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Understanding the molecular events underpinning cultivar differences in the physiological performance and heat tolerance of cotton (*Gossypium hirsutum*)

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Abstract. Diurnal or prolonged exposure to air temperatures above the thermal optimum for a plant can impair physiological performance and reduce crop yields. This study investigated the molecular response to heat stress of two high-yielding cotton (*Gossypium hirsutum* L.) cultivars with contrasting heat tolerance. Using global gene profiling, 575 of 21854 genes assayed were affected by heat stress, ~60% of which were induced. Genes encoding heat shock proteins, transcription factors and protein cleavage enzymes were induced, whereas genes encoding proteins associated with electron flow, photosynthesis, glycolysis, cell wall synthesis and secondary metabolism were generally repressed under heat stress. Cultivar differences for the expression profiles of a subset of heat-responsive genes analysed using quantitative PCR over a 7-h heat stress period were associated with expression level changes rather than the presence or absence of transcripts. Expression differences reflected previously determined differences for yield, photosynthesis, electron transport rate, quenching, membrane integrity and enzyme viability under growth cabinet and field-generated heat stress, and may explain cultivar differences in leaf-level heat tolerance. This study provides a platform for understanding the molecular changes associated with the physiological performance and heat tolerance of cotton cultivars that may aid breeding for improved performance in warm and hot field environments.

Additional keywords: electron transport, heat stress, membrane integrity, microarray, Rubisco, quantitative real-time polymerase chain reaction.

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Introduction

Exposure to temperatures exceeding the optimal range for plant growth and development occurs frequently for crops grown under both irrigated and dryland systems. Plants have evolved a complex array of tolerance and avoidance mechanisms from a multitude of metabolic pathways to prevent or mitigate damage caused by heat stress. These mechanisms ensure plant survival during daily fluctuations in temperature, but severe and chronic exposure to high temperatures, particularly during heat waves, may significantly reduce yields. Cultivar selection for specific heat tolerance in hot environments may reduce the severity of heat-induced yield losses. Current breeding programs are evaluating yield in warm and hot growing regions, but this approach can be slow, due to variable climate. Enhanced understanding of the molecular processes by which plants adapt to and cope with heat stress may provide the foundation for a trait-based approach to support the breeding of plants with superior physiological and yield performance (Tardieu and Hammer 2012).

Plant responses to high temperature stress at a molecular level have been studied predominantly in model systems such as *Arabidopsis thaliana* (L.), although some crop species with a higher inherent heat tolerance such as sunflower (*Helianthus annuus* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.) have also been studied (Rizhsky *et al*. 2002; Rensink *et al*. 2005; Hewezi *et al*. 2008) and show similar responses to abiotic stress as model species (Rizhsky *et al*. 2004). Molecular analysis indicates that the heat response is complex and varied, involving significant changes in global gene expression (Rizhsky *et al*. 2004; Busch *et al*. 2005; Rensink *et al*. 2005; Kant *et al*. 2008; Larkindale and Vierling 2008) and multigene interactions (Humphreys and Humphreys 2005). The initial heat stress response occurs within the first 0.5–1 h (Busch *et al*. 2005; Kant *et al*. 2008) after exposure to temperatures exceeding the thermal kinetic window that permits normal enzyme function in plants (Burke *et al*. 1988) but may vary between species.

The short-term heat shock response is characterised by the synthesis of heat shock proteins (HSPs) (Malik *et al*. 1999; Hong and Vierling 2000; Nieto-Sotelo *et al*. 2002; Busch *et al*. 2005) and heat shock transcription factors (Mishra *et al*. 2002; Panchuk *et al*. 2002; Ogawa *et al*. 2007; Kant *et al*. 2008; Larkindale and

Vierling 2008; Yokotani *et al*. 2008). Initial induction of genes encoding heat stress transcription factors and HSPs is accompanied by alteration of genes associated with protein biosynthesis, folding and processing (Busch *et al*. 2005; Hewezi *et al*. 2008; Larkindale and Vierling 2008; Yokotani *et al*. 2008) and protein degradation (Rizhsky *et al*. 2002; Hewezi *et al*. 2008; Kant *et al*. 2008; Tian *et al*. 2009). Increases in respiration are associated with the upregulation of genes encoding proteins involved in the catabolism of assimilates and glycolysis (Rizhsky *et al*. 2002; Kant *et al*. 2008; Larkindale and Vierling 2008). Despite a potential decrease in energy availability, genes associated with transport (Busch *et al*. 2005; Hewezi *et al*. 2008), signalling (Busch *et al*. 2005) and overall metabolism (Busch *et al*. 2005; Hewezi *et al*. 2008), specifically nitrogen and sulfur (Kant *et al*. 2008), are induced under heat stress.

Although the optimal temperature range of 20–25°C used for *A. thaliana* (Busch *et al*. 2005; Kant *et al*. 2008; Larkindale and Vierling 2008) is considerably lower compared with an optimal temperature range of 28–32°C for cotton (*Gossypium hirsutum* L.) (Burke *et al*. 1988), HSPs and heat shock factors are induced after short-term exposure to high temperatures or water deficit stress for both species (Voloudakis *et al*. 2002; Sotirios *et al*. 2006). Studies comparing relative photosynthesis, chlorophyll fluorescence, membrane integrity, enzyme viability and stomatal conductance in cotton cultivars under high temperatures in a growth cabinet or field situation have shown that significant genotypic variation for plant physiological performance, which may contribute to heat resistance (de Ronde and van der Mescht 1997; Pettigrew and Turley 1998; ur Rahman *et al*. 2004; Rahman *et al*. 2005; Bibi *et al*. 2008; Cottee *et al*. 2010). Identification of genes that contribute to genotypic differences in higher order physiological and biochemical function may provide an opportunity to understand the molecular basis of acquired thermotolerance (c.f. Rizhsky *et al*. 2002, 2004). Although global changes in gene expression for cotton tissue under heat stress are not well understood, previous research has shown changes in the expression of single genes for cotton under heat stress. For example, Rubisco α 2 mRNA is related to decreased photosynthetic efficiency of cotton leaves under high temperatures (DeRidder and Salvucci 2007) and may contribute to compromised heat tolerance. Furthermore, inherent genotypic variability for expression of these heat-inducible genes has been largely uncharacterised between cotton cultivars.

To understand the mechanisms underpinning the plant physiological performance that may contribute to heat tolerance, the global gene expression profiles of leaf tissue exposed to 1 h of high temperature stress were compared for high-yielding cotton cultivars Sicot 53 and Sicala 45, which have dissimilar ancestry and contrasting relative heat tolerance. A recent study by Cottee *et al*. (2012) found that Sicot 53 had greater heat tolerance for components of photosynthesis including electron transport rate, photochemical efficiency of PSII, nonphotochemical quenching, membrane integrity and expression of a Rubisco-activase associated gene, than Sicala 45 under high temperatures (42°C) in a growth cabinet. Furthermore, these cultivar differences in heat tolerance were consistent with previously determined performance under hot field conditions (Cottee *et al*. 2010).

In this study, global transcriptome analysis identified 575 genes (2.6% of genes assayed), spanning a wide variety of regulatory and metabolic processes with altered expression under high temperature stress but with few expression differences between cultivars. A subset of genes differentially regulated by heat stress and associated with physiological pathways known to affect heat tolerance were further analysed using quantitative real-time PCR (Q-PCR) over a 7-h heat stress period. These genes were associated with HSPs, electron flow and ATP production, and protein and secondary metabolism. They were also differentially expressed between the two cultivars, with implications for leaf-level physiological performance and genotype-specific heat tolerance. This finding complements previous research showing genotypic differences for the molecular abiotic stress response in various cropping species (Sahi *et al*. 2003; Zhang *et al*. 2005; Dev Sharma *et al*. 2006) and suggests genotypic differences for the relative expression of genes associated with various physiological pathways under heat stress for cotton cultivars. Identification of genotype-specific responses to heat stress for cultivars that have been previously physiologically characterised for heat tolerance provides a framework for further studies using molecular and physiological profiling for identification of and breeding for improved performance in warm and hot environments.

Materials and methods

Site description and pot management

Plants were established in a glasshouse at the Australian Cotton Research Institute, Narrabri, Australia. Cotton (*Gossypium hirsutum* L.) plants were grown in, 250 mm diameter, 9-L pots, filled with a grey cracking clay soil taken from a nearby field. Each pot contained two plants (of the same cultivar) and all pots were arranged in a completely randomised design with four replicates. Pots were watered at 0700 hours daily for 2 min by drip irrigation delivering 4–4.5 L h^{-1} and water was non-limited. Nutrients were applied fortnightly with 500 mL Miracle-grow all-purpose water soluble fertiliser (Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia) at a rate of 0.013 g mL^{-1} with 2.0 g magnesium sulfate heptahydrate ($MgSO_4.7H_2O$). At the first square physiological stage, plants were transferred to a growth cabinet (a 14-h photoperiod commencing at 0600 hours with 32 ± 3°C, 64 ± 15% relative humidity (RH) and a maximum 800 µmol m^{-2} s^{-1} of PAR, and a 10-h dark period at 25 ± 5°C and 85 ± 15% RH), running on a Maxim 510 controller (Innotech Trading Australia Pty Ltd, Forest Lake, Qld, Australia). Light intensity increased stepwise from the start of the photoperiod by 30% each 30 min to a steady maximum of 800 µmol PAR m^{-2} s^{-1}. Likewise, light intensity decreased stepwise by 30% each 30 min to the end of the photoperiod. RH was allowed to follow external atmospheric conditions and hence was largely a function of temperature, with a daily average of 65%. Carbon dioxide concentration similarly reflected atmospheric conditions, with a daily average of ~360 µL L^{-1}. Plants were hand watered daily to maintain adequate soil moisture levels.

Genotypes

Normal leaf, medium maturity and high yielding (>1500 kg lint ha^{-1}) genotypes of upland cotton were established under

glasshouse conditions. Cultivar Sicot 53 was selected as a relatively thermotolerant genotype, whereas Sicala 45 was chosen as a cultivar with lower relative heat tolerance. Cultivar Sicot 53 (Constable 2000) was developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) between 1989 and 1995, and was selected over several generations for adaptation to warmer production regions in Australia (cf. Reid *et al*. 1989). Cultivar Sicala 45 (Reid 2004) was developed by CSIRO between 1996 and 2004, and was selected over several generations for earlier crop maturity and resistance to *Fusarium* wilt and, as such, the selection was predominately done in the cooler cotton production regions of Australia. Cultivar differences in heat tolerance were validated under *in situ* high temperature stress, whereby various combinations of yield, physiological and biochemical performance were decreased to a greater extent for Sicala 45 compared with Sicot 53 under high water bath temperatures (40°C), hot tents in the field (6–20°C above ambient) (Cottee *et al*. 2010) and high-temperature growth cabinets (42°C) (Cottee *et al*. 2012). Although the genotypes were morphologically similar, the coefficient of parentage (where 1.0 represents identical genotypes) was 0.109, indicating dissimilar ancestry (Bernardo 2002).

Temperature treatments

Plants were acclimated for 4 days at optimal conditions (32 : 25°C day : night) in the growth cabinet before initiation of the temperature treatment. For the control (32°C), cabinet conditions were left unchanged during the treatment period. Due to limited growth cabinet space, plants of a comparative physiological age were transferred to the controlled environment growth cabinet after the control plants had been removed. Well watered plants were exposed to heat stress by increasing the ambient air temperature to 42°C (67% RH) during the light period and 25°C (40% RH) during the dark period. The optimal (control) temperature treatment (32°C) was selected as the upper limit of the thermal kinetic window for cotton (Burke *et al*. 1988); the high temperature treatment of 42°C was selected to impose a sufficient heat load to adversely affect plant physiology and biochemistry (Reddy *et al*. 1991; Bibi *et al*. 2008).

Tissue collection and processing

The third youngest fully expanded leaf of plants at the first square physiological age was sampled for all experiments. Three leaf tissue samples were collected for each genotype under ambient or hot growth cabinet conditions (12 samples total) at 0.5 h, 1 h, 3 h and 7 h into the heat stress period, where the 0.5 h samples were collected at 0930 hours, which equated to 3.5 h into the light period. For RNA preparations, whole leaves were excised at the junction of the lamina and petiole, immediately snap frozen in liquid nitrogen and stored at –80°C. Leaves were ground to a fine powder in liquid nitrogen, a 0.1-g subsample was taken and total RNA was extracted using a hot borate buffer (Cadman *et al*. 2006).

For microarray analysis, leaf tissues sampled at 1 h were chosen based on the observation that maximum cultivar differentiation for expression of *GhRCAα2* occurred at this stage of the heat stress period (Cottee *et al*. 2012). From the genes that were significantly altered in expression after 1 h of exposure to high temperatures in the growth cabinet, 12 genes with known associations with physiological pathways implicated in heat tolerance (Table 1) were assayed at 0.5 h, 1 h, 3 h and 7 h after initiation of the high temperature stress via Q-PCR, using the optimal (32°C) cabinet data at a comparative time point as a control reference.

Microarray

RNA quality was determined on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA). Array processing was performed by The Walter and Eliza Hall Institute of Medical Research at the Australian Genome Research Facility, Parkville, Victoria, Australia. Relative expression was determined using an Affymetrix GeneChip Cotton Genome Array (Affymetrix Inc. Santa Clara, CA, USA) containing specific probes for 21 854 genes. The microarray data were submitted to the Plant Expression Database (Dash *et al*. 2012), under PLEXdb accession number GO12 and to the public National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO accession GSE41725).

Quantitative real-time PCR

Q-PCR was used to validate the treatment and cultivar differences found using microarray analysis (Busch *et al*. 2005; Ogawa *et al*. 2007; Hewezi *et al*. 2008) and for a detailed evaluation of gene expression over a short (7 h) high-temperature treatment period. Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used in accordance with the manufacturer's instructions with 5μg total RNA and oligo (dT) primers to generate cDNA. Q-PCR was performed for each gene of interest using 20 ng cDNA, 0.4 U Platinum Taq (Invitrogen), 3.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate (dNTP) and 0.5 μM primers. All three biological replicates were run with three technical replicates using a Rotor-Gene 2000 real-time cycler (Corbette Research, Sydney, NSW, Australia) with SYBR green to detect amplified product. Sequences for primers are presented in Supplementary Table S2 (available as Supplementary Material to this paper). All expression values for each gene were normalised to the expression of β-tubulin (*CO492249*, Table S2), which has been previously been found to be unaffected by heat stress (Cottee *et al*. 2012). Analysis of the Q-PCR data was performed as described previously (Cottee *et al*. 2012). For all Q-PCR data, the relative expression ratio refers to the expression of a specific gene under high (42°C) compared with ambient (32°C) temperatures for the nominated cultivar. Relative expression ratios were calculated as normalised gene expression for the heat-treated sample divided by normalised gene expression for the control sample. Relative expression ratios are indicative of the degree of change in gene expression in response to high temperatures for a nominated cultivar. A heat map was created using HeatMapper Plus (www.bar.utoronto.ca) for the expression ratios of selected genes under control compared with high temperatures, sampled at various times during the heat stress period. Data for this heat map are presented in Table S3.

Table 1. Relative expression ratios (log_2) for selected high temperature responsive genes in cotton

All genes assayed by quantitative real-time PCR are highlighted in bold. n.s., relative expression ratios that are not statistically different between treatments at $P \leq 0.05$; HSP, heat shock protein

Accession number	UniGene ID	Gene description[A]	Heat *vs* control[B,C]	Control Sicala 45 *vs* control Sicot 53[B]	Heat Sicala 45 *vs* heat Sicot 53[B]
Cell wall synthesis					
CO122215	Gra.2095	UDPglucose 6-dehydrogenase	–1.3	n.s.	n.s.
CO122431	Gra.2478	Xyloglucan endotransglycosylase	–1.7	n.s.	n.s.
CO076413	Gra.1108	Thiamine biosynthesis protein	–4.2	n.s.	n.s.
Electron flow and ATP production					
AI731438	**–**	**Mitochondrian uncoupling protein (complex IV)**	**–1.1**	**n.s.**	**n.s.**
DN800322	**Ghi.10785**	**PSII oxygen-evolving enhancer protein**	**1.1**	**n.s.**	**n.s.**
DT049835	Ghi.9450	Adenosine triphosphate citrate lyase b-subunit	–1.1	n.s.	n.s.
DT053705	Ghi.482	Phospholipid-hydroperoxide glutathione peroxidase	–2.4	n.s.	n.s.
DW499824	**Ghi.13891**	**Pyrophosphate-dependent phosphofructo-1-kinase**	**–1.3**	**n.s.**	**n.s.**
Heat stress					
CA992712	Ghi.12591	HSP 81–1; ATP binding or unfolded protein binding	2.9	n.s.	n.s.
CO072814	**Gra.3235**	**Rubisco subunit binding protein**	**1.3**	**n.s.**	**n.s.**
DT049115	**Ghi.8379**	**Low molecular weight HSP 6**	**3.2**	**n.s.**	**n.s.**
DT050385	Ghi.4817	HSP 60; ATP binding or protein binding	2.3	n.s.	n.s.
DT467180	**Ghi.5136**	**Low molecular weight HSP 3**	**2.4**	**n.s.**	**n.s.**
DT545357	**Ghi.9821**	**Mitochondrion-localised small HSP 23.6**	**1.8**	**n.s.**	**n.s.**
Redox					
CO121107	Gra.2199	Protein disulfide isomerase	1.3	n.s.	n.s.
DN757535	Ghi.10450	Glutarexodin	1.1	n.s.	n.s.
DT463913	Ghi.17876	Ethylene responsive transcriptional protein	4.1	n.s.	n.s.
DW520074.1	Ghi.7786	Ascorbate or glutathionine	–1.2	n.s.	n.s.
Lipid metabolism					
DT048069	**Ghi.3704**	**Glycerol-3-phosphate dehydrogenase (NAD^+)**	**1.4**	**n.s.**	**n.s.**
DT048453	**Ghi.4752**	**Oxidoreductase or sphingolipid delta-4 desaturase**	**–2.2**	**n.s.**	**n.s.**
DT463798	Ghi.6402	Acyl-CoA oxidase 2	1.1	n.s.	n.s.
Protein metabolism					
AJ513700	–	Small ubiquitin-like modifier polypeptide	n.s.	3.5	3.6
CO124594	**–**	**Ribonuclease H-like superfamily protein**	**n.s.**	**n.s.**	**–2.6**
DT047015	Ghi.5645	Chaperonin 20; calmodulin binding	1.8	n.s.	n.s.
DT049001	Ghi.9336	Nucleic acid binding	n.s.	4.5	4.6
DT465471	**Ghi.3468**	**Serine-type endopeptidase activity**	**n.s.**	**n.s.**	**4.9**
Secondary metabolism					
AI729300	Ghi.12062	Wax metabolism: CER1 protein	–2.0	n.s.	n.s.
AI731478	Ghi.3260	ABA-responsive protein	2.4	n.s.	n.s.
DN781087	Ghi.1492	Cytochrome P450	n.s.	2.5	2.6
DV850132	**Ghi.10747**	**Ethylene responsive element binding factor**	**3.3**	**n.s.**	**n.s.**
DW520051.1	Ghi.1874	GA regulated (GASA2-like) protein	–2.4	n.s.	n.s.
Major carbohydrate metabolism					
CO125157	Gra.2087	Hexokinase	–1.2	n.s.	n.s.
DT047184	Ghi.9897	Fructokinase-like protein	–1.0	n.s.	n.s.
DW496260.1	Ghi.11895	4-α-glucanotransterase	–1.8	n.s.	n.s.
DW515652.1	Ghi.13597	Glucose 6 phosphate or phosphate translocator 2	–2.5	n.s.	n.s.
Miscellaneous or not assigned					
DR457397	Ghi.586	Transcribed locus	n.s.	–3.9	–3.9
DT455706	Ghi.4298	Transcribed locus	n.s.	2.3	n.s.
DT458096	Ghi.11010	S-adenosyl-L-homocysteine hydrolase 2	n.s.	–3.2	–2.3
DT463647	Ghi.17858	Transcribed locus	n.s.	–3.5	–2.8
DW225231.1	Ghi.15678	Transcribed locus	n.s.	4.8	3.5
DW497887.1	Ghi.15537	HGWP repeat containing protein	n.s.	1.7	n.s.
DW509834.1	–	–	n.s.	–0.7	–4.4

[A]Gene title annotations were based on the closest *A. thaliana* sequence matching using the The Arabidopsis Information Resource (TAIR 8). All matches used for annotations had a BLASTN e-value $\leq 1 \times 10^{-10}$.

[B]Leaf tissue expression values were determined from microarrays. Expression values are ratio in the fold change between each treatment, with an adjusted *P* value of <0.05.

[C]Expression values are pooled for both cultivars (Sicot 53 and Sicala 45 ($n = 6$)) either under heat stress or at optimal temperatures.

Statistical analysis

For microarray analysis, a twofold induction or repression limit and an adjusted *P*-value of <0.05 were used to identify genes that were significantly induced or repressed by exposure to high temperatures (42°C) in the growth cabinet in comparison to a control temperature regime (32°C). Microarray expression data were assigned functional bins (Table S1) using a mapping file created by Christianson *et al*. (2010). Microarray data were visualized using MapMan software (Fig. 1) and functional bins were identified as being significantly responsive to heat stress using the Wilcoxon rank sum test with Benjamini–Yekutieli-corrected *P*-values (Usadel *et al*. 2005). For heat and control comparisons (Table 1), data presented are pooled for cultivars Sicot 53 and Sicala 45 (six biological replicates per treatment temperature) to increase the sample size for looking at large-scale changes in the relative expression of a particular gene under high (42°C) temperatures compared with expression at optimal (32°C) temperatures. For cultivar comparisons (control Sicala 45 compared with control Sicot 53, heat Sicala 45 compared with heat Sicot 53; Table 1), the data presented are pooled for three biological replicates for each cultivar.

For Q-PCR temperature comparisons, restricted maximum likelihood was conducted separately for Sicala 45 or Sicot 53 for normalised expression under high and control temperature conditions over the heat stress period (0.5 h, 1 h, 3 h and 7 h), where the fixed model was temperature × time and the random model was biological replicate within technical replicate.

Cultivar differences for expression under heat stress are indicated by asterisks (*) in Fig. 2. For these cultivar comparisons, restricted maximum likelihood was conducted for the relative expression ratio (ratio of expression under high and control conditions) of a nominated gene for each time point during the heat stress period (0.5 h, 1 h, 3 h and 7 h), where the fixed model was cultivar by time and the random model was time, biological replicate or technical replicate. Significant cultivar differences indicate a large magnitude of difference between the relative expression ratio for cultivar Sicot 53 and that of Sicala 45. For Fig. 2, mean relative expression ratios are presented on a $\log_2$ scale.

Results

Leaf tissue (three biological replicates per treatment) obtained from cotton cultivars Sicot 53 and Sicala 45 under optimal (32°C) or high (42°C) temperatures in growth cabinets was evaluated to determine the molecular response of cotton leaf tissue to short-term heat stress. Leaf tissue was collected from plants at the first square stage, which is the start of reproductive development in cotton. Initially, transcriptome analysis was conducted for

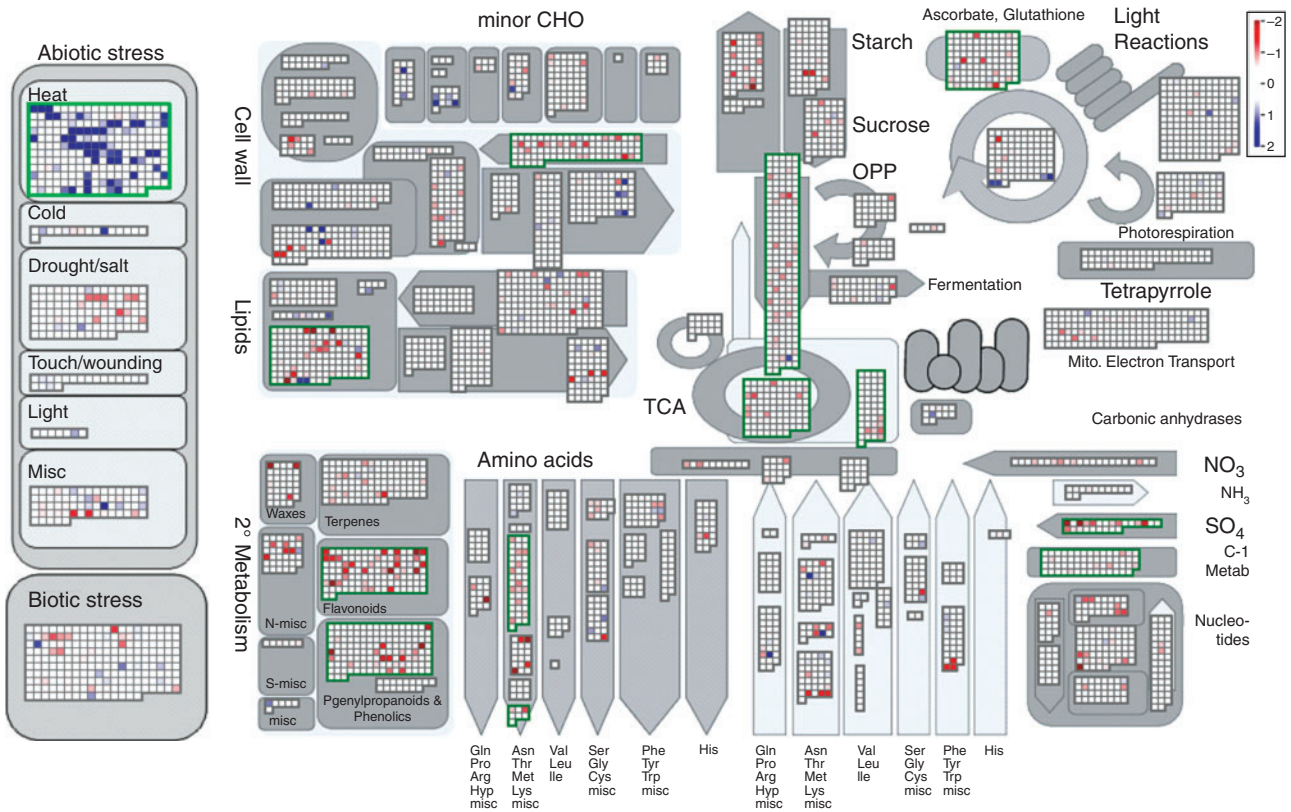

Fig. 1. Graphical representation of the relative expression of metabolism and stress genes for cotton grown under control (32°C) and high (42°C) temperatures. Red squares represent genes for which gene expression decreased; blue squares represent genes for which expression was upregulated on a $\log_2$ scale for plants grown under heat stress, compared with plants grown under optimal temperatures. Colour intensity reflects the extent of change in expression, with a more intense colour meaning a larger change in abundance. Functional bins identified as being significantly changed by high temperatures are outlined in green. TCA, tricarboxylic acid; OPP, oxidative pentose-phosphate.

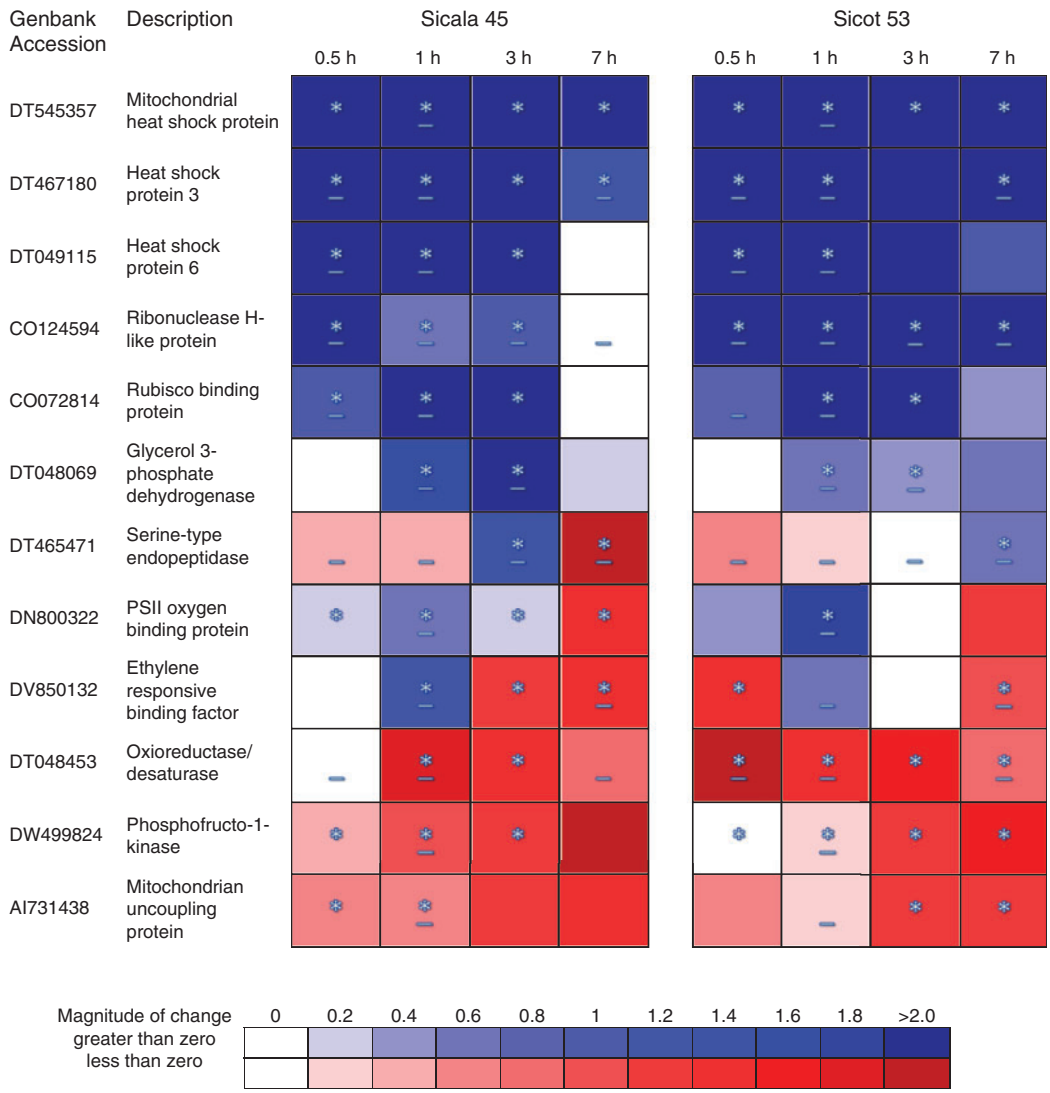

Fig. 2. Heat map of gene expression in heat-stressed cotton leaves over time. Selected genes were assayed by quantitative real-time PCR for expression in leaf tissue of cultivars Sicot 53 and Sicala 45 at various time points under heat stress. Mean relative expression ratios are presented on a $\log_2$ scale. Blue boxes indicate increased expression under high (42°C) temperatures compared with control (32°C) temperatures, and red boxes indicate decreased expression. Colour intensity reflects the degree of change in expression ($\log_2$). Times during the heat stress period where $P \leq 0.05$ for Sicot 53 transcript levels compared with Sicala 45 are represented by asterisks for the control or by a white dash for those under heat stress . Expression ratios are presented in Table S2.

leaf tissue at 1 h into the heat stress period to determine general responses to heat stress and then cultivar-specific responses to heat stress. Q-PCR was then used to determine the expression of selected genes associated with specific physiological processes over the entire 7-h heat stress period.

Heat stress caused changes in global gene transcription in cotton leaf tissue

To identify genes and gene pathways involved in the heat stress response, leaf tissue of cultivars Sicot 53 and Sicala 45 were analysed for global gene transcription after exposure to 1 h of optimal (32°C) or high (42°C) temperatures using Affymetrix Cotton GeneChip Genome arrays. The 1-h time point was chosen for global gene profiling based on cultivar differentiation for expression of the Rubisco α2-associated gene (*GhRCAα2*) at this stage during a 7-h high-temperature (42°C) treatment (Cottee *et al*. 2012). Genes altered in expression were expected to be associated with one of three classes: general heat stress responsive (altered in both cultivars by heat stress), cultivar-specific responses to heat stress or constitutive differences between cultivars unrelated to heat stress.

To determine genes consistently altered by general heat stress, data from both cultivars were pooled (six biological replicates) and differentially expressed genes were identified using a induction or repression ratios ≥2 and an adjusted *P* of <0.050.

A total of 575 of the 21 854 genes assayed on the GeneChip (Affymetrix) (2.6%) were differentially expressed. Heat-responsive genes identified spanned a wide variety of regulatory and metabolic process, whereby ~60% were induced and 40% were downregulated under heat stress. Approximately one-third (30%) of genes altered in response to heat stress had no assigned function for cotton (Fig. 1). Pathways involved in abiotic stress (11%), protein metabolism (24%), carbohydrate metabolism (6%), signalling and transport (9%), cell and development (7%), secondary metabolism (7%), and electron transport and ATP production (2%) comprised the major proportion of known gene groups affected by heat stress (Fig. 1). Genes involved in the abiotic stress pathway were induced ($P < 1 \times 10^{-20}$, Table S1) with 49 of the 61 abiotic stress-related genes altered being specifically associated with heat stress mediation (Fig. 1).

Physiological and biochemical pathways associated with growth and development such as secondary metabolism, cell wall synthesis, sulfur assimilation, glycolysis and the tricarboxylic acid (TCA) cycle were generally downregulated in the response to heat stress (Table S1). Expression for 8 out of 14 genes associated with the electron flow and the ATP pathway was downregulated under heat stress, including the genes associated with the glycolysis, TCA cycle and oxidative phosphate pathways (Fig. 1). In conjunction with increased transcript levels for genes regulating lipid and protein degradation (Table 1), this implies that a decreased capacity for photosynthesis and electron transport for leaves exposed to high temperatures was associated with a heat stress response.

Heat stress caused cultivar-specific gene expression changes

To investigate specific cultivar responses to high temperatures, the 1-h gene expression data were analysed by directly comparing heat-stressed Sicot 53 leaf tissue to heat-stressed Sicala 45 leaf tissue. Twelve genes altered in expression between Sicot 53 and Sicala 45 under control temperatures or under heat stress (Table 1); these were predominantly involved in protein and lipid metabolism or were of unknown function. Of these, eight genes were constitutively different and four genes were heat-responsive (Table 1). A HGWP repeat protein-associated gene (*DW497887.1*) and a gene with unknown function (*DT455706*) were heat-responsive, but cultivar differences were only determined under control (32°C) temperatures. Under heat stress, five genes were downregulated and five genes were induced for Sicala 45 compared with Sicot 53 (Table 1). However, only the expression of the serine-type endopeptidase gene (*DT465471*) and a ribonuclease H-like protein-associated gene (*CO124594*) were not significant when the cultivars' expression profiles were compared under control (32°C) temperatures (Table 1), indicating that the expression of the majority of these gene responses represent constitutive genotypic differences that may be associated with differing heat stress tolerance but are not exclusively heat stress-related.

Expression analysis on a 7-h heat stress time course

To validate and extend the microarray data, the serine-type endopeptidase-related (*DT465471*) and ribonuclease H-like superfamily protein (*CO124694*) genes that possessed differential expression between the two cultivars, and a selection of 10 genes that were significantly altered in their expression following 1 h of heat stress, which have either a known or hypothesised role in the response to heat stress, were assayed over a 7-h heat stress time course by Q-PCR. Three biological replicates with three technical replicates were performed on Sicot 53 and Sicala 45 tissue taken from both control (32°C) and high temperatures (42°C) over four different time points (0.5 h, 1 h, 3 h and 7 h). Representative genes chosen for analysis were involved in lipid and protein metabolism as well as protective proteins (Table 1). Protective proteins specifically associated with Rubisco (*CO072814*) and mitochondria (*DT545357*) were selected as important contributors to the maintenance of photosynthesis and electron transport under heat stress.

Apart from the gene expression of the ethylene responsive binding factor gene (*DV850132*) and the mitochondrion uncoupling protein gene (*DI731438*) for Sicot 53, all genes assayed using Q-PCR possessed significantly differential heat stress gene expression at 1 h for both Sicot 53 and Sicala 45, confirming the microarray data (Fig. 2).

Expression of genes associated with HSPs, PSII oxygen-evolving protein and glycerol 3-phosphate dehydrogenase was significantly induced under heat stress for both cultivars throughout the time course with the degree of differential expression decreasing by 7 h (Fig. 2). Expression was highest for the HSPs located at the mitochondrial membrane, reaching a peak relative expression ratio of 47 for Sicot 53 and 48 for Sicala 45 at 3 h (Fig. 2). Genes associated with metabolism were mostly significantly downregulated in response to high temperature stress throughout the time course for both cultivars (Fig. 2).

The serine-type endopeptidase related gene (*DT465471*) and the ribonuclease H-like superfamily protein gene (*CO124594*) were selected for further analysis based on significant differences in expression between heat-stressed Sicot 53 and Sicala 45 at 1 h (Table 1). This cultivar difference in expression was validated by Q-PCR and also occurred at later time points at 3 h and 7 h for both genes (Fig. 2). The increased sensitivity and specificity of Q-PCR also revealed significantly different cultivar expression levels at 1 h of heat stress for the other 10 selected genes (Fig. 2), which were not found using global gene profiling (Table 1). Cultivar differences for expression after 1 h heat stress were generally continued throughout the entire 7 h time course and were primarily associated with the magnitude of differences in relative gene expression ratios rather than absolute differences (presence *vs* absence) between the cultivars under heat stress. Relative differences in expression between the optimal and hot temperature treatments generally reached a maximum at 3 h and declined to a minimum at the end of the photoperiod, and cultivar differences in relative expression were generally more apparent at the beginning of the photoperiod (Fig. 2). As such, cultivar differences for heat-induced expression were most easily identified after 1 h or 3 h of heat stress.

Discussion

When cotton plants are grown in hot environments, their ability to withstand temperatures exceeding a thermal optimum of 32°C contributes significantly to growth, development and

subsequently yield in cropping systems. Although cotton-specific heat-responsive genes have been identified under drought stress at a molecular level (Rodriguez-Uribe and Zhang 2009; Payton *et al*. 2011; Padmalatha *et al*. 2012; Park *et al*. 2012), the heat stress response of cotton plants under elevated temperature and well watered conditions has not yet been characterised. To determine the heat stress response of cotton, heat-responsive genes and pathways were identified for leaves sampled under after 1 h incubation at either control (32°C) or elevated (42°C) temperatures in the growth cabinet. This high temperature stress reflects the temperatures frequently experienced in cotton production areas. Changes in gene expression under the heat stress treatment are thus physiologically relevant to commercial crop production systems. Furthermore, to reflect the heat stress experienced under irrigated cotton production systems, soil moisture was not limited in this experiment, ensuring that all treatment differences were attributed specifically to high temperature stress and are hence indicative of heat tolerance.

Gene transcription changes in leaves of heat stressed cotton plants

Global gene transcription for plants grown under high temperatures significantly altered ~2.6% of the genes assayed, which is considerably lower than 11% (Busch *et al*. 2005) and 21% (Larkindale and Vierling 2008) changes in expression under heat stress for *A. thaliana*. These differences may be partially attributed to a higher magnitude of the heat stress imposed (38–45°C) relative to a thermal optimum of 20–25°C for *A. thaliana* (Busch *et al*. 2005; Kant *et al*. 2008; Larkindale and Vierling 2008). Alterations in gene expression under high temperatures were associated with a range of regulatory metabolic pathways, with a significant upregulation of genes primarily involved in protection proteins and transcription factors, as well as downregulation of genes involved in growth and development (Fig. 1). Widespread suppression of genes involved in the electron flow and energy transfer pathway in conjunction, with downregulation of genes associated with metabolism, cell wall synthesis and sulfur assimilation may partially explain the decreased capacity for photosynthesis (30%), electron transport (12%) and membrane integrity (23%) under heat stress (Cottee *et al*. 2012). Additionally, induction of genes encoding redox regulating enzymes indicate increased oxidative stress and are consistent with large increases in non-photochemical quenching (42%) under heat stress (Cottee *et al*. 2012). These trends are consistent with the initial heat stress response of potato (Rensink *et al*. 2005), tobacco (Rizhsky *et al*. 2002), sunflower (Hewezi *et al*. 2008), *Festuca* sp. (Zhang *et al*. 2005) and *A. thaliana* (Rizhsky *et al*. 2004; Busch *et al*. 2005; Lim *et al*. 2006; Kant *et al*. 2008), and indicate that plants respond at a molecular level in a similar manner to temperatures well above their thermal optimum.

Gene transcription changes differed for heat-stressed cotton cultivars

In addition to changes in the general heat stress response, cultivar-specific heat responsive genes and related pathways were identified for high-yielding cotton cultivars Sicot 53 and Sicala 45, which have dissimilar ancestry and differing heat tolerance performance (Cottee *et al*. 2010). Subsequent analysis in controlled growth cabinets revealed that Sicot 53 had higher heat tolerance for components of photosynthesis including electron transport rate, the photochemical efficiency of PSII, nonphotochemical quenching, membrane integrity and expression of a Rubisco-activase associated gene than Sicala 45 under high (42°C) temperatures (Cottee *et al*. 2012). Large-scale transcriptome analysis of these two cultivars under high temperature stress identified genes that were differentially regulated by heat stress but can also potentially reveal genes whose expression are significantly different between the cultivars and so may be associated with cultivar performance under heat stress.

Twelve genes were found to possess significant cultivar expression difference, eight of which were constitutive and four of which were heat responsive. Of these 12 genes, 10 were found to possess significant cultivar expression differences when the heat-stressed cultivar leaf samples were directly compared. Furthermore, only two were specific to heat stress (*DT465471* and *CO124594*) and did not represent inherent differences for cultivar expression under control temperatures. For these two genes, Q-PCR verification confirmed cultivar differences in transcript levels over the entire heat stress period. Furthermore, for 10 additional genes that were differentially expressed under heat stress but not between cultivars, further analysis using Q-PCR found cultivar differences for expression that were associated with changes in magnitude of the differential expression rather than the presence or absence of differential expression. Therefore, due to its relative lack of sensitivity and specificity compared with Q-PCR in tetraploid cotton (Christianson *et al*. 2010), our GeneChip (Affymetrix) data may underestimate the extent of significant cultivar differences for heat tolerance.

Genes associated with HSPs were induced under heat stress

Large differences in expression were observed for genes associated with HSPs and heat shock transcription factors (77% of induced abiotic stress genes), indicating that the assay imposed heat stress in the absence of water deficit stress (Fig. 1). This data aligns with previous research showing widespread upregulation of HSP and heat shock transcription factors genes in *A. thaliana* (Kant *et al*. 2008; Larkindale and Vierling 2008) and are commensurate with a reported 10-fold increase in HSP synthesis for cotton plants grown in hot environments (de Ronde *et al*. 1993). Previous research undertaken for other plant species suggests that both HSPs and heat shock transcription factors contribute to short-term heat tolerance (Howarth 1991; Mishra *et al*. 2002; Charng *et al*. 2007) as well as acclimation to heat stress (Heckathorn *et al*. 1998; Larkindale and Vierling 2008). This tolerance may be attributed to HSP-dependent protection from proteotoxic effects (Busch *et al*. 2005), as well as mitigating the effects of heat stress on whole-plant physiological and biochemical function (Heckathorn *et al*. 1998; Leone *et al*. 2003), in addition to protection against other abiotic stresses (Rizhsky *et al*. 2004; Busch *et al*. 2005; Kant *et al*. 2008; Larkindale and Vierling 2008).

Cultivar specificity for HSP gene expression at the 1-h time point reflected increased transcript levels over the 7-h time course

(Fig. 2) for Sicala 45, which is consistent with previous research detecting cultivar specificity for HSP levels in glasshouse-grown cotton using a protein extraction assay (de Ronde *et al*. 1993) and *Festuca* species using global transcript profiling (Zhang *et al*. 2005). Furthermore, transcript levels for genes encoding HSPs that interact specifically with electron transfer through photosynthetic (*CO072814*) and respiratory (*DT545357*) pathways were higher for Sicala 45 compared with Sicot 53 during the initial stages of heat stress (Fig. 2). This may facilitate protection of photosynthesis and electron transport under high temperatures (Heckathorn *et al*. 1998; Salvucci 2008). However, increases in the synthesis and activity of HSPs and transcription factors are not always sufficient to protect and maintain energy production during sustained heat (Taiz and Zeiger 2006; Kant *et al*. 2008). In this study, increased transcript levels for the Rubisco-interacting HSP gene (*CO072814*) for Sicala 45 compared with Sicot 53 were not sufficient to protect photosynthetic enzymes from cultivar-specific heat-induced damage; transcript levels for Rubisco activase were previously shown to be lower for Sicala 45 compared with Sicot 53 after 0.5 h and 1 h of heat stress (Cottee *et al*. 2012).

Cultivar differences for expression of energy production-associated genes were consistent with previously measured physiological performance

Genes involved in energy production were largely downregulated and consistent with those seen in *A. thaliana* (Kant *et al*. 2008), and contributed to a ~5% overall change in gene expression under heat stress. Although protective proteins involved in photosynthetic and respiratory pathways were upregulated under heat stress (Table 1), the decreased expression of 90 genes associated with the electron flow and ATP pathways (Fig. 1) implied a decreased capacity for photosynthesis and energy availability during heat stress (Rizhsky *et al*. 2002; Zhang *et al*. 2005). These data reflect previously measured decreased electron transport, photosynthesis and Rubisco activase (Cottee *et al*. 2012). Furthermore, using PCR validation, genes associated with energy production and availability following 1 h of heat stress were more strongly upregulated and more moderately downregulated for Sicot 53 compared with Sicala 45, which reflects previously measured decreases in electron transport and photosynthesis for Sicala 45 compared with Sicot 53 during extended periods of heat stress. This occurred despite a disassociation in the time taken for maximal genotypic differentiation using molecular (1 h) and physiological (3 days) techniques, suggesting that the initial heat shock and complex heat stress responses are similar for cotton genotypes Sicot 53 and Sicala 45 (c.f. Shavrukov 2013).

Increased photoinhibition (non-photochemical quenching) for Sicala 45 under heat stress compared with Sicot 53 (Cottee *et al*. 2012) may contribute to decreased photosynthetic capacity. This may be partially attributed to the lower viability of serine-type endopeptidase, which is responsible for the cleavage of heat-denatured D1 protein in the thylakoid membrane, as well as increased downregulation of mitochondrial uncoupling proteins (Fig. 2), thus resulting in a disruption of linear electron flow and a reduced capacity for restricting the generation of reactive oxygen species, and restricting PSII (Haußühl *et al*. 2001) and the TCA cycle (Smith *et al*. 2004) in heat-damaged plants. Upregulation of genes associated with PSII enhancer proteins (Fig. 2) may also contribute to genotype-specific cellular heat tolerance and acclimation under moderate heat stress by increasing the thermal stability of the heat sensitive oxygen-evolving complex for transporting electrons through PSII and thus photosynthetic capacity (Kimura *et al*. 2002).

Correlated with observed decreases in electron transport rate and photosynthesis, increased upregulation of a gene associated with glycerol-3-phosphate dehydrogenase (Fig. 2) and greater downregulation of a gene associated with phosphofructo-1-kinase for Sicala 45 (Fig. 2) compared with Sicot 53 may indicate differences in the reliance on alternative energy pathways such as glycolysis and lipid degradation for heat-sensitive cotton cultivars under high temperature stress (Zhang *et al*. 2005). Downregulation of genes associated with glycolysis and the oxidative pentose pathway under heat stress in this experiment (Table 1) is in contrast to increased reliance on these pathways for tobacco under heat stress (Rizhsky *et al*. 2002), indicating alternate mechanisms of heat alleviation in various plant species and highlighting the importance of profiling various crop species for performance under heat stress (Boscaiu *et al*. 2012).

Decreases in transcript levels for genes associated with photosynthesis and the electron transport rate were reflected in decreased expression of genes associated with growth and development, and increased expression of genes associated with protein, lipid and RNA degradation. Expression of a large proportion of genes associated with growth and development, such as secondary metabolism, cell wall synthesis and sulfur assimilation, were generally downregulated in response to high temperature (Fig. 1), which corresponds to similar decreases for these pathways in heat-stressed *A. thaliana* (Kant *et al*. 2008) and tobacco plants (Rizhsky *et al*. 2002). Furthermore, decreases in transcript levels for genes associated with protein and secondary metabolism under high temperatures were greater for Sicala 45 across the 7-h time course compared with Sicot 53. Although the exact mechanism is still undefined, reduced expression of the genes associated with ethylene-responsive binding factor 2, and oxioreductase or sphingolipid delta-8 desaturase may reduce cell expansion (Fujimoto *et al*. 2000). This may also contribute to reduced membrane integrity, which is associated with ion leakage due to increased Bax-induced cell death (Ogawa *et al*. 2005) and changes in membrane lipid composition (Ryan *et al*. 2007). Reduced expression of the genes associated with secondary metabolism may partially contribute to the observed decrease in membrane integrity under high temperatures for Sicala 45 (Cottee *et al*. 2012). This may limit photosynthetic capacity (Sullivan 1971) and subsequently reduced tolerance to nutrient (Ryan *et al*. 2007) and heat stress (Tanaka *et al*. 2005).

Conclusions

Using global gene profiling, this research indicates significant changes to molecular pathways involving the regulation of protective proteins, electron flow and energy production, metabolism, and cell wall synthesis and modification. These results suggest a complex response of cotton plants to high temperature stress that is similar to that found for other plants,

such as *A. thaliana*. By comparing two high-yielding cultivars with established differences in their physiological responses to heat stress, genes associated with heat tolerance mechanisms were identified, including genes associated with protective proteins, electron transport and energy availability, and lipid, protein and secondary metabolism. Q-PCR data indicate that the differences in these cultivar expression profiles represent magnitude differences rather than the presence or absence of expression. As a result, absolute levels of expression of genes may be correlated with heat tolerance. Determination of small yet consistent and physiologically relevant cultivar differences at a molecular level indicates that the mechanisms of heat tolerance may not be so genetically complex and may hence be amendable to selective breeding. This study provides a platform for understanding plant performance under heat stress in cotton and could potentially aid in the breeding for improved performance in warm and hot environments via marker-assisted selection.

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