linked to the putative impairment in the synthesis of aromatic amino acids, which may also be affected (Supplementary Fig. S3). In fact, two other studies have linked changes in aromatic amino acid levels with HS in cowpea cells (Mayer et al. 1990) and concomitantly with phenylpropanoid pathway intermediates in Arabidopsis (Kaplan et al. 2004). On the other hand, many genes of S-Locus, LRR and receptor-like cytoplasmic kinases were repressed by heat (Supplementary Fig. S5) which can account for changes in signal transduction pathways (Fig. 2B) linked to a quick and successful heat responsiveness in N22.

General vs. specific responses to heat-stress in rice

The rationale behind improving rice germplasm successfully to face challenging conditions such as heat waves due to global climate changes requires a thorough and systematic understanding of general and specific stress responses. These responses can differ based on the cultivar subjected to study, the intensity and duration of HS, and the developmental stage in which the stress is applied. However, comparison of a number of reports in this field allowed us to identify common patterns of molecular heat responses even in conditions that seem a priori not to be comparable. Our comparison of data sets obtained from heat-treated N22 plants during anthesis with two other data sets from varieties 996 and Nipponbare during reproductive development showed that we identified 630 DEGs during heat stress, whereas Endo et al. (2009) report that >1,400 genes, and Zhang et al. (2012) that almost 2,500 genes, were deregulated. The fact that we have found a lower number of DEGs, despite having performed a genome-wide analysis, whereas both Endo et al. and Zhang et al. used microarrays, might be explained by the different statistical analyses that were applied to these methods and by the fact that Endo and colleagues used whole anthers and Zhang et al. young florets. For our analysis, we used pollinated pistils, but probably in the pistil tissue heat does not cause many transcriptional changes since the DEGs that we identified are mostly expressed in pollen. Another explanation might be that the durations of the applied heat treatment were different; Zhang and co-workers studied short/middle-term responses, between 20 min and 8 h, and Endo et al. applied the heat treatment for 2, 3, and 4 d, whereas we examined a short-term (30 min) HS response.

Comparison of data sets showed a higher overlap of deregulated genes with those published by Zhang et al. (2012) than those reported by Endo et al. (2009) (Fig. 4). This fact could be explained in three different ways. First, the duration of the applied heat treatment was different, as previously discussed. Secondly, Zhang et al. also worked with a heat-tolerant cultivar whereas Endo et al. used heat-sensitive Nipponbare (japonica type). Finally, phylogenic proximity may also provide an explanation, since Indica-type 996 is more closely related to aus-type N22 than to japonica-type Nipponbare. The differentially regulated genes shared between our study and that of Zhang et al. might help the identification of novel candidate genes conferring increased heat tolerance in rice. Moreover, some of them may be used as molecular markers to evaluate tolerance in rice at reproductive stages. During the preparation of this manuscript, another microarray-based study using tolerant and susceptible rice cultivars has been published with the transcript data used to support the metabolic programming of reproductive organs (Li et al. 2015). In that study, the authors provide a list of the 20 most up-regulated genes in pollinated pistils under combined drought–heat stress conditions in the tolerant variety N22. Fifteen out of those 20 genes (marked in red in Supplementary Table S1) are also highly up-regulated in our RNA-Seq analysis, supporting our data, while the remaining five genes are probably more specific to drought or combined drought–heat stress. In addition, Li and co-workers (2015) suggest that regulation of sugar and amino acid metabolism makes a major contribution to heat tolerance. The DEGs related to these functional categories that we found in our data sets (Supplementary Fig. S3) might participate in this regulation.

It is worth mentioning that RNA-Seq analysis performed in our study enabled us to study the whole transcriptome, in contrast to microarray analyses with a limitation in the number of genes detected. Thus, this work represents a source of novel heat-responsive genes that have not been found before, including many TFs (Supplementary Table S2). These may be interesting candidates for further functional analysis to evaluate their role in HS responses. Remarkably, only a small set of genes take part in the basal response to HS (Supplementary Table S5). Twenty-two out of 37 genes are part of a tissue-independent general response as they were also identified in another global expression profiling using heat-treated seedlings (Mittal et al. 2012). The remaining 15 genes did not behave similarly compared with heat-responsive genes in Mittal et al. suggesting that these might be involved in HS responses in reproductive organs. This small subset includes uncharacterized *bHLH* and *NAC/NAM* TF-encoding genes and *BTBA4*, the closest homolog of the salicylic acid (SA) receptor gene *NPR3* from Arabidopsis (Fu et al. 2012). Several studies have demonstrated the physiological effect of SA on heat tolerance in plants (reviewed in Horvath et al. 2007); however, no clear mechanistic interaction between SA-mediated biotic and abiotic stresses has been determined in rice. These two TFs and the putative SA receptor may constitute interesting candidates genes for further study.

Responses in the rice anther determine heat stress damage

There are many physiological processes occurring simultaneously in a very short span of time during anthesis: pollen swelling in anthers, anther dehiscence, pollen deposition on the stigmas, germination of pollen grains, and pollen tube elongation until they reach the ovaries for fertilization. All these processes are affected by increasing temperatures in

plants. Although some authors claim that pollen development and fertilization are often the critical heat-sensitive stages in plants (Zinn et al. 2010), others have suggested that anther dehiscence and pollen shedding is actually the crucial stage affected by high temperatures (Matsui et al. 1997, Matsui et al. 2001). In this work, a physiological and molecular approach was carried out in order to compare heat responses in the tolerant N22 19379 and two heat-sensitive cultivars (IR64 and N22 6264). Heat-treated IR64 plants showed an overall decrease in spikelet fertility probably due to a limited pollen production or poor viability and/or poor anther dehiscence, as the number of pollen grains counted on the stigma was low (around 11 per stigma) compared with the control conditions (>25 per stigma) and only a small percentage could germinate (**Fig. 4**). Satake and Yoshida (1978), Matsui et al. (2000 and 2001) and Jagadish et al. (2010) have shown that individual spikelets require a critical minimum number of between 10 and 20 germinated pollen grains on stigmas to be fertile. Additionally, pollen tube growth in heat-treated IR64 was poor, with the majority of tubes growing very slowly, and most of them did not reach the ovule (Jagadish et al. 2010). Similarly, heat-treated N22 6264 showed a reduced number of pollen grains on the stigmas (<20 per stigma), which suggests that processes such as anther dehiscence before pollen shedding are impaired. Although no pollen germination problems were observed in this cultivar, the reduced number of pollen grains and the slow growth of pollen tubes, as seen in Jagadish et al. (2010), might explain the observed low fertility. Additionally, the heat-sensitive N22 6264 pollen tube development is also affected under HS conditions (**Supplementary Table S7**). These results, together with previously published data, suggest that the number of viable pollen grains deposited on the stigma under HS conditions is the main factor that influences spikelet fertility. Therefore, pollen maturation in anthers before anthesis will probably have a significant role in determining the overall spikelet fertility.

On the other hand, the high proportion of heat-induced genes in N22 that are also expressed at high levels at the later stages of anther development is remarkable (**Supplementary Fig. S1**; **Supplementary Table S3**). Note that many of these genes are also up-regulated in earlier stages (0.3–0.6 mm anther length) coinciding with the establishment of pollen mother cells and meiosis, stages An3 and An4 described by Itoh et al. (2005). Microsporogenesis is also a heat-sensitive stage during pollen development (Endo et al. 2009, Zhang et al. 2012, Jagadish et al. 2013). Thus, anthers constitute a highly dynamic floral tissue that adjusts the levels of key components during development to succeed in the generation of fertile pollen.

Nineteen common heat-responsive genes were chosen for further molecular analysis of anthers before anthesis. Eighteen of these genes showed higher induction levels in the tolerant N22 than in IR64 or N22 6264 during heat treatment in anthers (**Fig. 5**). Remarkably, heat-responsive genes such as *HSFA2a*, *OsFKBP62b* or *OsHSP17.9A* are only slightly induced in IR64 and N22 6264. *HSFA2a* expression increased by 268-, 15- and 3.2-fold under HS exposure in N22 19379, N22 6264 and IR64, respectively. In similar conditions, this gene was induced >70-fold in Zhang's work and around 10-fold in Mittal's work. Similarly, there is a 108-, 10- and 3-fold induction in *OsFKBP62b* from N22 19379, N22 6264 and IR64, respectively, while an induction of 60- and 6-fold can be seen in Zhang's and Mittal's work, respectively. Also, another canonical heat-responsive gene, *Hsp101*, is not significantly induced in IR64. Taken together, these results suggest that molecular heat responses are quantitatively impaired in heat-sensitive cultivars. In summary, here we show that induction of expression levels during HS is qualitatively and quantitatively correlated with heat tolerance and can be used to evaluate tolerance to heat in other rice cultivars.

Strikingly, there are massive background levels of *OsFKBP62b* in IR64's anthers and, to a lower extent, in N22 6264. ROF1, the closest homolog of OsFKBP62b from Arabidopsis, is a peptidyl prolyl isomerase that has been described as a modulator of thermotolerance by interacting with HSP90 and affecting the accumulation of HSFA2-regulated HSPs (Meiri and Breiman 2009). Arabidopsis ROF2, the closest homolog of ROF1, can physically bind to ROF1 and be part of the complex together with HSP90 and HSFA2, but its action is opposite to that of ROF1, negatively regulating expression of small chaperones (Meiri et al. 2010). These data led us to speculate that high levels of OsFKBP62b in IR64 and N22 6264 anthers can have detrimental effects on the molecular response to HS, probably saturating the chaperone system and altering its efficiency to respond to temperature changes. It would be interesting to know if expression levels of *OsFKBP62b* correlate with heat susceptibility in other cultivars in order to use it as a reference molecular marker of heat tolerance. Furthermore, generating rice lines with altered expression levels will be informative to test whether it can confer tolerance to HS.

In conclusion, the data reported here represent a valuable resource for candidate heat tolerance gene identification, and therefore this study provides important information for breeding for heat tolerance in rice.

Materials and Methods

Plant material and growing conditions

Three *Oryza sativa* subspecies with contrasting tolerance to heat stress (N22 IRGC accession 19379, tolerant; N22 IRGC accession 6264, susceptible; and IR64, susceptible) were used in the study. Seeds were pre-germinated and sown in seeding trays with clay loam soil after breaking dormancy at 50°C for 3 d. A single 14-day-old seedling was transplanted into each pot filled with 6.0 kg of the same clay loam soil with 2 g of $(NH_4)_2SO_4$, 1 g of muriate of potash (KCl) and 1 g of single super phosphate (SSP). An additional 2.5 g of $(NH_4)_2SO_4$ was top dressed 25–30 d after transplanting.

Plants were grown in a temperature-controlled greenhouse maintained at 29/21°C day/night temperature and day/night relative humidity of 75–85% under natural sunlight conditions. HS treatments (38°C) were carried out in indoor controlled-environment or walk-in chambers (Thermoline), and a photosynthetic photon flux density of 650 µmol m^{-2} s^{-1} was supplied. Further details can be found in **Supplementary Materials S1**.

Sample collection

Between 15 and 35 plants for each experiment were used for sample collection. For HS treatment, plants were transferred to the growth chambers when three

or more primary tillers began to flower. Since plants did not flower on the same day, independent sets of plants were transferred to the growth chambers and samples were collected and pooled. For both control and stress treatments, spikelets flowering during the treatment period were collected in ice, after which spikelets were dissected to separate the pollinated pistils or anthers. Dissected floral organs were collected in tubes suspended in liquid nitrogen and stored at −80°C until further use.

Pollen count and pollen germination

Spikelets were randomly collected with minimum disturbance from primary tillers within the flowering period. Spikelets were sampled 1 h after flowering and collected into vials filled with FAA (50% absolute ethanol, 5% acetic acid, 27% formaldehyde and 18% sterilized water) as fixative. Spikelets were dissected under a stereomicroscope (Olympus SZX7; Olympus Corp.) and processed following the protocol of Lawas et al. (2013). The stigmas were cleared using 8 N NaOH for 24 h and subsequently stained with 0.2% aniline blue. The number of pollen and germinated pollen on the stigma were viewed and recorded at ×100. Images were taken with a DP70 digital camera attached to an Axioplane 2 microscope (Carl Zeiss) at ×50.

Spikelet fertility

Approximately 7–9 main tillers from three plants were used to estimate spikelet fertility for both control and stress treatments. Flowering spikelets were marked with acrylic paint following the protocol of Jagadish et al. (2007). At physiological maturity, the total number of marked spikelets and the number of grains formed were recorded and used to determine spikelet fertility.

Total RNA extraction and expression analysis

Total RNA was extracted from pools of pollinated pistils (Experiment 1) or anthers (Experiment 2) by using TRIzol® (Invitrogen). Three biological replicates were collected for each treatment (HS and C). The quality and quantity of RNA samples were assessed by gel electrophoresis and Nanodrop quantification.

A 1 μg aliquot of DNase I-treated RNA was retro-transcribed with an iScript cDNA synthesis kit (Bio-Rad). For selected genes, specific primers (listed in **Supplementary Table S8**) were designed and tested by reverse transcription-quantitative real-time PCR (RT–qPCR). Standard RT–qPCR was performed using iQ SYBR Green SuperMix (Bio-Rad) on a CFX96 Real Time System (Bio-Rad). Primer set efficiency was calculated with the CFX Manager 2.1 software. Data were normalized using *OsEF1* (LOC_Os03g08010) and *NABP* (LOC_Os06g11170) genes as reference.

Large-scale RT–qPCR was used to study the expression of heat-responsive genes from anther samples by taking advantage of the Microfluidic Dynamic Array developed by Fluidigm Corporation (Spurgeon et al. 2008). The 48 × 48 Dynamic Array Integrated Fluidic Circuit was loaded with cDNAs (sample inlets) and primer combinations (assay inlets) after specific target amplification (STA) and exonuclease I treatment. A fast cycling protocol and EvaGreen (Bio-Ras) as dye was used on a BioMark machine. The experiment was performed at the Genomics Platform of CRAG (Barcelona, Spain), following the workflow provided by the manufacturer. Three biological replicates with three technical replicates were performed for each sample. Data were normalized using the *OsUBQ* (LOC_Os02g06640) gene as reference.

Illumina sequencing

Upon treatment with TURBO DNase I (Ambion), 4 μg of RNA from each sample were used to produce sequencing libraries with the TruSeq mRNA sample preparation kit (Illumina). Sequencing of poly(A) RNA samples was carried out in multiplex (six samples per lane, single 50 bp reads) with the Illumina Hi-seq 2000 platform. Quality control of the raw sequence data was done using FastQC (Babraham Bioinformatics).

Mapping of short reads, quality analysis and assessment of gene expression analysis for RNA-Seq

Evaluation and processing of raw data was performed on the commercially available CLC Genomics Workbench v.4.7.1 (http://www.clcbio.com/genomics/). After trimming, the resulting high-quality reads were mapped onto the *Oryza* MSU7.0 database. More than 20 million reads for each sample that mapped with ≤2 mismatches were used for further analyses. The read number of each gene model was computed based on the co-ordinates of the mapped reads. A read was counted if any portion of that read's co-ordinates was included within a gene model. As CLC Genomics Workbench v.4.7.1 distributes multi-reads at similar loci in proportion to the number of unique reads recorded and normalized by transcript length, we included both unique reads and reads that occur up to 10 times in the analysis to avoid undercounting for genes that have closely related paralogs. Gene expression values were based on RPKM values. FC and $\log_2$FC were calculated in terms of RPKM of the corresponding transcripts. To obtain statistical confirmation of the differences in gene expression, *P*- and FDR values were computed using Baggerley's test on expression proportions. We applied a threshold value of $P < 0.05$ and FDR < 0.05 to ensure that differential gene expression was maintained at a significant level (5%) for the individual statistical tests. Absolute FC ≥ 2 was set as the threshold limit to obtain the DEGs. To gain insight into the biological processes associated with the regulated genes, we determined which GO annotation terms were over-represented, in both up- and down-regulated gene lists. Gene set enrichment analysis was performed with the agriGO database using singular enrichment analysis (SEA).

De novo assembly of rice promoters from IR64 and N22

Genome sequence data from IR64 (ID: CAAS_CX403) and two different accessions of N22 (IDs: IRIS_313-10150; CAAS_CX368) were downloaded from gigadb.org/dataset/200001. Sequence reads were de novo assembled using default parameters by CLC Genomics Workbench v.4.7.1. Contigs assembled were mapped to 2 kb upstream sequences of genes from reference Nipponbare (IRGSP-1.0, http://rapdb.dna.affrc.go.jp/download/irgsp1.html) and analyzed with the same software.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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