THERMOTOLERANCE OF COTTON

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Declaration of Originality

This thesis reports the original work of the author, except as otherwise stated. It has not been submitted previously for a degree at this or any other university.

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Abstract

The Australian cotton industry has developed high yielding and high quality fibre production systems and attributes a significant contribution of this achievement to highly innovative breeding programs, specifically focused on the production of premium quality lint for the export market. Breeding programs have recently shifted attention to the development of new germplasm with superior stress tolerance to minimise yield losses attributed to adverse environmental conditions and inputs such as irrigation, fertilisers and pesticides. Various contributors to yield, such as physiology, biochemistry and gene expression have been implemented as screening tools for tolerance to high temperatures under growth cabinet and laboratory conditions but there has been little extension of these mechanisms to field based systems.

This study evaluates tools for the identification of specific genotypic thermotolerance under field conditions using a multi-level 'top down' approach from crop to gene level. Field experiments were conducted in seasons 1 (2006) and 3 (2007) at Narrabri (Australia) and season 2 (2006) in Texas (The United States of America) and were supplemented by growth cabinet experiments to quantify cultivar differences in yield, physiology, biochemical function and gene expression under high temperatures. Whole plants were subjected to high temperatures in the field through the construction of Solarweave® tents and in the growth cabinet at a temperature of 42 ^{o}C. The effectiveness of these methods was then evaluated to establish a rapid and reliable screening tool for genotype specific thermotolerance that could potentially improve the efficiency of breeding programs and aid the development to high yielding cultivars for hot growing regions.

Cotton cultivars Sicot 53 and Sicala 45 were evaluated for thermotolerance using crop level measurements (yield and fibre quality) and whole plant measurements (fruit retention) to determine the efficacy of these measurements as screening tools for thermotolerance under field conditions. Sicot 53 was selected as a relatively thermotolerant cultivar whereas Sicala 45 was selected as a cultivar with a lower relative thermotolerance and this assumption was made on the basis of yield in hot and cool environments under the CSIRO Australian cotton breeding program. Yield and fruit retention were lower under tents compared with ambient conditions in all 3 seasons. Yield and fruit retention were highly correlated in season 1 and were higher for Sicot 53 compared to Sicala 45 suggesting that fruit retention is a primary limitation to yield in a hot season. Thus yield and fruit retention are good indicators of thermotolerance in a hot season. Temperature treatment and cultivar differences were determined for fibre quality in seasons 1 and 3; however, quality exceeded the industry minimum thereby indicating that fibre quality is not a good determinant of thermotolerance.

Physiological determinants of plant functionality such as photosynthesis, electron transport rate, stomatal conductance and transpiration rate were determined for cultivars Sicot 53 and Sicala 45 under the tents and an index of these parameters was also analysed to determine overall plant physiological capacity in the field. Physiological capacity was also determined under high temperatures in the growth cabinet using a light response curve at various levels of photosynthetically active radiation (PAR). Photosynthesis and electron transport rate decreased, whilst stomatal conductance and transpiration rate increased under the tents as well as under high temperatures in the growth cabinet. Photosynthesis and electron transport rate were higher for Sicot 53 but stomatal conductance and transpiration rate were higher for Sicala 45 under the tents. No cultivar differentiation was evident for plants grown under high temperatures in the growth

cabinet. Temperature treatment and cultivar differences in physiological function were greater in a hot year (season 1), thereby indicating the importance of cultivar selection for thermotolerance in the presence of stress. Electron transport rate was correlated with yield in season 1, thus suggesting the suitability of this method for broad genotypic screening for thermotolerance under field conditions.

Biochemical processes such as membrane integrity and enzyme viability were used to determine cultivar specific thermotolerance under high temperature stress in the laboratory, field and growth cabinet. Electrolyte leakage is an indicator of decreased membrane integrity and may be estimated by the relative electrical conductivity or relative cellular injury assays. The heat sensitivity of dehydrogenase activity, a proxy for cytochrome functionality and capacity for mitochondrial electron transport, may be quantified spectrophotometrically. Cellular membrane integrity and enzyme viability decreased sigmoidally with exposure to increasing temperatures in a water bath. Membrane integrity was higher for Sicot 53 compared with Sicala 45 under the tents and under high temperatures in the growth cabinet. No temperature treatment or cultivar differences were found for enzyme viability under the tents; however, enzyme viability for Sicala 45 was higher in the growth cabinet compared with Sicot 53. Relative electrical conductivity was strongly correlated with yield under ambient field conditions and under the tents, suggesting impairment of electron flow through photosynthetic and/or respiratory pathways, thus contributing to lower potential for ATP production and energy generation for yield contribution. Thus, the membrane integrity assay was considered to be a rapid and reliable tool for thermotolerance screening in cotton cultivars.

Gene expression was examined for cultivars Sicot 53 and Sicala 45 grown under high (42 ^{o}C) temperatures in the growth cabinet. Rubisco activase expression was quantified using

quantitative real-time polymerase chain reaction analysis and was decreased under high temperatures and was lower for Sicala 45 than Sicot 53. Maximum cultivar differentiation was found after 1.0 h exposure to high temperatures and hence, leaf tissue sampled from this time point was further analysed for global gene profiling using cDNA microarrays. Genes involved in metabolism, heat shock protein generation, electron flow and ATP generation were down-regulated under high temperatures in the growth cabinet and a greater number of genes were differentially expressed for Sicala 45, thereby indicating a higher level of heat stress and a greater requirement for mobilisation of protective and compensatory mechanisms compared with Sicot 53. Cultivar specific thermotolerance determination using gene profiling may be a useful tool for understanding the underlying basis of physiological and biochemical responses to high temperature stress in the growth cabinet. There is future opportunity for profiling genes associated with heat stress and heat tolerance for identification of key genes associated with superior cultivar performance under high temperature stress and characterisation of these genes under field conditions.

This research has identified cultivar differences in yield under field conditions and has identified multiple physiological and biochemical pathways that may contribute to these differences. Future characterisation of genes associated with heat stress and heat tolerance under growth cabinet conditions may be extended to field conditions, thus providing the underlying basis of the response of cotton to high temperature stress. Electron transport rate and relative electrical conductivity were found to be rapid and reliable determinants of cultivar specific thermotolerance and hence may be extended to broad-spectrum screening of a range of cotton cultivars and species and under a range of abiotic stress. This will enable the identification of superior cotton cultivars for incorporation into local breeding programs for Australian and American cotton production systems.

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Abbreviations

Abbreviation	Term Represented
Abs	Absorbance at 530 nm
AHTT	Acquired high temperature tolerance
DNA	Deoxyribonucleic acid
ETR	Electron transport rate ($\mu mol\ e^{-1}\ m^{-2}\ s^{-1}$)
FEC	Final electrical conductivity
HSP	Heat shock protein
IEC	Initial electrical conductivity
PAR	Photosynthetically active radiation
PSII	Photosystem II
qRT-PCR	Quantitative real-time polymerase chain reaction
RCI	Relative cellular injury
REC	Relative electrical conductivity
RH	Relative humidity (%)
RNA	Ribonucleic acid
RNAse	Ribonuclease
SCY	Seed cotton yield
TTC	2, 3, 5-triphenyl tetrazolium chloride

Publications by the Candidate Relevant to the Thesis

Cottee NS, Bange M, Tan D, Campbell L (2008) Identifying cotton cultivars for hotter temperatures. In 'Proceedings of the 14th Australian Cotton Conference'. Broadbeach, Gold Coast, 12 – 14th August, 2008.

Cottee NS, Bange MP, Tan DKY, Campbell LC (2006) Identification of thermotolerance in cotton. In 'Proceedings of the 13th Australian Cotton Conference'. Broadbeach, Gold Coast, 8 – 10th August, 2006.

Cottee NS, Tan DKY, Bange MP, Cheetham JA (2007) Simple electrolyte leakage protocols to detect chilling tolerance in cotton genotypes. In 'Proceedings of the 4th World Cotton Research Conference'. Lubbock, TX, USA, 10 – 14th September, 2007.

Cottee NS, Tan DKY, Cothren JT, Bange MP, Campbell LC (2007) Screening cotton cultivars for thermotolerance under field conditions In 'Proceedings of the 4th World Cotton Research Conference'. Lubbock, TX, USA, 10 – 14th September, 2007.

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Chapter 1 Introduction

1.1 Cotton production systems

Cotton production for fibre and oilseed extends across five continents, encompassing more than 100 nations and constitutes a total land area in excess of 30 million hectares of dryland and irrigated farming systems (Australian Bureau of Agricultural and Resource Economics 2008).

The total land base used for cotton production has remained fairly stable since the 1950's but global production has increased (Australian Bureau of Agricultural and Resource Economics 2008). This may be attributed to improved yields on a per hectare basis as a direct result of specific breeding programs and best management practises encompassing new technologies. Increased market competition from synthetic fibres and increased competition for land from biofuels and other commodities has meant that the industry as a whole needs to set new targets for production, quality and price to ensure the sustainability of cotton communities.

The United States of America, India and China are the largest producers of cotton. Although Australia contributes less than 1% of global cotton production (Australian Bureau of Agricultural and Resource Economics 2008), the industry focus is on lint quality and plays an integral role in exports to high quality markets. The Australian cotton industry is highly innovative and achieves high yields that are most likely attributed to rapid uptake of research underlying high input agronomic management (Hearn and Fitt 1992) and plant breeding for local environments.

Current aims of the Australian cotton industry include the development of superior germplasm for lint quality and contribution to a sustainable production system including high water use efficiency, high and low temperature tolerance, and pest and disease

resistance (Constable *et al.* 2001; Cotton Research and Development Corporation 1995; Thomson *et al.* 2004; Whyte and Conlon 1990).

1.2 **Breeding for high temperature tolerance**

Broad acre cotton production occurs in both the tropical and temperate regions of Australia extending from Emerald, QLD (23.57 ^{o}S) in the north to Hillston, NSW (33.48 ^{o}S) in the south and is greatly limited by climatic factors. High temperatures (> 35 ^{o}C) throughout the growing season are commonplace among the cotton production areas of Australia (Table 1-1) and exceed the thermal kinetic window for which metabolic activity is most efficient in cotton plants (Burke *et al.* 1988), thereby limiting the growth and development of the crop and hence yield (Hodges *et al.* 1993). Fibre quality may also be adversely affected by high temperatures during fibre development (Constable and Shaw 1988).

Table 1-1 Mean number of days equal to or exceeding 35 ^{o}C and 45 ^{o}C for Australian cotton growing regions during the cotton season, between the months of October and April.

Location	Latitude	Longitude	Mean no of days ≥ 35 ^{o}C	Mean no of days ≥ 40 ^{o}C
Emerald	23.57 °S	148.18 °E	66.2	5.5
St George	28.04 °S	148.58 °E	53.4	4.8
Bourke	30.09 °S	145.94 °E	77.1	21.7
Narrabri	30.34 °S	149.76 °E	42.4	2.8

Preferential selection for heat tolerant cultivars may delay the onset of heat stress in the plant throughout the season, thereby minimising yield loss whilst maintaining fibre quality in a hot year. Breeding programs have principally relied on yield and fibre quality as screening tools in local environments and hence, screening for thermotolerance has been largely incidental (Constable *et al.* 2001). However, this approach involves high environmental variability and a long lag-time between intergeneration analyses. Hence, the development of a rapid and reliable screening tool for genotype specific

thermotolerance could potentially improve the efficiency of breeding programs and aid the development of high-yield cultivars for hot growing regions.

Cotton plants possess mechanisms to buffer the effects of short term high temperature stress. This is generally the result of a cascade of physiological and biochemical alterations, generated by the up- or down-regulation of stress responsive genes which permit survival under unfavourable conditions and genotypic differences in expression of the mechanisms under stress may be used to select for thermotolerance. Photosynthesis and respiration are decreased under high temperature stress (Reddy *et al.* 1991a). Growth cabinet and laboratory experiments have shown that analysis of the underlying components of these processes such as electron flow through the photosystem (Wise *et al.* 2004), photosynthesis (Salvucci and Crafts-Brandner 2004b), respiratory enzymes viability (de Ronde and van der Mescht 1997) and cell membrane disruption (Sullivan 1971) may be used to quantify heat stress in plants and gene profiling may explain the mechanisms underlying these physiological and biochemical processes.

The repeatability of biochemical assays for heat tolerance is primarily attributed to the imposition of a consistent high temperature stress that can be generated to screen multiple generations under identical environmental conditions. However, there are few reports on whether assumptions of heat tolerance based on growth cabinet and glasshouse experiments are applicable to field conditions. High temperature stress under field conditions varies both daily and seasonally and is generally confounded by concurrent high light, low humidity and drought stress (Marcum 1998). Furthermore, biotic stresses such as insect, disease and weed pressure may contribute to low yields under field conditions (Hearn and Fitt 1992).

1.3 **Central research question**

Is there genetic variation for thermotolerance in cotton and can a simple, reliable and repeatable method be developed to assess this tolerance for breeding programs?

1.4 **Objectives**

The aim of this study was to understand cultivar differences in heat tolerance by:

(a) quantifying the effects of high temperatures on cotton grown at various levels of plant functioning under field and growth cabinet conditions;

(b) evaluating and developing rapid and reliable methods for detecting high temperature tolerance in cotton cultivars

A series of field and glasshouse experiments was conducted to evaluate methodologies to determine cotton thermotolerance under high temperature stress. High temperature stress was imposed in the field by construction of radiation permissible, solar weave tents over the crop. Whole plants were also subjected to high temperatures using thermally regulated growth cabinets. Leaf samples were incubated in distilled water and subject to high temperatures using a thermally controlled water baths in the laboratory.

Cultivar specific thermotolerance was determined through a series of screening assays and measurements at a crop, whole plant, leaf, cell and individual gene level, thus providing a multi-scale analysis of thermotolerance in cotton under field and growth cabinet conditions (Figure 1-1). The approach applies both a top-down and bottom-up evaluation of plant function under high temperature stress to better understand heat tolerance and to clearly identify appropriate methodologies for detecting differences in cultivars. Crop level measurements of yield and fibre quality were determined and whole plant fruit retention measured under field conditions to identify thermotolerant cultivars (Chapter 4). Leaf level physiological measurements which may contribute to yield, such as

photosynthesis, electron transport, stomatal conductance and transpiration rate were measured under field and growth cabinet conditions (Chapter 5). Biochemical assays evaluating membrane integrity and enzyme viability were determined under field and growth cabinet conditions (Chapter 6) as potential limitations to photosynthesis and electron transport rate. Expression of genes associated with metabolic, photosynthetic and electron transport pathways were determined under growth cabinet conditions to determine potential genetic limitations to biochemical function under high temperature stress (Chapter 7). This overall approach facilitated the investigation of the underlying contributions to yield under high temperatures, thus allowing evaluation of measurements and assays for future thermotolerance determination (discussed in Chapter 8).

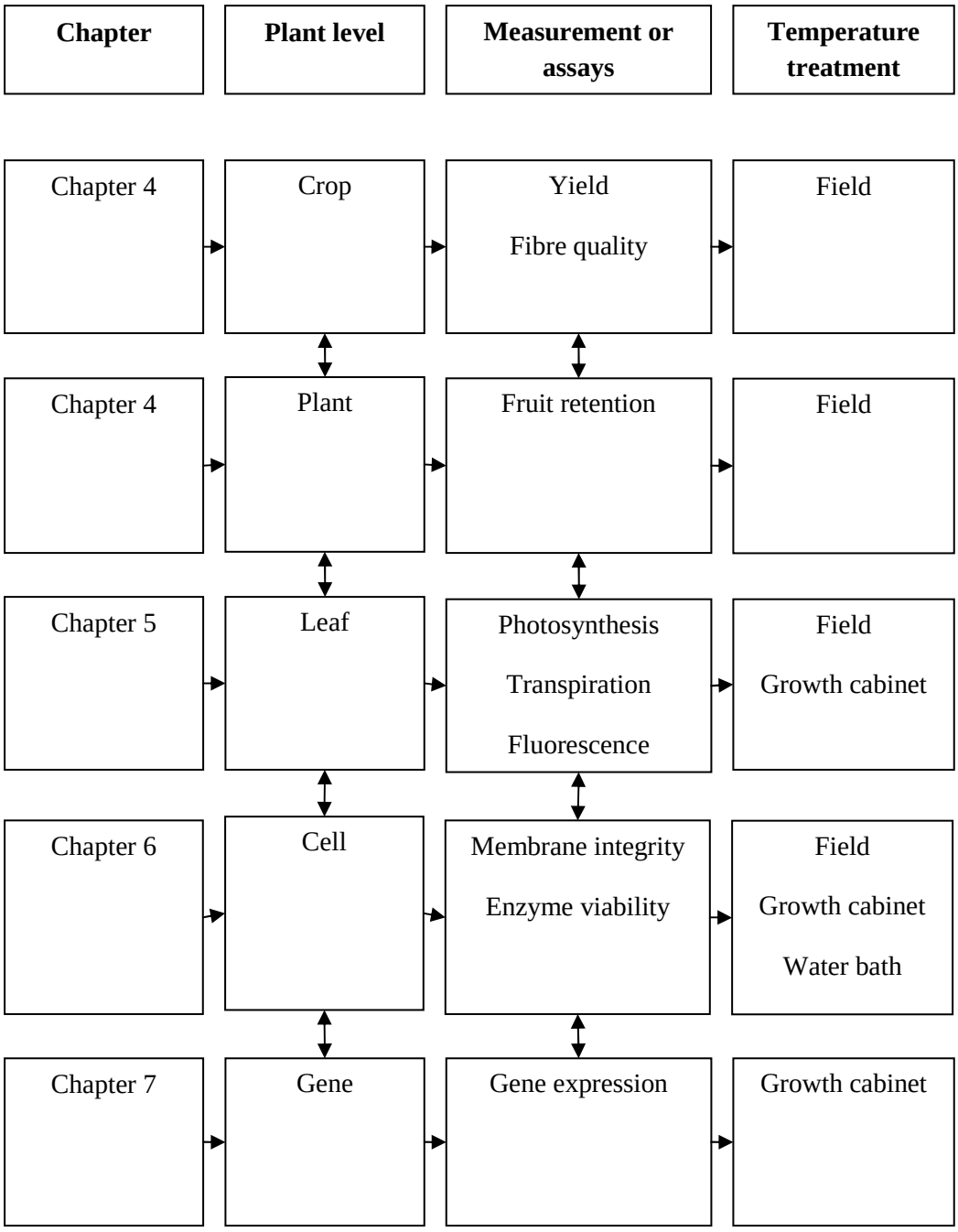

Figure 1-1 Schematic diagram of multi-scale analysis of methods for screening for heat tolerance in cotton under field, growth cabinet or laboratory conditions

Chapter 2 Review of Literature

2.1 **Introduction**

The Australian cotton industry has developed high yielding and high quality fibre production systems and attributes a significant contribution of this achievement to highly innovative breeding programs, specifically focused on the production of premium lint for the export market. However, recent pressure has been placed on the industry for the development of high yielding and low cost production systems to compete for land effectively with other food and bio-fuel commodities, as well synthetic fibres through the textile market.

Breeding programs have recently shifted attention to the development of new germplasm with superior stress tolerance to minimise yield losses attributed to adverse environmental conditions and unreliable inputs such as irrigation, fertilisers and pesticides. Various contributors to yield, such as physiology, biochemistry and gene expression have been implemented as screening tools for high temperature tolerance under growth cabinet and laboratory conditions but there has been little extension of these mechanisms for analysis under field based systems.

This review examine currents literature on the effects of high temperature stress on cotton and other crop species, as well as the identification of thermotolerant cultivars in response to heat stress. The review summarises the effects of high temperatures on cotton at a crop, whole plant, leaf, cell and gene level and reviews screening mechanisms for cultivar specific thermotolerance determination using agronomic, physiological, biochemical and molecular tools. This review also highlights the potential for the validation of growth cabinet screening mechanisms under field conditions to ascertain the validity of using growth cabinet based screening methods to identify stress tolerant cultivars.

2.2 **Effects of temperature on growth and development of cotton**

2.2.1 **Heat stress overview**

Cotton is generally grown in warm to hot regions of New South Wales and Queensland. Throughout these regions, temperature and water availability are primary regulators of plant growth and production and are often indistinguishable in terms of influence. Temperature determines the commencement of the growing season, with most producers observing a 14 ^{o}C soil temperature minimum at 10 cm depth for a minimum of 3 days before planting a crop (Constable and Shaw 1988). Temperature is also a primary determinant of season length as delayed maturity increases yield potential (Bange and Milroy 2004).

In Australian cotton cropping systems, the temperature requirement of a cotton crop for morphological development may be described as the thermal time function and is a measure of degree days based on minimum and maximum daily air temperatures (Constable and Shaw 1988). Under limiting conditions such as water deficit and heat stress, the leaf or canopy temperatures are required to explain yield whereby yield decreases as the leaf temperature and air temperature differential increases (Idso *et al.* 1979). This relationship forms the foundation for the development of a crop water stress index which accounts for external environmental variables and has applications for irrigation scheduling for heat and water deficit stress minimisation (Idso *et al.* 1981). Species and cultivar specificity for leaf temperature of cotton grown under irrigated systems indicates that leaf temperatures may vary for individual plants exposed to similar air temperatures (Ehrler 1973), depending on plant morphology and environmental variables (Gates 1968) and this may in turn affect a range of morphological, physiological and biochemical processes (Burke and Hatfield 1987). Cotton has an optimal thermal kinetic window of 23 to 32 ^{o}C in which metabolic activity is most efficient (Burke *et al.*

1988). High temperatures (>35 ^{o}C) throughout the growing season are common among the cotton production areas of Australia and may adversely affect the growth and development potential of the crop and ultimately yield (Hodges *et al.* 1993). High temperatures may induce a heat shock response, which may involve various tolerance or avoidance mechanisms.

Heat shock infers the sudden exposure of leaf tissue to supraoptimal temperatures. Under field conditions, the heat shock response is generated at around 10 ^{o}C above the ambient temperature (Gallie 2001) and initiates multiple genes encoding a cascade of physiological and biochemical changes associated with acquired thermotolerance pathways, to compensate for the increase in temperature (Leone *et al.* 2003). Such changes include modification in enzymatic and membrane composition, photosynthetic apparatus and the synthesis of heat-protecting molecules such as heat shock proteins, chaperones and free-radical scavengers. The capacity for acquired thermotolerance in plants varies greatly between species and cultivars and screening genotypes for different physiological or biochemical processes may confer tolerance or susceptibility within a plant population (Blum and Ebercon 1981), thereby providing a basis for the development of stress breeding programs (Klueva *et al.* 2001).

Conversely, heat avoidant pathways may be induced under heat shock to protect yield under high temperature stress. Avoidance mechanisms include the ability to maintain tissue temperature by increasing water uptake or reducing water loss through stomatal regulation (Radin *et al.* 1994), and completion of critical stages of growth and development before damage due to abiotic stress is incurred (Turner and Kramer 1980). By selecting for yield in hot environments, traditional plant breeding programs have inadvertently selected for high temperature avoidance (Radin *et al.* 1994). Although heat

avoidant genotypes typically yield higher (Lu *et al.* 1997), avoidance mechanisms such as increasing heat dissipation through evaporation are largely ineffective in humid or water limited environments and are hence not suitable as selection criteria for screening thermotolerant cotton cultivars.

Whole and partial plant physiological measurements and assays must be implemented during periods of high temperature stress to ascertain whether a specific cultivar predominantly relies on tolerance or avoidance mechanisms to achieve final yield. It is important that plants selected for inclusion in breeding programs for production under high abiotic stress be moderate in performance for both heat avoidance and tolerance, rather than in just one or the other (Sullivan 1971).

Prolonged exposure to high temperatures throughout the season may result in the development of thermal acclimation associated with inherent thermotolerance, which enables survival under subsequent supraoptimal and potentially lethal temperatures (Klueva *et al.* 2001). This adaptation is induced by environmental factors and counterbalanced by the acclimation potential which is a direct function of genotypic composition of the plant (Berry and Bjorkman 1980). Hence, the physiological and morphological changes in a plant under high temperature stress largely correspond to the daytime growth temperature under which the plant has been previously grown (Bednarz and van Iersel 2001). In plants, this adaptation is predominantly associated with an increase in the stability of various components of the chloroplast, under high temperature stress (Bjorkman *et al.* 1980). However, this type of thermotolerance does not usually vary greatly within a species and is less effective in terms of targeting for thermotolerance breeding programs (Klueva *et al.* 2001).

Genes associated with inherent and acquired tolerance may be identified using molecular techniques and can be further quantified via various assays and plant physiological measurements. There exists great potential for the identification and insertion of novel genes into heat susceptible species and cultivars that regulate the production of protective proteins, enzymes and molecules under heat stress to maintain plant physiological process and yield under high stress conditions (Leone *et al.* 2003).

2.2.2 **Fibre development**

High temperatures throughout the boll filling and fibre development stage may reduce fibre quality (Mauney 1984). The effect of high temperature on fibre quality has been determined under field conditions, through the use of staggered planting date (Rahman 2006) and heated mats to increase temperatures by 1 ^{o}C (Pettigrew 2008) in the field, as well as under high (30 ^{o}C) compared with low (26 ^{o}C) temperatures in the growth cabinet (30 ^{o}C) (Roussopoulos *et al.* 1998). Rahman (2006) found cultivar differentiation for the number of spinnable fibres on a per seed and per surface area basis resulting from additive genetic variability under high temperatures in the field. Decreases in spinnable fibres may be attributed to decreases in fibre length (Roussopoulos *et al.* 1998), strength (Pettigrew 2008; Roussopoulos *et al.* 1998) and maturity (Pettigrew 2008; Reddy *et al.* 1999) under high temperature stress. Furthermore, the genetic variation and heredity of fibre quality parameters is sufficient under control (Cheatham *et al.* 2003) and high (Rahman 2006) temperatures for consideration in breeding programs for fibre quality. However temperature responsive decreases in fibre quality are not typically severe enough to result in a discount in current markets (Pettigrew 2008). Furthermore, quality parameters such as maturity, elongation and micronaire have variable responses to high temperatures (Pettigrew 2008; Roussopoulos *et al.* 1998). Hence, evaluation of fibre quality parameters

under high temperature stress may not be a good screening tool for determination of high temperature tolerance in the field.

There are few reports of fibre quality discounts associated with high temperature stress (Pettigrew 2008; Rahman 2006; Roussopoulos *et al.* 1998) and these treatments are typically subtle in the context of anticipated fluctuations in temperature across a growing season. Furthermore, the effect of prolonged *in-situ* high temperature stress has not been described for cotton cultivars grown under field conditions and must be validated to determine whether fibre quality parameters may be employed as screening tools for cultivar specific thermotolerance.

2.2.3 **Yield and fruit retention**

High temperatures throughout the flowering period may result in low fruit set due to pollen infertility (Burke 2004; Kakani *et al.* 2005; Marshall *et al.* 1974), low boll size (Reddy *et al.* 1999; Roussopoulos *et al.* 1998) and seeds per boll (Pettigrew 2008) or high rates of fruit abscission (Reddy *et al.* 1999; Zhao *et al.* 2005), thereby limiting yield potential (Mauney 1984). Thus, yield and yield component analysis may be used to screen for stress tolerance under field conditions.

Boll size and number are primary determinants of yield potential (Brook *et al.* 1992). High temperature stress may decrease pollen germination and tube elongation at temperatures greater than 32 ^{o}C (Burke *et al.* 2004; Kakani *et al.* 2005; Liu *et al.* 2006), limit the number of ovules laid down during carpel formation (Hearn and Constable 1984; Mauney 1984) and limit fertilisation percentage of formed ovules (Pettigrew 2008), thereby reducing seed numbers per boll and boll dry weight (Reddy *et al.* 1991b), and ultimately yield (Mauney 1984).

Boll retention is the primary determinant of yield under stress in the field (Reddy *et al.* 1999). Reproductive structures compete directly with leaves for imported assimilates and in the event of shortage, assimilates are preferentially directed to leaves to further generate energy at the expense of squares (Hearn and Constable 1984). Cotton plants can abscise up to 80% of squares and young bolls throughout a growing season (Hearn and Constable 1984; Oosterhuis 1990). This natural shedding may be accentuated by temperatures exceeding 30 ^{o}C (Reddy *et al.* 1991b), insufficient solar radiation interception or moisture and nutrient availability and insect damage (Hearn and Constable 1984; Oosterhuis 1990).

Hence, yield and determinants of yield such as pollen viability and fruit retention may be used to detect high temperature stress in cotton and thus, provide potential for development as methods for determination of thermotolerance (Liu *et al.* 2006).

2.2.4 **Vegetative growth**

Protective and avoidance mechanisms are insufficient to completely protect a plant against the deleterious effects of prolonged high temperature stress. Temperatures exceeding the optimal thermal kinetic window for cotton (Burke *et al.* 1988) may severely limit plant growth and development. Heat stressed cotton plants typically exhibit a lower number of branches per plant with a lower branch length, fewer nodes and shorter internodal length (Abrol and Ingram 1996; Reddy *et al.* 1992). Furthermore, these plants are unable to achieve their reproductive potential as dry matter is preferentially accumulated in monopodial branches rather than in sympodial branches (Reddy *et al.* 1991b).

Sympodial branches support 50 to 60 % of total plant leaf area and leaf expansion and development may be reduced under high temperatures throughout the growing season (Roussopoulos *et al.* 1998). Reductions in the light harvesting potential of the plant may limit energy generation via photosynthetic and respiratory electron transport chains

thereby reducing vegetative dry matter accumulation and fruit load development for less thermotolerant cultivars (Roussopoulos *et al.* 1998). Crafts-Brander and Salvucci (2004) found exposure of cotton plants to 37 or 40 ^{o}C night temperatures over a 4 day period significantly decreased dry matter accumulation and leaf area production, whilst root growth was inhibited in the 40 ^{o}C treatment groups. Reddy *et al*. (1991b) also found suppressed biomass accumulation at 40/30 ^{o}C day/night temperatures in cotton, thereby resulting in lower main stem leaf area and variable plant height. This suppressed dry matter production may be partly attributed to elevated respiration rates and an increase photosynthetic requirement (Reddy *et al.* 1992; Reddy *et al.* 1991b).

2.2.5 **Heat stress proteins**

Heat shock protein (HSP) synthesis is a primary protective buffer against the deleterious effects of heat shock in field grown plants. Although the exact mechanism of heat tolerance in cotton is unknown, the synthesis and accumulation of heat stress proteins during rapid or gradual stress may contribute to thermotolerance (Klueva *et al.* 2001) as well as tolerance to other abiotic stresses (Busch *et al.* 2005; Larkindale and Vierling 2008; Piper *et al.* 1997). Genes regulating the expression of heat shock proteins under high temperatures have been identified (Larkindale and Vierling 2008; Lohmann *et al.* 2004) and provide potential for targeted breeding programs.

Under maximal heat stress, the synthesis and accumulation of high molecular weight heat shock proteins such as HSP70 and HSP90 and mRNA may increase up to ten-fold (de Ronde *et al.* 1993) and may be cultivar dependent (Fender and O'Connell 1989). It is proposed that HSP70 may prevent protein denaturation during stress and plants with blocked or inactivated HSP70 synthesis are susceptible to heat injury (Burke *et al.* 1985).

Although the exact mechanism of protection against heat stress is unknown for many heat shock proteins (Malik *et al.* 1999), heat shock proteins associated with chloroplasts, ribosomes or mitochondria may also contribute to thermotolerance. For example, the small, methionine-rich chloroplast heat-shock protein protected PSII and the electron transport chain to an extent that it completely accounted for heat acclimation in pre-heat-stressed tomato plants (Heckathorn *et al.* 1998). Differences in cultivar thermotolerance attributed to heat shock protein expression provide further evidence that breeders may utilise genetic resources and crop management to develop thermotolerance in new crop cultivars.

2.2.6 **Photosynthesis and electron transport**

Photosynthesis is largely regulated by temperature and is particularly heat sensitive (Burke *et al.* 1988; Hodges *et al.* 1993; Lu *et al.* 1997; Perry *et al.* 1983; Reddy *et al.* 1991a; Schrader *et al.* 2004; Wise *et al.* 2004). The optimal temperature for gross photosynthesis in cotton is approximately 30 ^{o}C (Bednarz and van Iersel 2001; Perry *et al.* 1983; Reddy *et al.* 1998) with an ideal range between 23 and 33 ^{o}C for metabolic activity and photosynthesis (Burke *et al.* 1988). Hence high temperatures (>35 ^{o}C) throughout the season may limit photosynthetic potential for plant growth and hence yield (Lu *et al.* 1997).

At higher temperatures, net photosynthesis decreases proportionally and inversely to photorespiration (Berry and Bjorkman 1980). This decrease may be attributed to both a decrease in the electron transport rate and a decline in activity of rate-limiting enzymes, particularly those associated with photosynthetic and respiratory channels (Bjorkman *et al.* 1980; Burke *et al.* 1988) such as Rubisco (Ribulose-1,5 biphosphate carboxylase/oxygenase).

Rubisco is a heat-labile enzyme (Law and Crafts-Brandner 1999; Salvucci and Crafts-Brandner 2004a) that is regulated by Rubisco 1,5-biphosphate (RuBP) and has potential to limit growth and development in C_3 and C_4 plants. It has been proposed that the regeneration of RuBP, via energy supplied from the electron transport chain, is the primary limitation to net photosynthesis (Salvucci and Crafts-Brandner 2004a; Stidham *et al.* 1982). Considering that stromal enzymes are generally more stable than PSII under heat stress, temperature-induced suppression of the electron transport chain may limit the functionality of these enzymes which themselves, are relatively stable under moderate heat stress (Wise *et al.* 2004). However, a model proposed by Crafts-Brander and Salvucci (2004) suggested that the primary biochemical limitation to photosynthesis at high temperatures and ambient CO_2 concentrations is in fact the activation state of Rubisco. This function is regulated by the activity of Rubisco activase and is not dependent on the electron transport energy pathway as chlorophyll fluorescence signals from PSII were not affected at these Rubisco-limiting temperatures. Cultivar specificity for Rubisco suggests that assays evaluating genes associated with Rubisco activity may be useful for screening programs for thermotolerance (Pettigrew and Turley 1998).

At high temperature, the down-regulation of PSII is most likely the prominent limitation to photosynthesis (Klueva *et al.* 2001). However, protein denaturation associated with PSII protein membrane complexes did not occur at temperatures below 45 ^{o}C (Al-Katib and Paulsen 1999). It has been proposed that the susceptibility of photosynthetic decline at high temperatures is mostly likely attributed to instability of PSII in the thylakoid membranes (Santarius 1973), particularly at the water-splitting complex and subsequently, for noncyclic photophosphorylation (Al-Katib and Paulsen 1999). Al-Katib and Paulsen (1999) found a similar response of protoplasts, chloroplasts and thylakoids at temperatures greater than 40 ^{o}C, whereas stomatal effects and stromal enzymatic activity remained

relatively stable across a range of high temperature treatments. This indicates that a common component of PSII is essential for high temperature tolerance.

High temperature stress (35 to 45 ^{o}C) can alter the conformation, composition and hence, permeability of the thylakoid membrane in the chloroplasts at temperatures that do not affect electron transport and ATP synthesis (Bukhov *et al.* 1999; Suss and Yordanov 1986). Cyclic phosphorylation is then unable to compensate for the leakiness of the lipid membrane resulting in disruption of electron transport between PSI and PSII thereby reducing energy availability (Stidham *et al.* 1982). This may further reduce or alter enzymatic activity and limit net photosynthesis at temperatures above 35 ^{o}C (Havaux *et al.* 1996).

Under high temperature stress, electron transport may be further directed to molecular oxygen, eliciting the generation of reactive oxygen species that may cause further damage to photosynthetic organelles (Cothren 1999). The onset of oxidative stress is rapid upon exposure to high temperatures and the effects of this stress are partially buffered through the scavenging and processing of active oxygen species by plant-based antioxidants and this process is generally known as quenching (Cothren 1999). Excited oxygen states are extremely reactive and cytotoxic and may cause peroxidation of the lipids in the plasmalemma and intracellular organelles resulting in degradation of cell structure and cytoplasmic leakage (Suss and Yordanov 1986) decreased viability of respiratory enzymes in the mitochondria, reduced affinity for chloroplastic carbon fixation, photoinhibition and photooxidation in the chloroplast. Although conversion of violaxanthin to zeathanthin via the xanthophyll cycle (Bilger and Bjorkman 1994) may moderate membrane fluidity under mild heat stress (Suss and Yordanov 1986) and enable the down-site synthesis of heat shock proteins (Wise *et al.* 2004), this protective mechanism may be insufficient at higher

temperatures that do not damage PSII (Salvucci and Crafts-Brandner 2004c). Hence, quenching analysis may be used to indicate species and cultivar specific thermotolerance, before damage to PSII is evident (Salvucci and Crafts-Brandner 2004c). Furthermore, genetic modification of the quenching pathway may be used to reduce oxidative stress and hence, increased thermotolerance of plant species (Kornyeyev *et al.* 2001).

Characteristics associated with high photosynthetic rates are cultivar specific (Pettigrew and Turley 1998; Reddy *et al.* 1991a) and highly heritable (Abdullaev *et al.* 2003), thus providing a potential target for breeding programs to increase the heat tolerance of commercial cotton cultivars (Lu *et al.* 1997). Although cultivar specificity for photosynthesis has been determined under high temperatures in the growth cabinet (Bibi *et al.* 2008) little information exists on cultivar specificity of photosynthesis and fluorescence under *in-situ* high temperature stress in field conditions. Hence, photosynthesis and fluorescence need to be evaluated for cotton cultivars grown under high temperatures in the field to enable gene expression of thermotolerance cultivars in the field for incorporation into breeding programs.

2.2.7 **Stomatal conductance and transpiration rate**

Transpiration is the primary contributor to maintenance of leaf temperature under high temperature stress in upland cotton (Lu *et al.* 1997; Radin *et al.* 1994; Rahman 2005). Leaf temperature is regulated by leaf area and boundary layer conductance, stomatal conductance, heliotropism and radiation interception (Ayeneh *et al.* 2002; Leidi *et al.* 1993; Lu *et al.* 1997; Radin *et al.* 1994). High temperatures, in the absence of drought stress, induce increased stomatal aperture thereby permitting heat loss through transpiration and reducing leaf temperature whilst still facilitating gaseous exchange (Bednarz and van Iersel 2001; Radin *et al.* 1994). In cotton, leaf temperature is very volatile and may change by more than 1 ^{o}C per second (Wise *et al.* 2004). Morning leaf

temperatures are generally within a few degrees of the ambient air temperature. Midday and afternoon temperatures that exceed 35 ^{o}C may result in variable leaf temperatures that may exceed or be lower than ambient temperatures (Wise *et al.* 2004).

Stomatal conductance and leaf temperature are correlated with yield (Lu *et al.* 1997; Radin *et al.* 1994). In fact, leaf temperatures can be up to 10 ^{o}C lower than ambient air temperatures (Radin *et al.* 1994). Hence, traditional breeding programs that select cotton cultivars for yield under hot environments, inadvertently and simultaneously select for high temperature avoidance through evaporative cooling potential (Rahman *et al.* 2004). However, highly conducting genotypes may not necessarily be high yielding under limiting and optimal environments (Leidi *et al.* 1993; Lopez *et al.* 1993) and hence yield should be included in breeding programs screening for stomatal conductance under stress.

Heat avoidance through evaporative cooling has some degree of species (Lu *et al.* 1994) and cultivar specificity under drought (Radin *et al.* 1994) and high temperature stress (Rahman 2005). However, little information is available on cultivar differences in stomatal conductance and transpiration rate under *in-situ* high temperature stress in the field. Identification of cultivar differences in stomatal conductance and transpiration under *in-situ* field high temperature stress may indicate the suitability of these methods as screening tools for cultivar specific thermotolerance under field conditions.

2.2.8 **Cellular membrane integrity**

Heat stress may cause irreversible disruption and damage to cell membranes. Under optimal conditions, cell membranes are freely permeable to CO_2 and O_2, slightly permeable to water and require transport proteins to carry inorganic ions and hydrophilic solutes (e.g. sucrose and amino acids) across the plasma membrane and tonoplast. High temperatures weaken the hydrogen bonds between polar groups of proteins within the

fluid bilayer of the membrane, thereby causing a decrease in the specificity of membrane permeability, a decrease in transport system activity and disruption and damage to the cell membrane (Gupta 2007). Cell membrane damage may result in leakage of internal cytoplasmic electrolytes to the surrounding environment, which may be quantified by directly measuring the changes in electrical conductivity of the solution containing plant tissue prior to, and after exposure to high temperatures (Rahman *et al.* 2004). The ability of the cell membrane to remain intact after exposure to high temperature stress may be indicative of species or cultivar specific thermotolerance (Raison *et al.* 1980).

The membrane integrity method has been used for discrimination between cultivars and hybrids of cotton (Ashraf *et al.* 1994; Blum and Ebercon 1981; Rahman *et al.* 2004; Sethar *et al.* 1997), soybeans (Martineau *et al.* 1979a; Sethar *et al.* 1997), sorghum (Sullivan 1971), bean (Schaff *et al.* 1987) and wheat (Saadalla *et al.* 1990b), under both greenhouse and field conditions (Ashraf *et al.* 1994; Rahman *et al.* 2004) and at various growth stages (Ashraf *et al.* 1994). Furthermore, Ashraf *et al.* (1994) noted that screening for thermotolerance at initial and latter growth stages were positively correlated, thus minimising the inter-generational time in any breeding program.

Cellular membrane thermostability (CMT) has been directly correlated to whole-plant high temperature tolerance in Kentucky bluegrass (Marcum 1998), soybean (Martineau *et al.* 1979a), wheat (Shanahan *et al.* 1990) and cotton (Rahman *et al.* 2004). Furthermore, this correlation in the presence of stress may be used to discriminate between cotton cultivars and hybrids for genetic inclusion in breeding programs (Rahman *et al.* 2004). However, cultivar discrimination for thermotolerance based on the CMT method is dependent on the further development of the method in terms of heritability and combining ability (Bajji *et al.* 2002; Martineau *et al.* 1979b; Rahman *et al.* 2004).

Breeding for cultivar specificity with specific biochemical pathways may provide viable mechanisms for the development of cultivars with superior thermotolerance. Heat shock proteins aid in the protection of biochemical pathways in heat stressed plants (Malik *et al.* 1999; Piper *et al.* 1997; Queitsch *et al.* 2000; Sotirios *et al.* 2006) but is not the sole mechanism of thermotolerance (Larkindale and Vierling 2008). Superior membrane integrity under stress may be attributed to increased membrane rigidity as a result of rapid isomerisation of naturally occurring *cis* to *trans* configuration unsaturated fatty acids (Murakami 2000). High antioxidant activity may minimise membrane damage as a result of phospholipid degradation associated with lipid peroxidation (Liu and Huang 2000) and heat-induced oxidative stress (Larkindale *et al.* 2005). Manipulation of calcium channels (Bhattacharjee 2008) and sterol conjugation under stress may also aid in breeding plants with optimal membrane fluidity and a higher relative thermotolerance (Gupta 2007). Assays based on cellular membrane integrity are considered easy, reliable, cost effective and repeatable and provide an invaluable resource to describe the underlying biochemical mechanisms of stress tolerance (Marcum 1998).

2.2.9 **Enzyme viability**

Damage to cell membranes is likely to reduce the efficacy of downstream respiratory enzymes and electron transport chains (Taiz and Zeiger 2006) at temperatures exceeding 40 ^{o}C (Burke *et al.* 1988).

The tetrazolium viability test is a simple assay that may be used to determine the physiological viability of a large number of plant samples at a particular point in time (Burke 2007; de Ronde and van der Mescht 1997) by assessment of dehydrogenase activity in mitochondrial respiratory electron transport chains. Heat tolerant plants are better able to reduce 2,3,5-triphenyltetrazolium salts in the mitochondria to an insoluble red formazan compound (de Ronde and van der Mescht 1997) by accepting electrons from

the electron transport chain (Towill and Mazur 1975) via the dehydrogenase pathway (Nachlas *et al.* 1960). This reduction can be correlated back to the level of enzyme viability (Towill and Mazur 1975). This assay can be used for screening a range of cotton cultivars for thermotolerance (de Ronde and van der Mescht 1997).

Cultivar specificity for enzyme viability has been similarly reported for water deficit stress (de Ronde and van der Mescht 1997), low (McDowell et al. 2007) and high temperature stress (Chen et al. 1982; de Ronde and van der Mescht 1997; Porter et al. 1995; Schaff et al. 1987). Thermotolerant cultivars have increased capacity for tetrazolium reduction under high temperature stress compared with the control (de Ronde and van der Mescht 1997). However, heat tolerance and drought tolerance can be either correlated positively or negatively, depending on environmental conditions. Hence, genetic selection for breeding programs should be undertaken with consideration for the interaction between drought and heat tolerance rather than either factor independently (de Ronde and van der Mescht 1997).

2.2.10 **Gene expression**

A cascade of morphological, physiological and biochemical responses are initiated under exposure of plants to short and long term high temperature stress. Molecular techniques provide an insight as to the genetic basis of responses to heat stress. DNA microarrays are a powerful tool for surveying the expression patterns of thousands of genes simultaneously. This enables rapid determination of differential gene expression between two RNA populations, thus providing a global and integrated analysis of biological processes in response to stress. Quantitative trait locus (QTL) mapping is also an effective method for identifying the underlying pathways contributing to changes in yield and physiology under abiotic stress in cotton (Saranga *et al.* 2004). However, the low level of DNA polymorphism in cotton limits the application of QTL mapping to interspecific

families and backcross lines (Chen *et al.* 2007). Key genes and gene families involved in the development of heat tolerance provide a platform for further characterisation and identification of stress-responsive genes which may be targeted for breeding programs for stress tolerance (Chinnusamy *et al.* 2005; Ishitani *et al.* 2004; Zhang and Blumwald 2001).

Abiotic stress and acclimation to abiotic stress induces a cascade of differential gene regulation (Busch *et al.* 2005) with respect to morphological, physiological and biochemical plant pathways and global gene profile may be used to identify the response of individual genes or general pathways to such stress (Busch *et al.* 2005; Klok *et al.* 2002) particularly when no obvious phenotype is attributed to the stress response (Kennedy and Wilson 2004).

Microarray analysis has been used to determine cultivar differences in gene expression for salt stress in rice (Sahi *et al.* 2003), drought stress in sorghum (Sharma *et al.* 2006) and heat stress in fescue (Zhang *et al.* 2005), drought and heat stress in *Arabidopsis* (Sakuma *et al.* 2006) and a range of abiotic stresses in potato (Rensink *et al.* 2005). However, genes associated with these stresses may confer overall stress tolerance and hence, may be used as a model for further investigation under heat stress (Piper *et al.* 1997; Rensink *et al.* 2005; Sakuma *et al.* 2006). Gene expression under high temperature stress has been described for a range of species including *Arabidopsis* (Busch *et al.* 2005; Sakuma *et al.* 2006), *Agrostis scabra* (Tian *et al.* 2009), sunflower (Hewezi *et al.* 2008) and potato (Rensink *et al.* 2005). Whilst these species can be used as a platform for research into gene expression in cotton, there is little specific research on gene functionality in cotton (Dowd *et al.* 2004).

Heat shock proteins and transcription factors play an integral role in thermotolerance (Gao *et al.* 2008; Larkindale *et al.* 2005; Lee *et al.* 1995; Lohmann *et al.* 2004; Lu *et al.* 1995;

Malik *et al.* 1999; Piper *et al.* 1997; Queitsch *et al.* 2000; Salvucci 2008; Schrader *et al.* 2004; Sotirios *et al.* 2006). Genes involved in osmotic adjustment (Alia *et al.* 1998), ethylene synthesis (Larkindale *et al.* 2005), amino acid synthesis (Fouad and Rathinasabapathi 2006), calcium dependent pathways (Lu *et al.* 1995; Sotirios *et al.* 2006), abscisic acid synthesis (Larkindale *et al.* 2005) and membrane protection (Lee *et al.* 1995) are thought to confer thermotolerance in some plant species.

Genes involved in energy conservation through ATPase activity and photosynthesis regulation via Rubisco viability may also contribute to energy generating pathways involved in thermotolerance. However, a large proportion of transcription factor and heat shock protein genes have been identified under high temperatures but have no known function (Malik *et al.* 1999). Furthermore, there has been little evaluation of the expression of genes involved in thermotolerance at high temperatures under field conditions. Hence, gene profiling for cotton cultivars may provide an overall indication of the viability of molecular tools for genotype screening under field conditions.

2.3 **Conclusion**

The major opportunities for research that emerge from this literature review are listed below. These need to be addressed in order to quantify the effects of high temperatures on cotton at various levels of plant function under field and growth cabinet conditions and to evaluate and develop rapid and reliable methods for detecting high temperature tolerance in cotton cultivars.

The physiological, biochemical and molecular basis of cultivar specific thermotolerance has not been described for Australian production systems. The Australian cotton system differs from production systems in the Northern hemisphere in terms of environmental conditions, crop management, germplasm and breeding objectives. Hence, cultivar

specific thermotolerance needs to be evaluated under local Australian production systems for future breeding programs.

Little is known about the cultivar specific changes in physiology and biochemistry that result from exposure of cotton plants to high temperature stress in the field. Changes in plant function have been described for many plant species, but there have been few comparisons between cotton cultivars.

The use of physiological and biochemical screening tools for thermotolerance have not been validated under *in-situ* high temperature stress in the field. Growth cabinet and laboratory based screening tools need to be validated under field conditions to ascertain their suitability for inclusion in plant breeding programs that incorporate screening for stress tolerance.

The molecular basis of cultivar specific heat tolerance in cotton has not been described. High instances of differential expression of genes with 'unknown function' limit progress in the identification of heat responsive genes. Linking this understanding with higher crop measurements such as yield and physiology may help to target specific genes for superior biochemical, physiological or morphological functioning under stress conditions.

Gene expression under high temperatures in the growth cabinet has not been validated under field conditions. The field is generally a more variable environment and hence gene expression in the field will most likely represent changes in the expression of whole groups of genes involved in a range of environmental stresses, rather than one specific gene.

Chapter 3 General Materials and Methods

3.1 **Introduction**

Experiments were conducted to investigate the effect of high temperature stress on cotton and to evaluate methods to determine thermotolerance in response to this stress. This chapter describes cotton cultivars, site and climate descriptions, experimental design, field plot and growth cabinet pot management, treatments and measurements that were common to experiments discussed in Chapters 4 to 7. Materials and methods specific to each chapter are described in the relevant chapter.

3.2 **Genotypes**

Normal leaf, medium maturity and non-transgenic cultivars of upland cotton (*Gossypium hirsutum* L.) were screened under glasshouse and field conditions for thermotolerance. Cultivar Sicot 53 and breeding line CSX 99209-376 were selected as relatively thermotolerant genotypes whereas Sicala 45 and Sicala V-2 were chosen as cultivars with lower relative thermotolerance, based on yield performance in warm and hot growing regions. Although these genotypes all originate from the CSIRO breeding program (Table 3-1) and share a number of common ancestors, the coefficients of parentage are low and thus are relatively diverse in respect to available commercial germplasm (Table 3-2). The four genotypes were grown under field conditions in all 3 growing seasons to enable yield comparisons across the sites and seasons. On the basis of preliminary experiments and data from the plant breeding program, Sicot 53 and Sicala 45 were used for all experiments as a model to evaluate methods for testing cultivars with differing levels of thermotolerance.

Table 3-1 Parentage for cotton genotypes Sicot 53, CSX 99209-376, Sicala 45 and Sicala V-2, where the asterisks (*) denote CSIRO breeding lines.

Cultivar	Citation	Parent 1	Parent 2
Sicot 53	(Constable 2000)	Sicot 50	83203-183*
CSX 99209-376*		Sicot 80	Delta Topaz
Sicala 45	(Reid 2004)	Sicala 40	Sicala V-1 x 84009-47*
Sicala V-2	(Reid 1995)	DP 90 x Tamcot SP37H	DP 190 x 75007-3*

Table 3-2 Coefficients of parentage for Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2, where 0.000 represents genotypes with no common ancestors and 1.000 represents genotypes that are identical.

	Sicot 53	Sicala 45	CSX 99209-376	Sicala V-2
Sicot 53	1.000	0.109	0.155	0.203
Sicala 45	0.109	1.000	0.063	0.094
CSX 99209-376	0.155	0.063	1.000	0.053
Sicala V-2	0.203	0.094	0.053	1.000

3.3 **Field experiments**

Field-based experiments were used to provide field grown plant tissue samples for yield, fibre quality, gas exchange, fluorescence, membrane stability and enzyme viability measurements.

3.3.1 **Site and climate descriptions**

Field experiments were conducted over three consecutive cotton growing seasons in two locations between 2005 and 2007 (Table 3-3). These two locations were selected to enable evaluation of cultivar thermotolerance in two distinct climates. The Narrabri (Australia) field site was a hot and dry climate, whereas the Texas (United States of America) field site was a hot and humid growing environment (Table 3-3).

Table 3-3 Location and year of each field experiment.

Experiment	Season	Year	Planting date	Harvest date	Location	Country	Latitude	Longitude
Field	1	2006	14-Oct-05	10-May-06	Narrabri	AUS	31°12'S	149°59'E
Field	2	2006	25-Apr-06	11-Sep-06	Texas	USA	30°32'N	96°26'W
Field	3	2007	18-Oct-06	20-Apr-07	Narrabri	AUS	31°12'S	149°59'E

Table 3-4 Seasonal climate data between sowing and harvest dates for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas.

Season	Average maximum temperature (°C)	Average minimum temperature (°C)	Average temperature (°C)	No days >35 °C	Accumulated day degrees	Average daily radiation (MJ m^{-2})	Photothermal quotient (Villalobos and Ritchie 1992)	Precipitation (mm)	Average daily relative humidity (%)
1	31.5	16.2	23.9	59.0	2580.3	24.6	2.2	532.8	74.9
2	33.6	20.8	27.2	55.0	2128.9	24.9	2.0	319.0	73.4
3	32.9	17.2	25.0	65.0	2443.0	25.0	1.7	182.0	67.8

Narrabri

Field experiments in seasons 1 and 3 were undertaken at the Australian Cotton Research Institute, Narrabri, Australia. The soil was a uniform grey cracking clay (Australian soil taxonomy: Grey Vertosol, USDA soil taxonomy: Typic Haplustert) with a clay fraction percentage of 60 to 65%, pH of 8.0 - 8.8 and has inherently low organic matter and nitrogen. Long term average annual rainfall is 643 mm with a mean maximum temperature of 26.7 ^{o}C and a mean minimum of 11.6 ^{o}C (Australian Government Bureau of Meteorology 2008). Season specific climatic data are presented in Table 3-4.

Texas

Experiment 2 was conducted at the Texas Agricultural Experiment Station located near College Station, Burleson County, Texas, USA during the 2006 cotton growing season. The soil was a Weswood silt loam (USDA Soil taxonomy: Udifluventric Halpustepts) with a pH of 8.3 and conductance of 193 μmho cm^{-1}. Season specific climatic data are presented in Table 3-4.

3.3.2 **Field experimental design and plot management**

Narrabri

A randomised complete block design with four replicates was used for field experiments in Narrabri during seasons 1 and 3. Blocks were replicates, blocked down the field from the head ditch to the tail drain. Plots were 19 m in length, with 9 raised beds at 1 m spacing. Samples were only taken from plants growing on the inner 7 rows and from the inner 17 m of the plot.

Nitrogen was applied at a rate of 200 kg ha^{-1} as anhydrous ammonia and phosphorous at a rate of 132 kg ha^{-1} as mono-ammonium phosphate prior to planting to ensure adequate nitrogen and phosphorous nutrition for the crop. Plots were pre-watered prior to planting

and maintained with full furrow irrigation utilising high input management and insect control throughout the season as described by Hearn and Fitt (1992).

All cotton seed was pre-treated with Dynasty® fungicide seed treatment (Syngenta) and planted with a commercial row crop planter (Kinze) at 12 seeds m^{-2}. Season 1 was planted on the 14th October, 2005 and season 2 was planted on the 18th October, 2006.

In-plot temperature and relative humidity were recorded on a Tiny Tag Ultra (TGX-3680), 10 cm below the maximal canopy height and at least 3 replicates were recorded. Complete meteorological data at a fully serviced weather station were measured 2 km from the field site.

Texas

A randomised complete block design with four replicates was used. Blocks were replicates, blocked down the field from the head ditch to the tail drain. Whole plots were 5.8 m long with 4 raised beds, 1 m apart. Samples were only taken from plants growing on the inner 2 rows of the plot, and from the inner 3.8 m of the row.

The soil was pre-prepared with nitrogen (134 kg ha^{-1}), Caparol® 4 L broadleaf herbicide (0.57 L ha^{-1}) and Dual® pre-emergent herbicide (0.26 L ha^{-1}). Treated cotton seed was planted on the 24th April, 2006 at 13 plants m^{-2} with a John Deere max emerge vacuum planter. Plots were furrow irrigated and pest pressure was rigorously controlled.

In-plot temperature and relative humidity data was collected with a Tiny Tag Ultra (TGX-3680), 10 cm below the canopy height, with at least 3 replicates. A full meteorological survey was recorded 0.5 km from the field site at a fully serviced weather station.

3.3.3 **Temperature treatments**

In-situ high temperature stress was imposed in the field by the construction of Solarweave® tents over the crop canopy (Figure 3-1) at multiple times during the season

(Table 3-5). Tents were erected at 4 d post irrigation to ensure adequate soil water and relatively uniform leaf temperature (Gardner *et al.* 1981), A 1 m buffer area was left between the alleyway and the front entrance to the tents, which were constructed at the front of the plot (Figure 3-1). The tents then covered the next 3 m of the row. A 1 m buffer was left between the tents and the control plants. The control plants were sampled from the next 3 m of the row. A 1 m buffer area was similarly left between the control plants and the alleyway at the back of the plot. All tents were assembled between the first square and cut-out stages of crop development. The tents dimensions were as follows; 2800 mm length, 2800 mm breadth and 2600 mm height. A 700 mm gap was left between the soil surface and the Solarweave® canvas at the front and back of the tent to permit air flow down the rows. At the end of the measurement period, the tents were removed and transferred to a different section of the plot, where similar measurements were taken on a later irrigation cycle.

Table 3-5 Dates of construction and dismantlement of the tents for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas and the associated day degrees associated with these dates.

Season	Tent	Construction		Dismantlement		No days under tent	No degree days under tent
		Date	Day Degrees	Date	Day Degrees		
1	1	3-Feb-06	1523.69	9-Feb-06	1635.11	6	111.42
1	2	23-Feb-06	1840.08	6-Mar-06	1974.07	11	133.99
2	1	13-Jul-06	1106.55	19-Jul-06	1213.85	6	107.3
3	1	17-Dec-06	537.72	22-Dec-06	811.89	5	274.17
3	2	16-Jan-07	1160.87	20-Jan-07	1227.41	4	66.54

Figure 3-1 Solarweave® tents used to impose in-situ high temperature stress in the field during season 3 (2006) at the Narrabri field site.

Solarweave® is a clear, radiation permissible fabric. It has a nominal shade value of 18 % and may increase the relative proportion of diffuse radiation reaching the canopy; it was considered an appropriate commercially available fabric for field experiments, particularly attributed to high durability under adverse climatic conditions (Healey and Rickert 1998; Healey *et al.* 1998). These tents were erected above the crop canopy to raise temperatures above the high temperature threshold for cotton (35 ^{o}C) under the fabric (Table 3-6) but still facilitate airflow down the rows to permit effective gaseous exchange (Lopez *et al.* 2003a). Mean temperature under the tent was higher than mean temperature under ambient field conditions in all 3 seasons (Table 3-7). However, relative humidity was inadvertently increased (Table 3-7). Although both sites experienced high temperatures throughout the cotton season, the Texas site had a higher average ambient temperature than the Narrabri site (Table 3-4). Air temperatures under the control and tents are presented in Figure 3-2 for seasons 1, 2 and 3.

Table 3-6 Number of h above the thermal kinetic window (32 °C) and high temperature threshold (35 °C) for cotton, under ambient field conditions and under the tents for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas.

Season	Tent no.	No hours > 32 °C	No hours > 35 °C	No hours > 40 °C	No hours > 45 °C	No hours > 50 °C
Control (ambient)						
1	1	57.3	35.2	6.3	0.0	0.0
1	2	26.8	6.5	0.0	0.0	0.0
2	1	82.8	66.3	20.7	0.0	0.0
3	1	26.3	17.7	3.5	0.0	0.0
3	2	44.0	17.3	0.0	0.0	0.0
Tent						
1	1	69.7	62.8	40.8	20.7	5.8
1	2	45.8	30.2	8.8	1.7	0.2
2	1	80.7	71.5	55.5	33.2	8.5
3	1	30.5	28.0	23.4	0.0	0.0
3	2	62.7	46.5	26.5	6.8	0.2

Table 3-7 Mean temperature and relative humidity under ambient (control) and tent temperature regimes in the field for seasons 1 (2005) and 3 (2006) in Narrabri and season 2 (2006) in Texas. The mean is taken from 3 replicates.

Season	Tent No	Control Mean	Tent Mean	F test P value	l.s.d.
Temperature (°C)					
1	1	29.9	33.7	<0.001	0.72
1	2	23.8	25.5	<0.001	0.41
2	1	31.3	33.1	<0.001	0.72
3	1	26.4	25.6	0.026	0.79
3	2	27.1	29.0	<0.001	0.62
Relative humidity (%)					
1	1	56.2	80.3	<0.001	1.09
1	2	70.6	74.7	<0.001	1.29
2	1	62.7	66.3	0.039	2.59
3	1	50.6	51.9	n.s.	1.97
3	2	61.6	69.3	<0.001	1.59

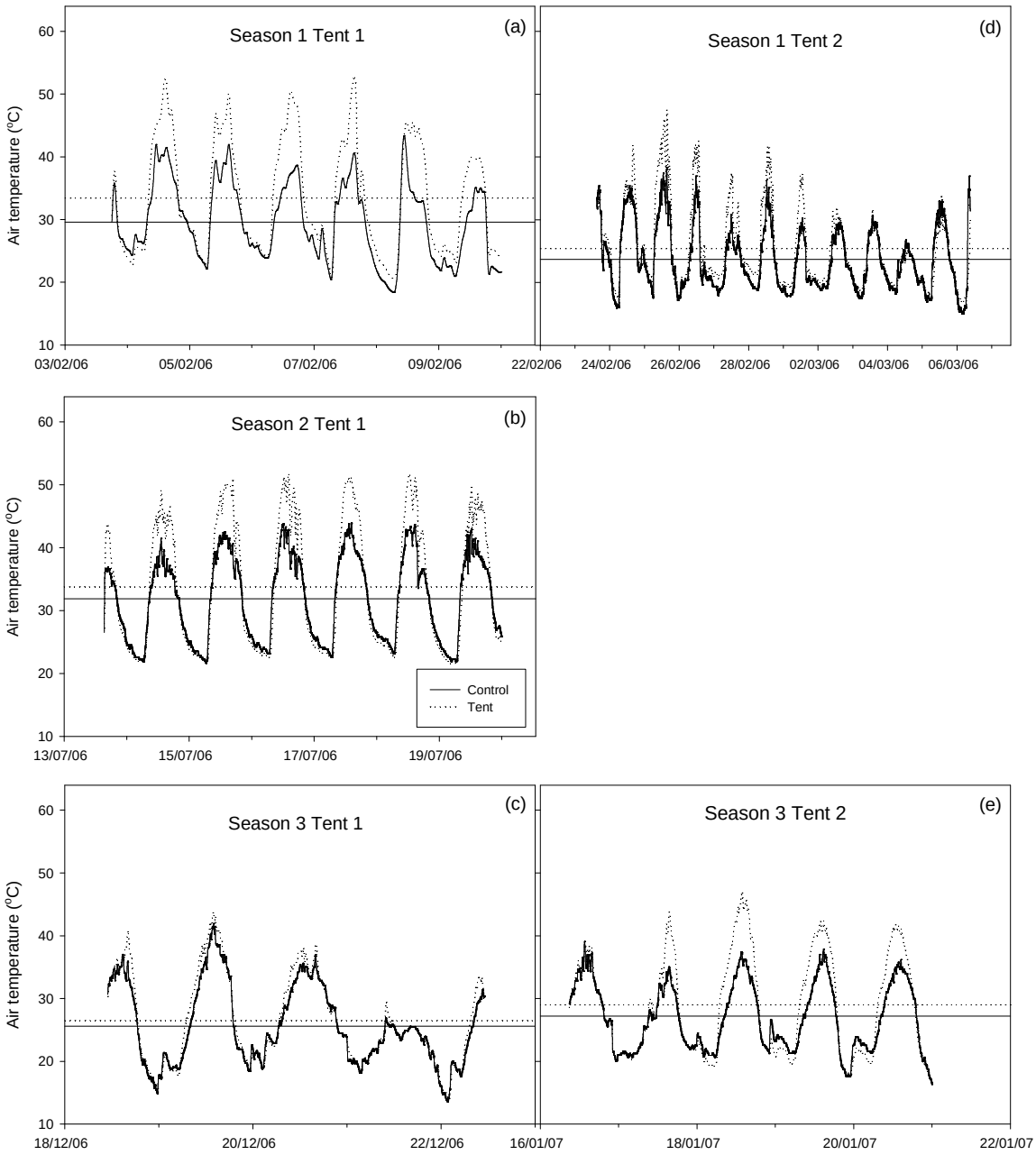

Figure 3-2 Air temperatures (°C) under the control and under tent 1 in (a) season 1 (Narrabri, 2006), (b) season 2 (Texas, 2006) and (c) season 3 (Narrabri, 2007 and under tent 2 in season 1 (Narrabri, 2006) and season 3 (Narrabri, 2007). The average temperature during the measurement period is represented by a solid line for the control and a dotted line for the tent.

3.3.4 **Measurements**

Under ambient (control) field conditions, leaves were sampled at least 1 m from the edge of the plot. For samples under high temperature (tent) conditions, leaves were sampled from the middle of three rows located under the tent, leaving a 1 m buffer on all sides. Photosynthesis, electron transport rate, stomatal conductance, transpiration rate,

membrane integrity and enzyme viability was measured on the third youngest fully expanded leaf of actively growing cotton plants under all temperature treatments. This minimised the effects of leaf age on various physiological and biochemical functioning under the various treatments (Perry *et al.* 1983).

3.4 **Growth cabinet experiments**

3.4.1 **Site description and pot management**

Plants were established in a glasshouse at the Australian Cotton Research Institute, Narrabri, Australia. Cotton plants were grown in 250 mm diameter, 9 L pots, filled with a grey cracking clay, taken from a nearby field. Each pot contained 2 plants and was arranged in a completely randomised design with 4 replicates. Pots were watered at 0700 h daily for 2 min by drip irrigation delivering 4 to 4.5 L h^{-1}. Nutrients were applied fortnightly with 500 mL, 0.013 g mL^{-1} Miracle-grow® all purpose water soluble fertiliser (Scotts Australia Pty Ltd, Baulkham Hills, Australia) (Table 3-8) with 2.0 g magnesium sulphate heptahydrate ($MgSO_4.7H_2O$).

Table 3-8 Composition of Miracle-grow fertiliser used in glasshouse and growth cabinet pot experiments (N:P:K = 15:13.1:12.4)

Nutrient	Compound	% (w/v)
Nitrogen	Mono-ammonium and di-ammonium phosphate	6.80
Nitrogen	Urea	8.20
Phosphorous	Mono-ammonium and di-ammonium phosphate	13.10
Potassium	Potassium chloride	12.40
Iron	Iron ethylenediaminetetraacetic acid	0.15
Copper	Copper sulfate	0.07
Zinc	Zinc sulfate	0.06
Manganese	Manganese ethylenediaminetetraacetic acid	0.05
Boron	Boric acid	0.02
Molybdate	Sodium molybdate	0.00

At first square, plants were transferred to a growth cabinet (14 h photoperiod commencing at 0600 h, 32 ± 3 ^{o}C, 45 ± 15% relative humidity, maximum 800 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) and 10 h dark period at 25 ± 5 ^{o}C and 85 ± 15% RH), running on a Maxim 510 controller (Innotech Trading Australia Pty Ltd, Forest Lake, QLD). 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. Relative humidity 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 approximately 360 μL/L. Plants were hand watered daily to maintain adequate soil moisture levels.

3.4.2 **Temperature treatments**

Plants were acclimated for 4 d at optimal conditions (32/25 ^{o}C day/night) in the growth cabinet before initiation of the temperature treatment. For the control (32 ^{o}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 ^{o}C (67% RH) during the photoperiod and 25 ^{o}C (40 % RH) during the dark period. Photosynthesis, electron transport rate, stomatal conductance, transpiration rate, membrane stability, enzyme viability and gene expression were then determined for the third youngest fully expanded leaf of cultivars Sicot 53 and Sicala 45. Details for specific measurements or assays are described in the respective chapters.

Chapter 4 Screening for Cultivar Specific Thermotolerance at the Crop and Whole Plant Level

4.1 Introduction

In-season crop exposure to high temperature may adversely affect cotton yield. This may be due to limited assimilate availability for growth and hence a reduction in the development of potential fruiting sites. High temperatures throughout the flowering period may result in low fruit set due to pollen infertility (Burke 2004; Kakani *et al.* 2005; Marshall *et al.* 1974), low boll weight and seeds per boll (Pettigrew 2008) or high rates of fruit abscission (Reddy *et al.* 1999; Zhao *et al.* 2005) thereby limiting yield potential. Thus yield and yield component analysis may be used to screen for stress tolerance under field conditions.

Plant breeding has traditionally focused on yield and yield components as parameters for species and cultivar selection for adaptation to local environments and management practices (Constable *et al.* 2001). However, the production of final harvestable yield and its components are the result of acclimation and exposure to biotic and abiotic factors affecting crop growth. While measuring yield does not enable cultivars to be evaluated for a specific tolerance, it still provides a description of the suitability of a particular genotype for economically viable, local production systems.

Yield-based cultivar selection in hot seasons may not only provide selection pressure for high temperature tolerance but also tolerance to other biotic or abiotic stresses associated with high temperatures such as drought, radiation stress, plant pests or pathogens prevalent in hot dry seasons. Therefore, to ensure that particular tolerances are entrenched in a breeding program, strategies must be developed to identify, isolate and target germplasm with specific tolerances.

Variability between seasons and the interaction between germplasm and biotic and abiotic stress may limit the repeatability of identification of superior cotton cultivars, particularly for temperature tolerance.

This chapter details a series of experiments, conducted to determine the effects of high temperature stress on yield, fruit retention and fibre quality and the suitability of using these measurements to determine cultivar-specific thermotolerance under field conditions. The aim of these experiments was to determine cultivar specific thermotolerance using crop and whole plant measurements for field grown plant material:

(a) under ambient temperatures (experiment 1); and

(b) under *in-situ* high temperature stress generated by tents (experiment 2)

Specifically the hypothesis tested was that yield, fruit retention and fibre qualities are effective methods of screening for cultivar specific tolerance to high temperature stress.

4.2 **Materials and methods**

4.2.1 **Site description**

Experiments 1 and 2 were conducted over three growing seasons at field sites located at The Australian Cotton Research Institute, Narrabri, Australia in 2006 (season 1) and 2007 (season 3), and at The Texas Agriculture and Experiment Station, College Station, Texas, USA in 2006 (season 2). Details for each site and season are presented in Chapter 3.

4.2.2 **Treatments**

Experiment 1

Cotton cultivars Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2 were established under field conditions in a randomised block design with four replicates. Plants were grown under ambient field conditions over three seasons under furrow irrigation and high

input management. No additional high temperature treatment was imposed on crops for experiment 1.

Experiment 2

Two cotton cultivars (Sicot 53 and Sicala 45) were grown in the field over three consecutive seasons in a randomised block design with four replicates. This experiment was conducted as a subset of experiment 1. High temperature stress was artificially imposed on field grown plants by the construction of Solarweave® tents over the crop canopy, 4 d post irrigation. Tents were removed from the field after a 7 d incubation period and transferred to a different section of the plot for further treatment. Tent 1 was assembled at during squaring. Tent 2 was assembled at approximately first flower. Yield data from the tents in season 2 were accidentally lost.

4.2.3 **Measurements**

For experiment 1, seed cotton yield was determined for cotton cultivars Sicot 53, CSX 99209-376, Sicala 45 and Sicala V-2 grown under ambient field conditions in seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas. For experiment 2, seed cotton yield, fruit retention, and fibre quality parameters; length, strength and micronaire were measured for cultivars Sicot 53 and Sicala 45 grown under ambient (control) or tent regimes in the field for seasons 1 (2006) and 2 (2007) in Narrabri.

Plants were defoliated when 60% of bolls were open and subsequently harvested at maturity. Plants were excised below the cotyledons from a 1 m section of a single row, and at a distance of at least 2 m from the border of each plot to measure yield, fruit retention and fibre quality. For samples under ambient (control) field conditions, plants were sampled from at least one metre from the edge of the plot. Under the tents in experiment 2, plants were sampled from the middle of three rows under the tent, leaving a

one metre buffer area to all sides. Plants were immediately transferred to the laboratory for processing.

Fruit retention was calculated as the percentage of final open bolls to total fruiting sites on sympodial branches. Seed cotton from each open boll was removed for yield determination. A subsample was taken and ginned for lint quality. Lint was excised from the seed coat in a Continental Eagle 20-saw gin. Lint yield was calculated from ginned lint samples. Fibre length, strength and micronaire were determined for ginned lint samples using a high volume instrument (HVI).

4.2.4 **Data analysis**

Experiment 1

One-way Analysis of Variance (ANOVA) was conducted for total seed cotton yield of cotton cultivars Sicot 53, CSX 99209-376, Sicala 45 and Sicala V-2, pooled across the 3 seasons. Two-way ANOVA (cultivar*season) was conducted for total seed cotton yield of cultivars grown under ambient field conditions in season 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas. One-way ANOVA was employed to determine cultivar specific differences in seed cotton yield for each individual season.

Experiment 2

One-way ANOVA was conducted for seed cotton yield, fruit retention and fibre quality parameters of cotton cultivars Sicot 53 and Sicala 45, pooled for seasons 1 (2006) and 3 (2007) in Narrabri and pooled for the ambient (control) and high temperature (tent) treatment regimes. Two-way ANOVA (cultivar*tent) was conducted for seed cotton yield, fruit retention and fibre quality of cotton cultivars under ambient (control) and high temperature (tent) regimes in the field and pooled for seasons 1 (2006) and 3 (2007) in Narrabri. Two-way ANOVA (cultivar*tent) was conducted for seed cotton yield, fruit

retention and fibre quality parameters of cotton cultivars under ambient (control) and high temperature (tent) conditions in the field, pooled for each season and data for each season were analysed independently. One-way ANOVA was conducted for seed cotton yield, fruit retention and fibre quality of cotton cultivars under ambient (control) field conditions or under the tents at an early (tent 1) or later (tent 2) growth stage.

4.3 **Results**

4.3.1 **Experiment 1**

Yield for Australian cotton cultivars, pooled over 3 seasons

Mean seed cotton yield of all four Australian cotton cultivars was higher (P<0.001) in seasons 1 and 3 compared with season 2. The cotton seed yield of Sicala V-2 plants was lower (P=0.026) than the other three cultivars when pooled over the 3 growing seasons (Figure 4-1). The seed cotton yield of Sicot 53, Sicala 45 and CSX 99209-376 did not differ.

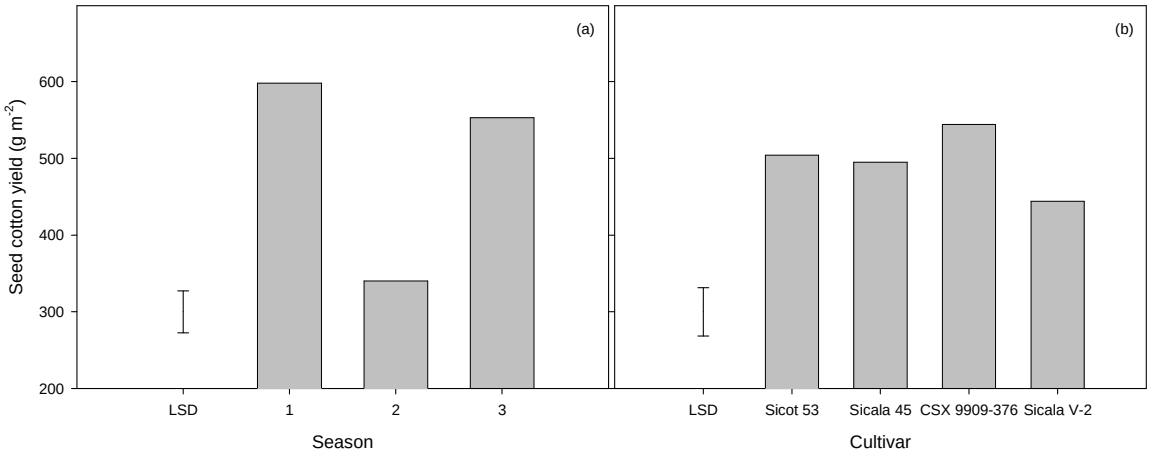

Figure 4-1 Mean seed cotton yield for (a) plants grown at the Narrabri field sites in seasons 1 (2006) and 3 (2007) and at the Texas field site in season 2 (2006) and (b) Australian cotton cultivars Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2, pooled over the three seasons. The vertical lines represent the l.s.d. for season or cultivar main effects at P<0.05.

Cotton seed yield was analysed for the four Australian cotton cultivars for each season independently. For season 1, the seed cotton yield of CSX 99209-376 was higher

(*P*=0.044) than the seed cotton yield for Sicala 45 and Sicala V-2 (Table 4-1). For season 2, the mean cotton seed yield was higher for Sicala 45 compared with Sicot 53 and mean seed cotton yield of Sicala V-2 plants was lower (*P*<0.001) than the other three cotton cultivars (Table 4-1). There were no cultivar differences for seed cotton yield in season 3 (Table 4-1).

Table 4-1 Seed cotton yield (g m^{-2}) for cultivars Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2 grown under ambient field conditions during season 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas, where n.s. represents F test P values that are not significant for *P*<0.05. Means followed by the same letter in the same row are not significantly different at *P* <0.05.

Season	Sicot 53	Sicala 45	CSX 99209-376	Sicala V-2	Grand Mean	Max L.S.D.	P value
1	621 ab	550 b	684 a	536 b	598	109	0.044
2	345 b	385 a	363 ab	268 c	340	35	<0.001
3	547	550	586	529	553	-	n.s.

4.3.2 **Experiment 2**

Yield, fruit retention and fibre quality response to heat stress, pooled over 2 seasons

Yield, fruit retention and fibre quality parameters were determined for cotton cultivars Sicot 53 and Sicala 45, grown under either ambient (control) or tent (tent 1 and tent 2) regimes in the field for seasons 1 and 3. Results are summarised in Table 4-2. Although cultivar differences were determined for fruit retention, fibre strength and micronaire in season 1 and temperature treatment differences were found for fruit retention, seed cotton yield and fibre length in season 2, an interaction between cultivar and temperature treatment was only determined for fruit retention in season 1.

Table 4-2 Probability of cultivar and temperature treatment main effects and cultivar by temperature treatment interaction for yield, fruit retention and fibre quality for seasons 1 (2006) and 3 (2007) in Narrabri, where n.s. represents F test P values where *P*>0.05.

	Cultivar	Temperature treatment	Cultivar * Temperature treatment
Season 1			
Seed cotton yield	n.s.	<0.001	n.s.
Fruit retention	0.002	<0.001	<0.001
Fibre length	n.s.	0.012	n.s.
Fibre strength	0.007	n.s.	n.s.
Micronaire	0.045	n.s.	n.s.
Season 3			
Seed cotton yield	n.s.	0.027	n.s.
Fruit retention	n.s.	0.022	n.s.
Fibre length	n.s.	0.049	n.s.
Fibre strength	n.s.	n.s.	n.s.
Micronaire	n.s.	n.s.	n.s.

Yield

The mean seed cotton yield of plants grown under tents were lower than plants grown under ambient (control) conditions for tent 1 in season 1 ($P<0.001$) and in tent 2 in season 3 ($P=0.026$) (Figure 4-2). There were no cultivar differences for yield in seasons 1 or 3.

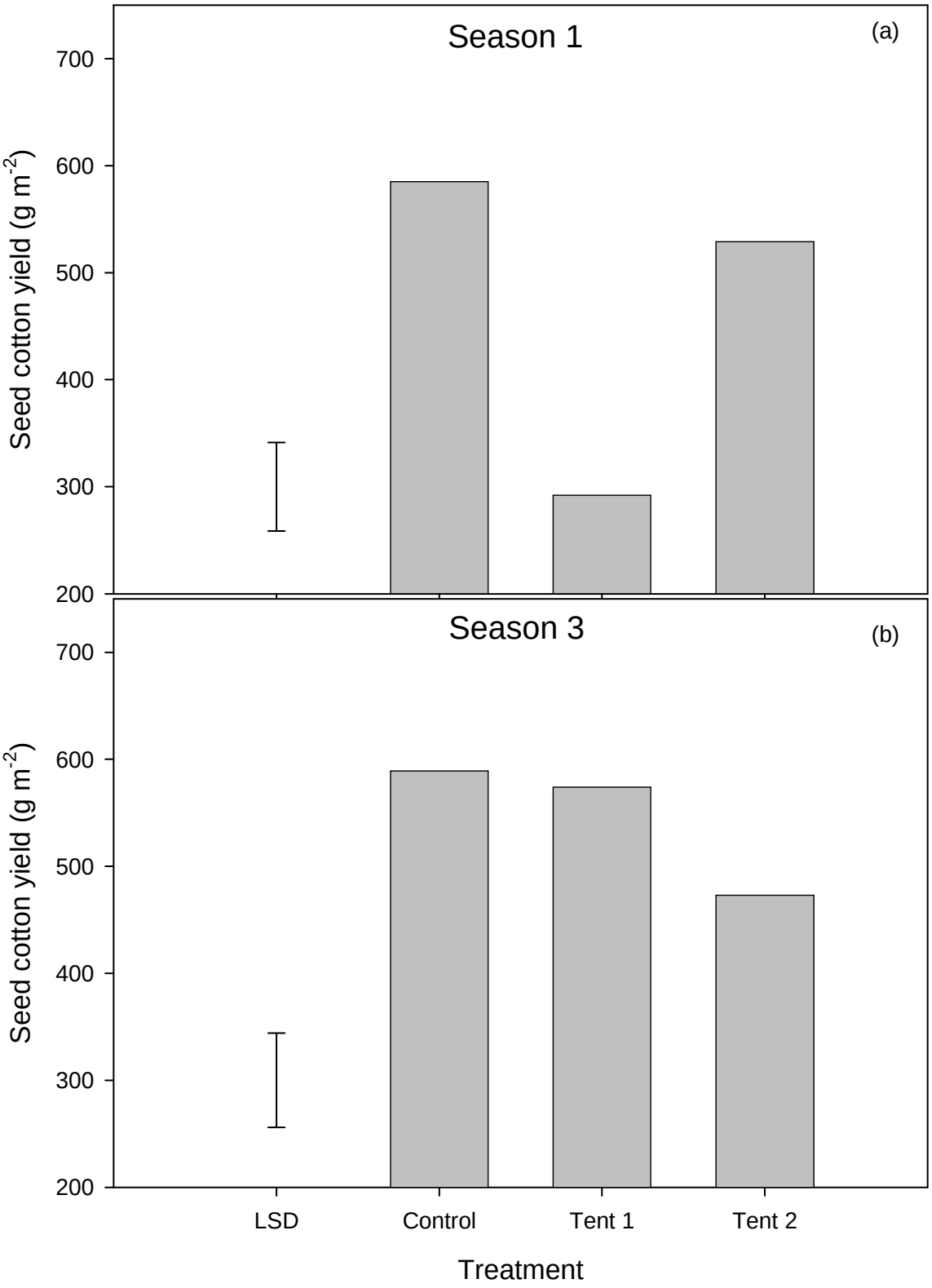

Figure 4-2 Mean seed cotton yield of cotton cultivars Sicot 53 and Sicala 45 under ambient (control) and under the tents at an early (tent 1) or later (tent 2) growth stage in (a) season 1 (2006) and (b) season 3 (2007) at Narrabri. The vertical lines indicate the l.s.d. value at *P*<0.05 for temperature treatment main effect.

Fruit retention

Overall fruit retention was lower (P<0.001) for season 1 than season 3 (Figure 4-3). For season 1, there was an interaction (P<0.001) between cultivar and treatment for fruit retention (Table 4-2). Under ambient (control) conditions in the field, fruit retention for Sicot 53 was higher than Sicala 45. There was no difference in fruit retention between the two cultivars under the tents. For season 1, fruit retention was lower for plants grown under tent 1 compared with tent 2 and under ambient (control) conditions in the field (Figure 4-3). For season 3 fruit retention under tent 2 was lower (P=0.026) than the fruit retention for plants growing under ambient (control) field conditions.

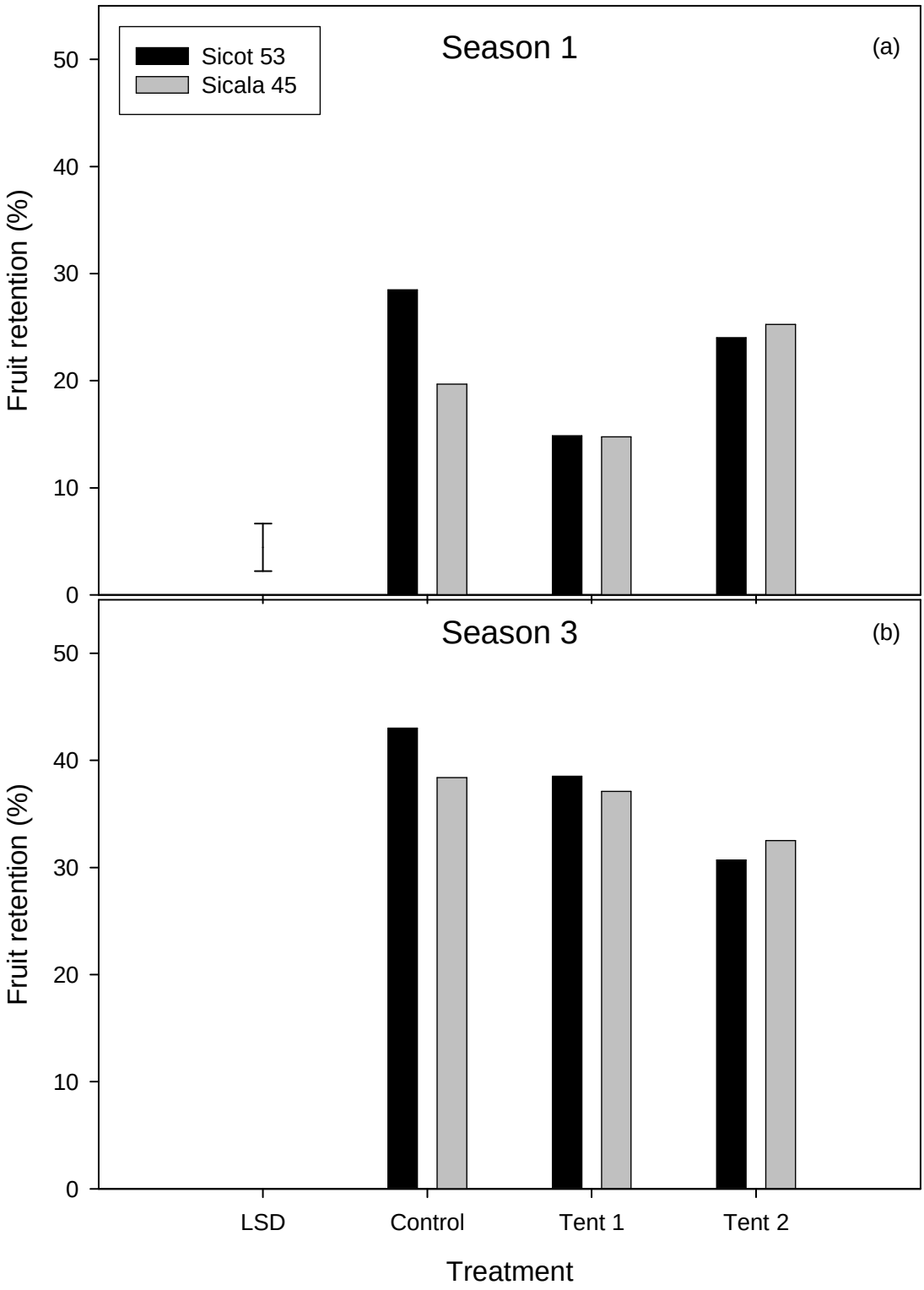

Figure 4-3 Fruit retention (%) of cotton cultivars Sicot 53 and Sicala 45 under ambient (control) and under the tents (tent 1 and tent 2) during (a) season 1 (2006) and (b) season 3 (2007) at Narrabri. The (a) vertical line represents the l.s.d. for temperature treatment by cultivar interaction at *P*<0.05.

A positive correlation (P<0.0001) existed between fruit retention and seed cotton yield under field conditions in season 1 (Figure 4-4) and accounted for up to 47 % of the variation (Table 4-3). In season 3, there was no correlation (P>0.05) between fruit retention and seed cotton yield (Figure 4-4).

Table 4-3 Correlation between fruit retention (%) and seed cotton yield (g m^{-2}) under ambient (control) and tent regimes in the field in season 1 (2006) and 3 (2007) in Narrabri where n.s. represents F test P values where P<0.05.

Season	n	Adjusted R^2	Equation	P value
1	24	0.47	y=132.31+15.83*x	<0.001
3	24	-	-	n.s.

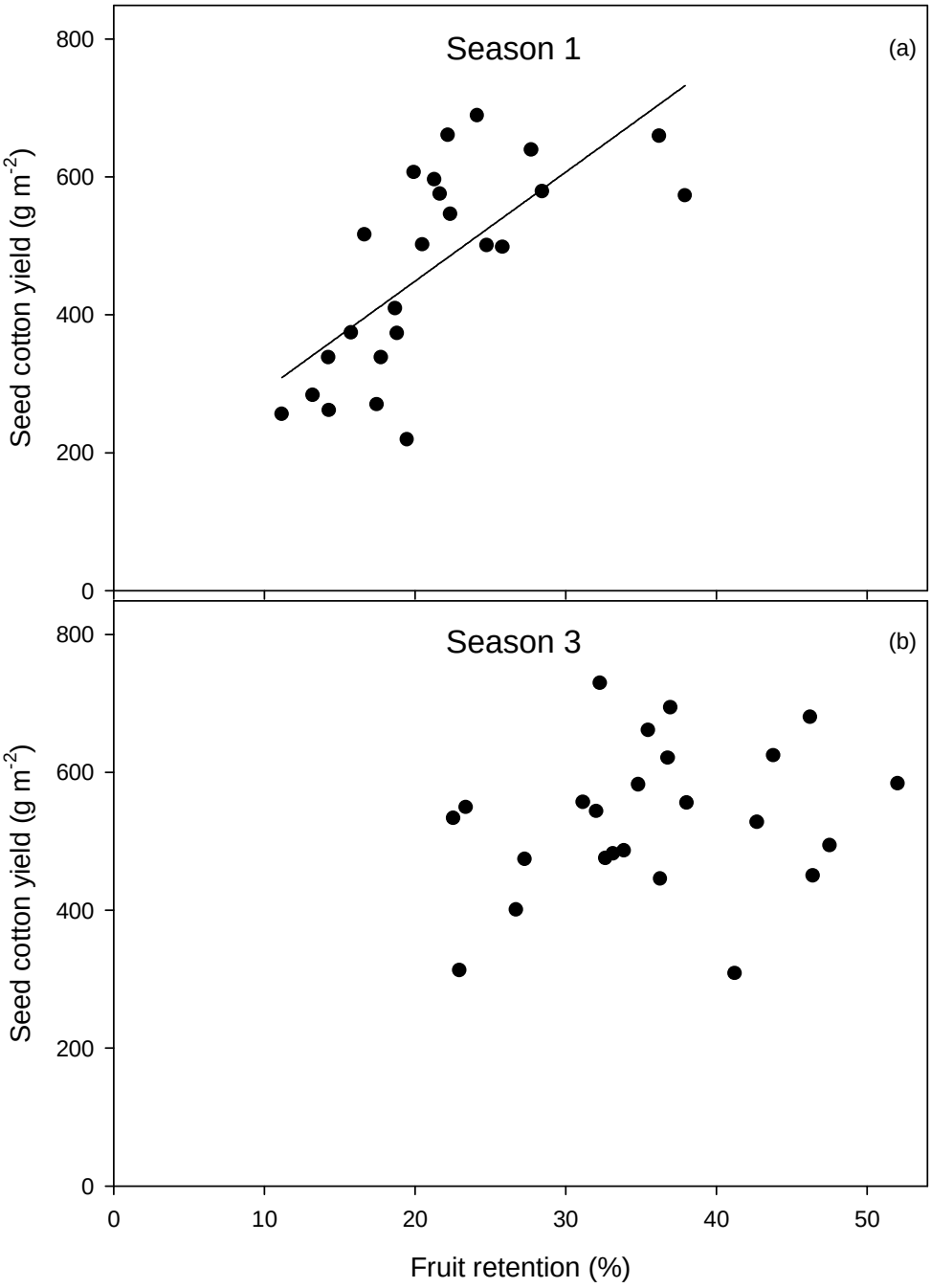

Figure 4-4 Correlation between fruit retention (%) and seed cotton yield (g m^{-2}) for cotton cultivars Sicot 53 and Sicala 45 grown under ambient and tent regimes in the field in (a) season 1 (2006) and (b) season 3 (2007) at Narrabri, replicated 4 times.

Fibre quality

Fibre length was lower (P=0.012) for plants grown under tents 1 and 2 compared with plants grown under ambient (control) field conditions in season 1 (Figure 4-5). For season 3, fibre length was lower (P=0.019) for plants grown under tent 1 compared with plants

grown under tent 2 and plants grown under ambient (control) field conditions (Figure 4-5). There were no cultivar differences for fibre length.

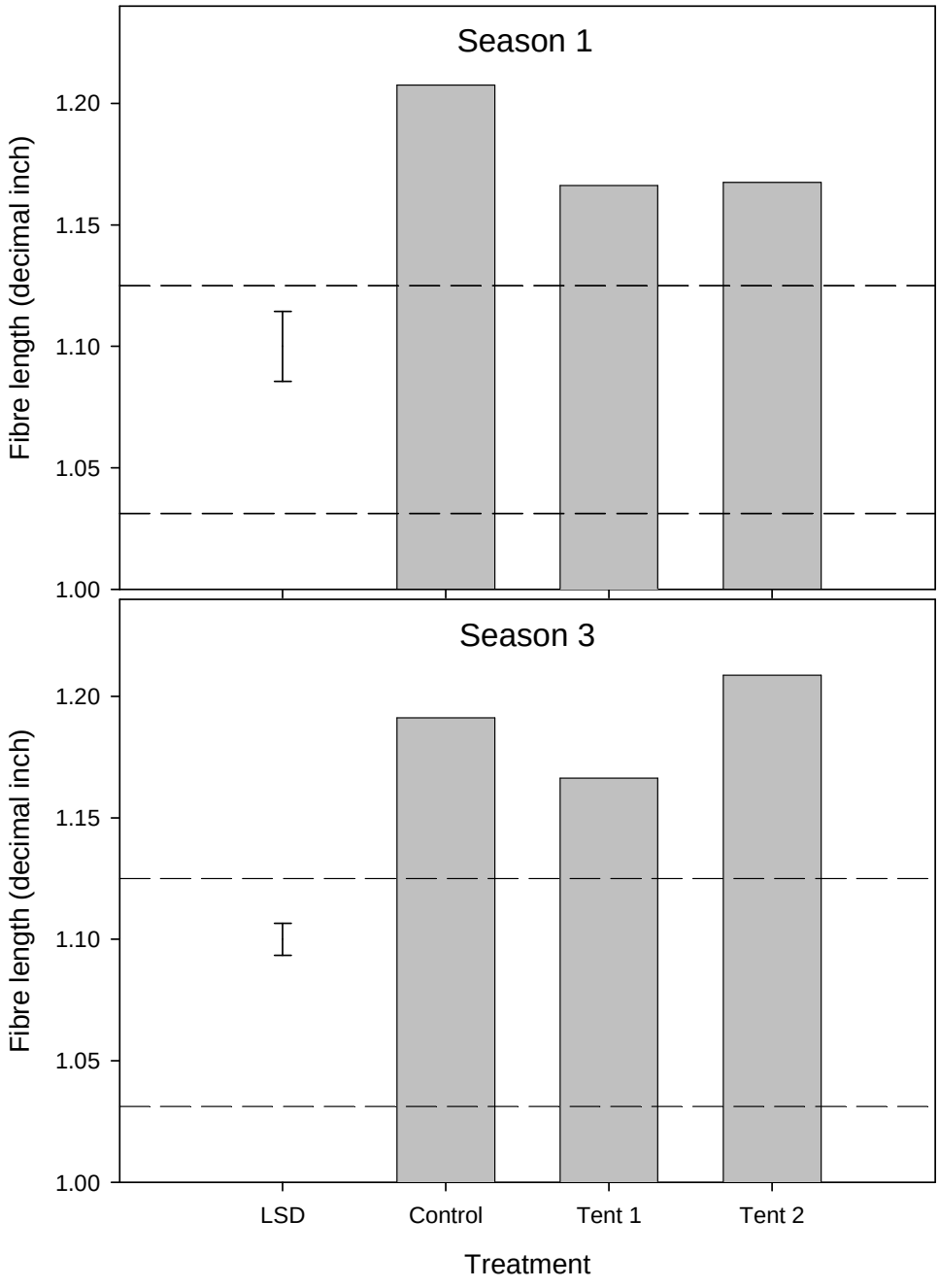

Figure 4-5 Mean fibre length (decimal inch) of plants grown under ambient (control) and under the tents (tent 1 and tent 2), grown during (a) season 1 (2006) and (b) season 3 (2007) at Narrabri. The vertical lines represent the l.s.d. for temperature treatment main effects at *P*<0.05. The upper line represents the ideal target for fibre length (1.125 inch). The lower line represents the minimum target for fibre length (1.031 inch) under which, significant financial penalties are incurred.

Fibre strength in season 1 was lower (P<0.001) than in season 3 (Figure 4-6). Fibre strength under ambient (control) conditions was higher (P=0.007) than under high temperature (tent) conditions during season 1 (Figure 4-6). There was no difference (P>0.05) in fibre strength under ambient (control) or under the tents during season 3. There were no cultivar differences (P>0.05) for fibre strength in seasons 1 or 3. The micronaire of cotton cultivars Sicot 53 and Sicala 45 did not differ under the tents compared with ambient (control) field conditions.

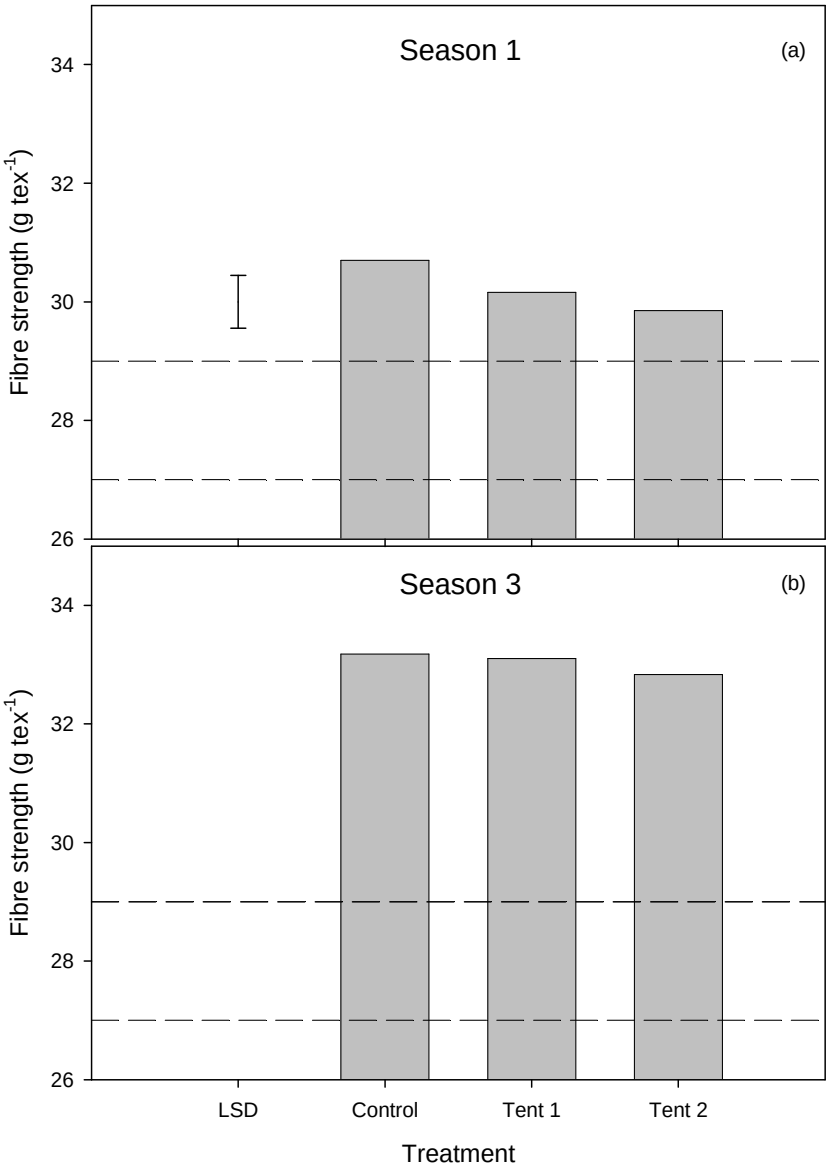

Figure 4-6 Mean fibre strength (g tex^{-1}) of Sicot 53 and Sicala 45 under ambient (control) and high temperature (tent 1 and tent 2) conditions during (a) season 1 (2006) and (b) season 3 (2007) at Narrabri. The vertical line represents the l.s.d. for temperature treatment main effects at *P*<0.05. The upper dashed line represents the industry target for fibre strength (29 g tex^{-1}) above which, small premiums are obtained for superior fibre quality. The lower dashed line represents the industry minimum for fibre strength (27 g tex^{-1}) under which discounts are incurred for sub-standard fibre quality.

4.4 **Discussion**

Yield has been traditionally employed by plant breeders as a primary screening parameter for determining the adaptability of cotton cultivars to local production systems (Constable *et al.* 2001). Yield evaluation of four cotton cultivars over three consecutive growing seasons showed that Sicala V-2 was consistently lower yielding than Sicot 53, Sicala 45

and CSX 99209-376. This cultivar discrimination indicates that that evaluation of yield and fruit retention under ambient field conditions provides a discriminatory mechanism for identification of cultivar specific adaptability to local conditions and higher yield potential thus validating traditional thinking in conventional plant breeding.

However, yield-based selection under favourable conditions may eventuate in the selection of genotypes that perform well under ambient conditions but fail to perform under stress conditions (Lopez *et al.* 2003b). Furthermore, due to the complex nature of yield and a multitude of selection pressures for yield across the growing season (Watson 1952), the exact mechanism for cultivar-specific yield differentiation cannot be concluded to be heat stress from this set of experiments. Hence, Solarweave® tents were employed to determine if temperature could be a primary determinant of cotton yield.

Seed cotton yield and fruit retention were lower for Sicot 53 and Sicala 45 under tent 1 in season 1 and tent 2 in season 3, compared with plants grown under ambient (control) conditions. This indicates that exposure of plants to temperatures exceeding 45 ^{o}C for at least 6 h under the tents (Table 3-6) provided sufficient exposure to abiotic stress to illicit a stress response and subsequent decreases in yield and fruit retention. Decreased yield and fruit retention under the tents on only one of two instances in both seasons 1 and 3 may be attributed to a high thermal sensitivity at the squaring and flowering developmental stages and exacerbated by environmental conditions at the time of tent installation.

Fruit retention is most likely the main factor associated with lower yields under the tents in a hot season; this agrees with previous research showing fruit shedding under high temperature stress (Reddy *et al.* 1999; Reddy *et al.* 1992; Zhao *et al.* 2005), and specifically for plants grown under tents (Dhopte and Eastin 1988; Lopez *et al.* 2003a).

Decreased yield may also be partially attributed to pollen infertility (Burke 2004; Kakani *et al.* 2005) or low boll weight and seeds per boll (Pettigrew 2008). Differences in plant physiological capacity under ambient (control) conditions may be exacerbated by inherent climatic differences between the Narrabri and Texas field sites, and physiological process under ambient and tent regimes in the field are potentially influenced by seasonal weather differences between seasons 1 and 3 at the Narrabri field site.

Cultivar rankings for yield varied between field sites and seasons. Previous reports for yield under tents are for a single season (Dhopte and Eastin 1988; Lopez *et al.* 2003a) thereby not accounting for seasonal influences on cultivar performance. Plants grown at the Texas site (season 2) were exposed to high average daily temperatures but a fewer number of days with temperatures exceeding the high temperature threshold for cotton (35 ^{o}C). Plants grown at Narrabri (seasons 1 and 3) were typically exposed to a lower average temperature but a higher number of days exceeding the high temperature threshold for cotton (35 ^{o}C) (Table 3-4). Hence it not surprising that the Australian-developed cultivars Sicot 53, Sicala 45, Sicala V-2 and CSX 99209-376 yielded highest in a mild season (Season 3) at the Narrabri field site; conditions for which they were specifically bred. The seasonal nature of yield indicates that screening for thermotolerance using yield both under ambient (control) conditions and under the tents is largely dependent on climate and in-season weather patterns and hence, requires sufficient replication in a local environment for the development of a reliable cultivar-specific heat tolerance index.

Fibre length and strength were reduced under the tents compared with plants grown under ambient (control) conditions. This is consistent with previous studies (Pettigrew 2008; Reddy *et al.* 1999) showing that exposure to high temperature stress limits fibre elongation and may ultimately have a deleterious effect on fibre quality. However, both

Sicot 53 and Sicala 45 plants still exceeded the minimum and recommended industry targets for all fibre quality parameters. Although fibre length and strength are not useful indicators of genotypic stress tolerance, these parameters need to be monitored during breeding programs to ensure that breeding for heat tolerance does not inadvertently reduce fibre quality.

4.5 **Conclusion**

Yield is an excellent predictor of overall plant functionality within a season and hence, is a useful selection tool for plant breeders, whilst also providing an industry recognised parameter and target for breeding improvement. Yield is also readily translatable to actual farming systems and hence is an important output parameter for any plant breeding program.

Quantification of yield, fruit retention and fibre quality under the tents provided little evidence for thermotolerance discrimination between Sicot 53 and Sicala 45 for thermotolerance. These results suggest that the mechanisms underlying yield in the field are a complex network of physiological changes involved with heat tolerance and heat avoidance. Heat tolerant plants may be better candidates for screening programs as heat avoidant plants are generally reliant on higher inputs such as water to maintain homeostasis under high temperature stress. Plant physiological, biochemical and molecular screening techniques should be concurrently investigated to provide an understanding of cultivar specific heat tolerance under field conditions and the genetic basis of thermotolerance in cotton cultivars. Furthermore, inclusion of a greater diversity of germplasm and a larger number of samples may aid in accounting for large biological variability in stress tolerance screening, thereby increasing the effectiveness of yield-based, in-field screening programs.

Chapter 5 Screening for Cultivar Specific Thermotolerance at the Leaf Level

5.1 Introduction

Temperature influences the growth and development of crops. It is a primary factor determining sowing date, seasonal development and harvest date. Cotton has an optimal thermal window of 23 to 32 ^{o}C in which metabolic activity is most efficient (Burke *et al.* 1988). Maximum daily temperatures exceeding 32 ^{o}C are common in many cotton growing regions and may limit growth, development and ultimately crop yield. Effects of high temperature stress include decreased plant growth, delayed development and increased fruit shedding (Hodges *et al.* 1993). This may be attributed to photosynthetic decline under high temperature stress (Reddy *et al.* 1991a), particularly attributed to decreased electron flow through the photosystem (Wise *et al.* 2004) through membrane disruption (Sullivan 1971) and decreased stability of photosynthetic (Salvucci and Crafts-Brandner 2004b) and respiratory enzymes (de Ronde and van der Mescht 1997).

Changes in plant physiological function under high temperature stress may be useful to identify heat tolerant genotypes for inclusion in future breeding programs (Constable *et al.* 2001) for production in the warmer cotton growing regions of the world. Point-in-time (survey) measurements for photosynthesis, electron transport rate, stomatal conductance and transpiration are established methods of screening for high and low temperature tolerance in cotton under greenhouse conditions (Bibi *et al.* 2004b; Brown and Oosterhuis 2004; McDowell *et al.* 2007) and for drought stress (Kitao and Lei 2007; Leidi *et al.* 1993) and waterlogging tolerance (Conaty *et al.* 2008) screening studies in the field. However, plant specific responses to high temperature stress may differ under greenhouse and field conditions due to additional environmental stress in the field (Watson 1952).

In-field high temperature stress has been achieved by staggering planting date (Rahman 2005), ambient temperature-dependent sampling (Bibi *et al.* 2004a) and the use of polyethylene shelters (Lopez *et al.* 2003a) for drought stress studies. Few studies evaluating heat tolerance under field conditions have been reported, primarily attributed to the difficulty in distinguishing between water stress tolerance and high temperature tolerance. Field studies are furthermore confounded by acclimation to high temperature stress in hot growing seasons.

This chapter examines the effectiveness of measuring rates of leaf gas exchange and chlorophyll fluorescence under high temperature stress to screen for cultivar specific thermotolerance both under field and glasshouse conditions. The aim of these experiments was to determine genotype specific thermotolerance of cotton cultivars Sicot 53 and Sicala 45 using gas exchange and fluorescence measurements for;

(a) field grown plant material under ambient (control) or high (tent) temperatures (Experiment 1), and correlate these measurements with yield; and

(b) growth-cabinet grown plant material under optimal (32 ^{o}C) or high (42 ^{o}C) temperature regimes by evaluation of light response curves [400, 800, 1200, 1600, 2000 μmol PAR m^{-2} s^{-1}] (Experiment 2)

Specifically the hypothesis tested was that genotypic differences in heat tolerance can be quantified by measuring photosynthesis, electron transport rate, stomatal conductance and transpiration rate.

5.2 **Materials and methods**

5.2.1 **Site description**

Experiment 1 was conducted over three growing seasons at two field sites. Seasons 1 (2006) and 3 (2007) were conducted at the Australian Cotton Research Institute, Narrabri. Season 2

(2006) was conducted at The Texas Agriculture and Experiment Station, Texas (Table 5-1). Experiment 2 was conducted at the Australian Cotton Research Institute, Narrabri. Plants were initially established in a glasshouse and then transferred to a growth cabinet. Details of each experiment are presented in Figure 5-1.

Table 5-1 Location and timing of experiments 1 and 2

Experiment	Site	Season	Year	Location
1	Field	1	2006	Narrabri
1	Field	2	2006	Texas
1	Field	3	2007	Narrabri
2	Growth cabinet	-	2007	Narrabri

5.2.2 **Treatments**

Experiment 1

Cotton cultivars Sicot 53 and Sicala 45 were grown under ambient (control) field conditions in a randomised block design with four replicates. The photosynthetic rate, electron transport rate, stomatal conductance and transpiration rate of the two cultivars was measured on various days under ambient (control) field conditions and also under the tents (Table 5-2).

Experiment 2

Plants of cotton cultivars Sicot 53 and Sicala 45 were established under glasshouse conditions in a completely randomised design with four plants per treatment and transferred to a growth cabinet at first square. Photosynthesis, electron transport rate, stomatal conductance and transpiration were measured under optimal (32 ^{o}C) and high temperature (42 ^{o}C) conditions in the growth cabinet at 1230 h and daily for a 3 d period. Measurements were taken at various light intensities (200, 400, 600, 800, 1000, 1200 µmol PAR m^{-2} s^{-1}) by implementation of a step-wise incremental light intensity auto-program for an internal light source in the sensor head of a Li-6400 portable photosynthesis system.

5.2.3 **Measurements**

Gas exchange & fluorescence

Measurements of photosynthesis, electron transport rate, stomatal conductance and transpiration were made using a Li-6400 portable photosynthesis system (Li-Cor Ltd, Lincoln, NE, USA), with a pulse-amplitude modulated (PAM) leaf chamber fluorometer sensor head. Environmental variables were highly controlled in the sensor head for effective comparison between samples and were set to approximately ambient external conditions for the day of sampling. The reference carbon dioxide concentration was set at 400 μmol CO_2 mol^{-1} using a CO_2 mixer. Relative humidity followed ambient conditions. The system flow rate was adjusted to maintain a vapour pressure deficit between 1.5 and 2.5 kPa. Light adapted fluorescence was measured using the fluorometer attachment, immediately following the photosynthesis measurement.

Experiment 1

Gas exchange and fluorescence were determined on multiple days throughout the season (Table 5-2). Initial photosynthetic rates and chlorophyll fluorescence were measured at ambient field conditions (Day 0). The tents were then erected and left over the crop canopy for a defined number of days (ranging from 2 to 5 d) whilst measurements were taken (Table 5-2). The tents were removed and a recovery measurement was taken two days later. The timing of tent construction and removal was dependent on irrigation cycles, rain events and availability of resources and therefore differed slightly between experiments. This treatment was repeated twice in seasons 1 (2006) and 3 (2007) in Narrabri. All measurements were taken between the developmental stages of pinhead square and cut-out. The physiological parameters were analysed for each day before, during and after tent installation to account for daily climatic variation and incremental exposure to high temperature stress under the tents. Single measurements were taken for 3 different plants per replicate over 4 replicates. All

measurements were taken between 1000 and 1230 h (Eastern Summer Time – Australia). Light intensity of the Li-6400 was set at 2000 μmol m^{-2} s^{-1}. The leaf chamber block temperature was maintained at 30 ^{o}C as it is considered within the optimal temperature range for photosynthesis (cf. Burke *et al.* 1988; Wise *et al.* 2004).

Table 5-2 Measurement dates for photosynthetic rate, electron transport rate, stomatal conductance and transpiration rate of cotton cultivars Sicot 53 and Sicala 45, grown under ambient (control) and tent regimes in the field in seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas.

Season	Tent number	Initial (Day 0) measurement	1st time of measurement	2nd time of measurement	Recovery measurement
1	1	3-Feb-06	5-Feb-06	9-Feb-06	11-Feb-06
1	2	23-Feb-06	25-Feb-06	6-Mar-06	-
2	1	13-Jul-06	15-Jul-06	19-Jul-06	21-Jul-06
3	1	17-Dec-06	20-Dec-06	22-Dec-06	15-Jan-07
3	2	16-Jan-07	18-Jan-07	20-Jan-07	-

Experiment 2

Gas exchange and fluorescence measurements were taken on the third youngest fully expanded leaf of 4 plants per treatment. Measurements were taken between 1000 h (4 h into the photoperiod) and 1400 h for 7 d. Measurements for gas exchange and fluorescence were taken at increasing light intensities at 200, 400, 600, 800, 1000 and 1200 μmol m^{-2} s^{-1} PAR by implementation of a step-wise incremental light intensity auto-program for the internal light source on the Li-6400 portable photosynthesis system. The leaf chamber block temperature of the Li-6400 was set to 32 ^{o}C in the optimal growth cabinet and 42 ^{o}C in the high temperature growth cabinet. Leaf temperature measurements were taken using a Mikron M100 series portable infrared thermometer.

5.2.4 **Data analysis**

Experiment 1

Two-way Analysis of Variance (ANOVA) (cultivar*temperature) was conducted for photosynthesis, electron transport rate, stomatal conductance and transpiration of cultivars

Sicot 53 and Sicala 45 under ambient (control) conditions and under the tents in the field and pooled for all 3 seasons.

An index of these 4 physiological parameters was then created by analysing the principal components (Manly 2005) using multivariate analysis (Genstat, 10th edition). The development of a single index for plant physiological function under abiotic stress may be more beneficial than analysis of single physiological measurements in isolation. Data from all 3 seasons was combined for this analysis.

Two-way ANOVA (cultivar*temperature) was conducted for photosynthesis, electron transport rate, stomatal conductance and transpiration rate of cultivars Sicot 53 and Sicala 45 under ambient (control) and tent regimes in the field independently for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas.

A linear regression was fitted to determine whether changes in stomatal conductance could predict photosynthesis under high temperature stress in the field. Analysis was run for Sicot 53 and Sicala 45 plants grown under ambient (control) conditions or under the tents for seasons 1, 2 and 3 separately.

Two-way ANOVA (cultivar*temperature) was conducted for gas exchange and fluorescence of cultivars Sicot 53 and Sicala 45, grown under ambient (control) and tent regimes in the field, where an independent ANOVA was conducted for each individual day under the tents.

A linear regression was fitted to correlate photosynthesis, electron transport rate, stomatal conductance, transpiration, principal component 1 and principal component 2 with seed cotton yield under ambient or tent regimes in the field. Analysis was run for Sicot 53 and Sicala 45 plants grown under ambient (control) conditions or under the tents for combined data from seasons 1 (2006) and 3 (2007) in Narrabri. Season 2 (2006) was excluded from the

analysis as seed cotton yield data from under the tents was misplaced. The means for each season, temperature treatment and cultivar were represented graphically.

Experiment 2

Waiting-in-line curve fit for light response curve

Electron transport rate and photosynthesis were plotted against irradiance (200, 400, 800, 1200, 1600, 2000 μmol PAR m^{-2} s^{-1}) and a waiting-in-line curve (Ritchie 2008) was fitted to the data for cotton cultivars Sicot 53 and Sicala 45 grown under optimal and high temperature regimes in the growth cabinet (Equation 5-1).

$$y=A*k*E*e^{-kE} \qquad \text{(Equation 5-1)}$$

Where y = photosynthesis measured as electron transport rate, A = maximum ETR as irradiance approaches infinity, k = irradiance at ½ A and E = irradiance.

The photosynthetic efficiency (α0), optimal irradiance for electron transport (optimum E) or maximum photosynthesis (Pmax) was determined for each cultivar and each temperature treatment. Data were loge transformed to account for increasing variance with increasing irradiance; however, this did not improve the fit of the curve and hence all data presented were on an untransformed basis.

A linear regression was fitted to determine whether stomatal conductance or transpiration rate changed with exposure to step-wise increases in irradiance. Analysis was run for cultivars Sicot 53 and Sicala 45 grown under optimal (32 ^{o}C) or high (42 ^{o}C) temperature regimes in the growth cabinet. Data presented is the mean of 4 replicates.

REML

A linear mixed model (REML) was fitted for photosynthesis, electron transport, stomatal conductance and transpiration rate of cultivars Sicot 53 and Sicala 45 at various rates of

irradiance (200, 400, 800, 1200, 1600, 2000 μmol PAR m^{-2} s^{-1}) under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet. Cultivar, growth cabinet temperature and irradiance were analysed for main effects and interactions (cultivar*cabinet temperature*irradiance). The REML was run using Genstat 10.0 and was used in preferences to ANOVA as the design of this experiment was unbalanced.

A linear mixed model was also fitted for leaf temperature of cultivars Sicot 53 and Sicala 45 under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet. Cultivar and growth cabinet temperature were analysed for main effects and interactions (cabinet temperature*cultivar). The REML was run using Genstat 10.0 in preference to ANOVA to include a random model incorporating day of measurement and replicate number (day*replicate).

5.3 **Results**

5.3.1 **Experiment 1**

Physiological response to heat stress, pooled over 3 seasons

The photosynthetic rate, electron transport rate, stomatal conductance and transpiration rate of Sicot 53 and Sicala 45 were measured under ambient (control) and tent regimes in the field and pooled for seasons 1, 2 and 3. All four physiological parameters were influenced by the imposition of the tents (Table 5-3). Although there were no cultivar effects evident for ETR, stomatal conductance or transpiration, an interaction was found between cultivar and temperature treatment for photosynthesis (Table 5-3).

Table 5-3 Probability of cultivar and temperature treatment (control, tents) main effects and cultivar by temperature treatment interaction for photosynthesis, electron transport rate, stomatal conductance and transpiration rate for cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) conditions and under the tents during seasons 1 (2006) and 3 (2007) at Narrabri and in and season 2 (2006) in Texas, where n.s. represents F test P values where *P*<0.05.

Measurement	Cultivar	Temperature treatment	Cultivar * Temperature treatment
Photosynthesis	n.s.	0.041	0.043
Electron transport rate	n.s.	<0.001	n.s.
Stomatal conductance	n.s.	<0.001	n.s.
Transpiration rate	n.s.	<0.001	n.s.

The photosynthetic rate of Sicala 45 was higher (*P*=0.043) (Table 5-3) under ambient (control) conditions but lower under the tents compared with Sicala 53 (Figure 5-1). The electron transport rate was lower (*P*<0.001) (Table 5-3) under the tents (Figure 5-2), whilst the stomatal conductance and transpiration rate were higher (*P*<0.001) (Table 5-3) under the tents compared with ambient (control) conditions (Figure 5-2), but these differences were not cultivar specific.

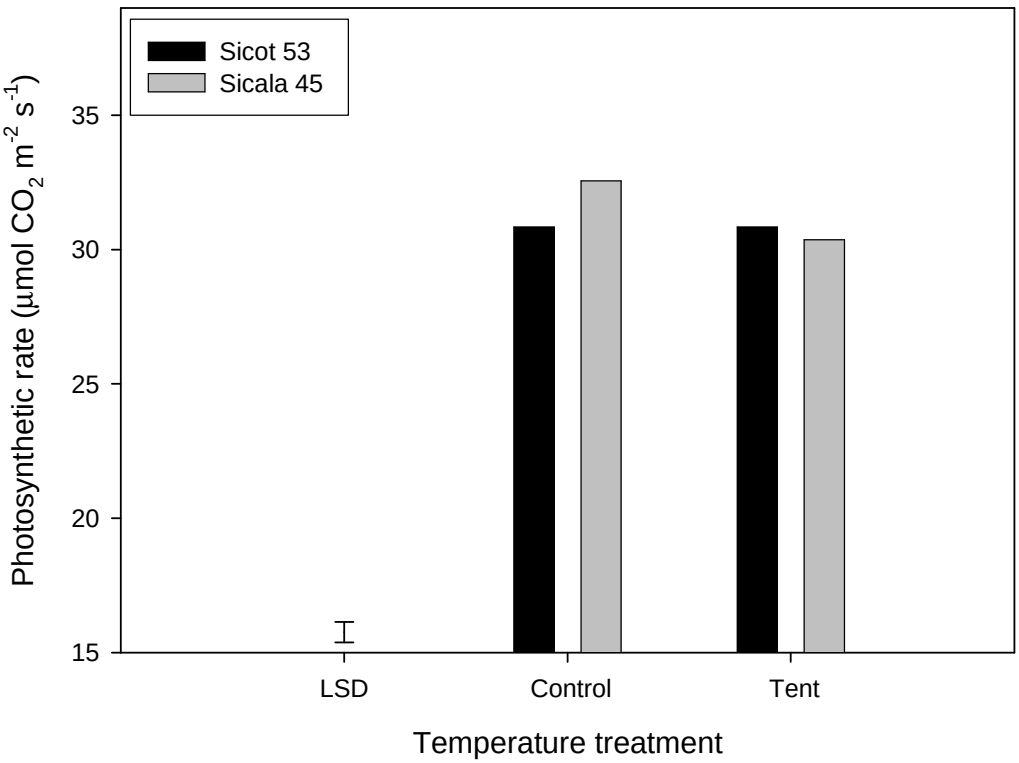

Figure 5-1 Mean photosynthetic rate for Sicot 53 and Sicala 45 plants grown under ambient (control) and tent regimes in the field and pooled for seasons 1 (2006) and 3 (2007) at Narrabri and season 2 (2006) in Texas. The (a) vertical line indicates the l.s.d. value for temperature treatment by cultivar interaction at *P*=0.05.

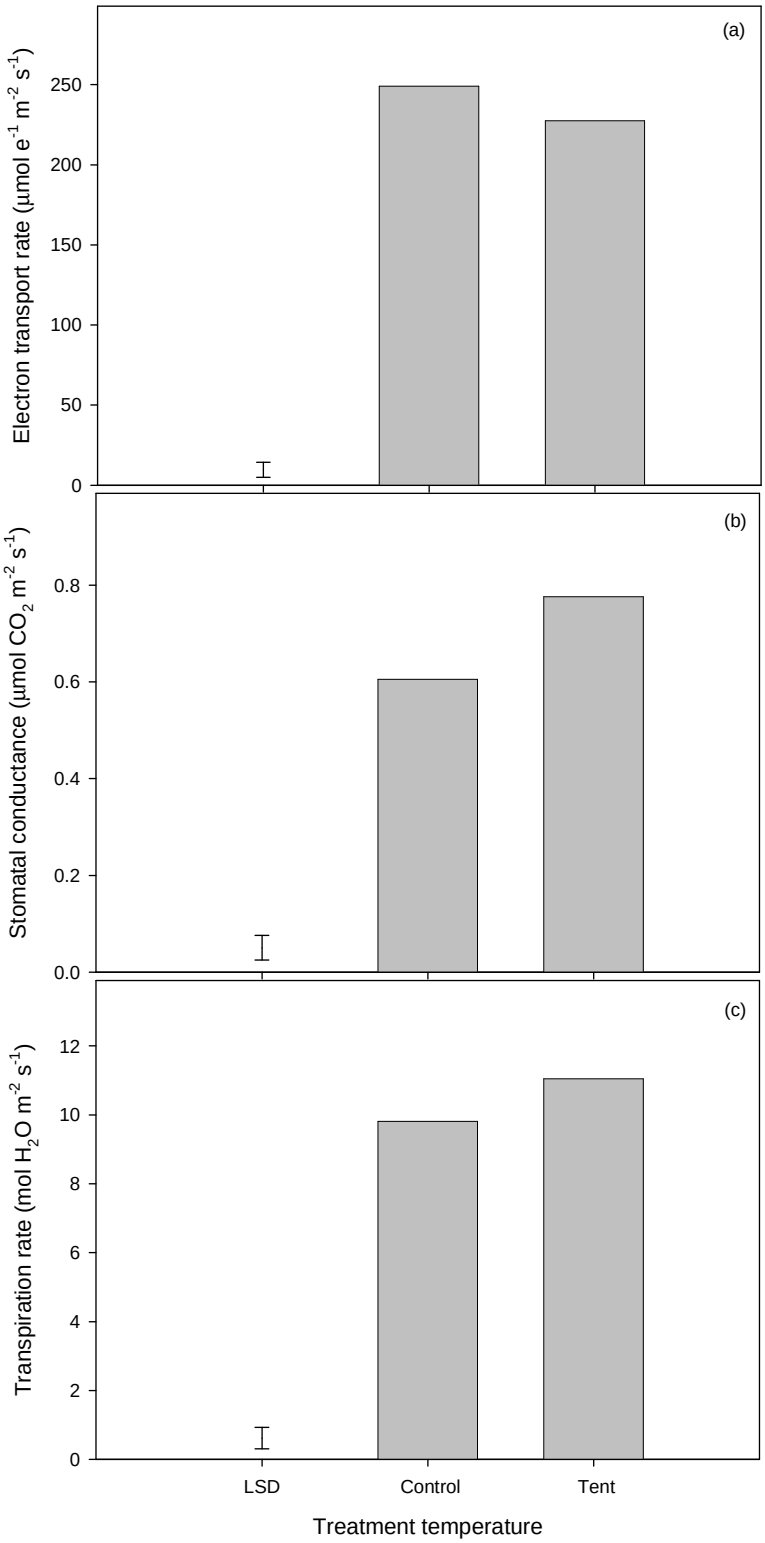

Figure 5-2 Mean (a) electron transport rate, (b) stomatal conductance and (c) transpiration rate for Sicot 53 and Sicala 45 plants grown under ambient (control) and tent regimes in the field and pooled for seasons 1 (2006) and 3 (2007) at Narrabri and season 2 (2006) in Texas. The (a) vertical line indicates the l.s.d. value for temperature treatment main effects at *P*=0.05.

A plant physiological capacity index was created by provide a simple index of physiological capacity under high temperature stress in the field. The eigenvalue for a principal component indicates the variance it accounts for out of a total of 4. The first (PC_1) and second (PC_2) principal components were considered for this analysis as they were the only components with an eigenvalue greater than 1 (Manly 2005) and accounted for 87 % of the variation. Eigenvalues were correlated for physiological indicators for carbon assimilation and heat dissipation (Figure 5-3). Photosynthesis and electron transport rate were closely correlated and had an inverse relationship to stomatal conductance and transpiration (Figure 5-3).

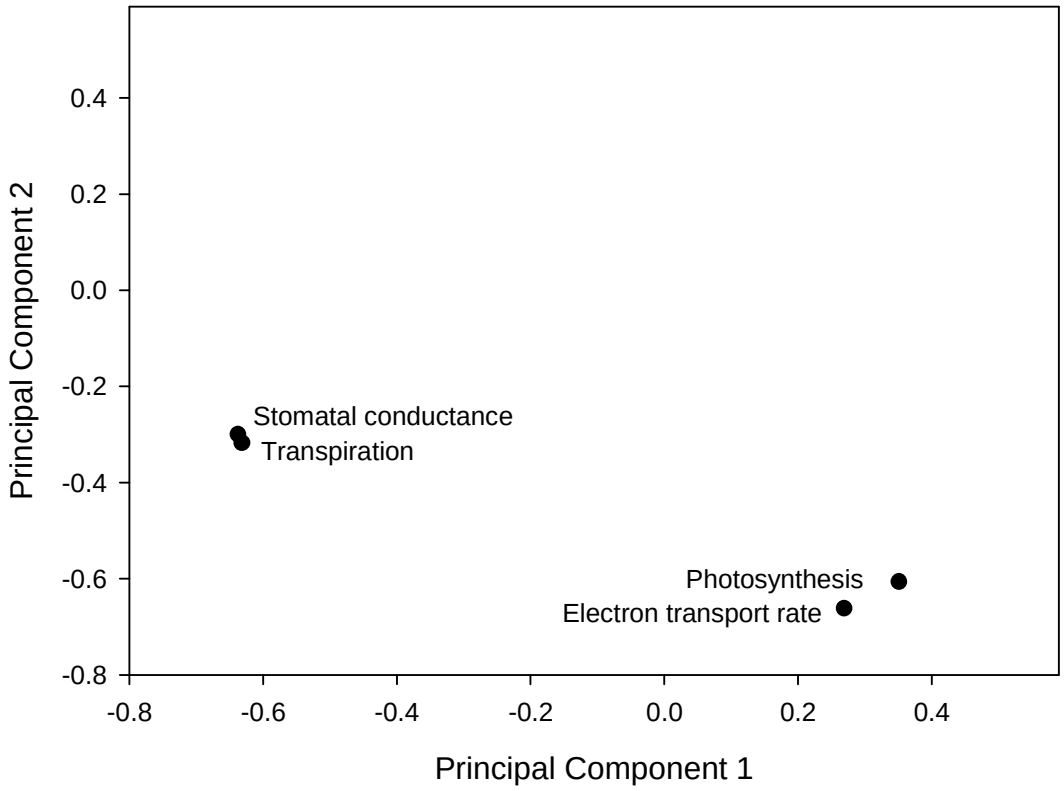

Figure 5-3 Mean correlation between eigenvalue for principal component 1 and eigenvalue for principal component 2 for photosynthesis, electron transport rate, stomatal conductance and transpiration. Means represent pooled averages for cultivars Sicot 53 and Sicala 45, grown under both ambient (control) and high temperature (tent) regimes in the field during season 1 (2006) and season 3 (2007) at the Narrabri and season 2 (2006) at the Texas field sites.

The first principal component accounted for 49 % of the variation (eigenvalue = 1.960) and was determined by the following index:

$$PC_1 = 0.352X_1 + 0.270X_2 - 0.637X_3 - 0.631X_4$$

Where X_1 is photosynthetic rate; X_2 is electron transport rate; X_3 is stomatal conductance and X_4 is transpiration rate. Photosynthesis and electron transport rate were high under ambient conditions and low under the tents. In contrast, stomatal conductance and transpiration were low under ambient conditions and high under the tents. As a result, PC_1 was high when photosynthesis and electron transport were high and low when stomatal conductance and transpiration rate were low (Figure 5-4). Hence PC_1 is an overall photosynthesis-conductance efficiency index under a treatment.

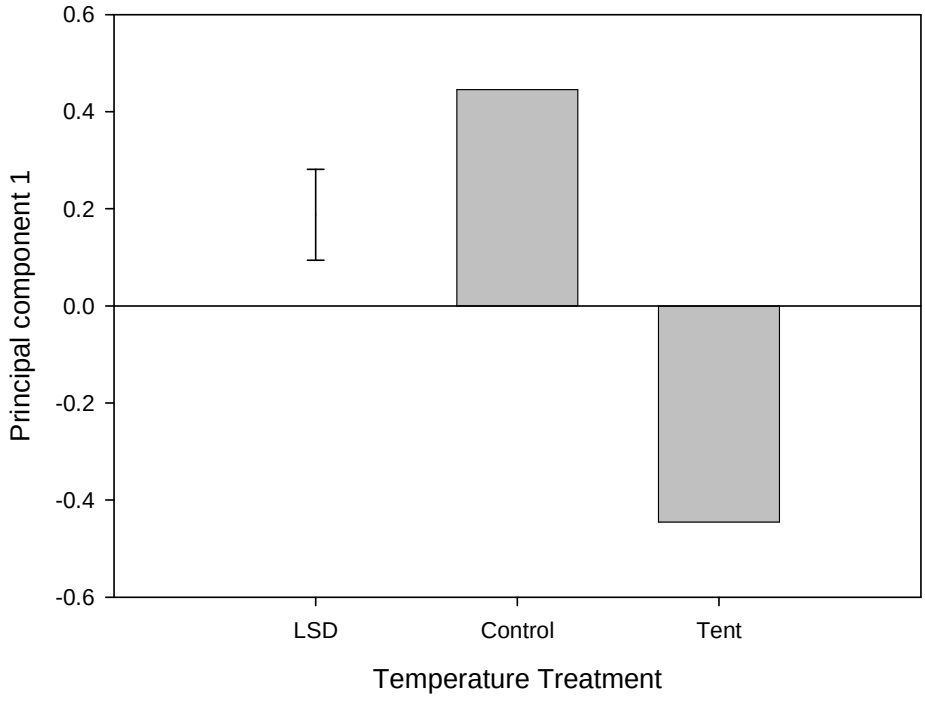

Figure 5-4 Principal component 1 for plants grown under ambient (control) and high temperature (tent) regimes in the field. Data are pooled for cotton cultivars Sicot 53 and Sicala 45 and also for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas. The vertical line represents the l.s.d. for temperature treatment main effects at the 95% confidence interval.

The second principal component (PC_2) accounted for 38 % of the variation (eigenvalue = 1.524) and is described by the following index:

$$PC_2 = -0.607X_1 - 0.663X_2 - 0.301X_3 - 0.318X_4$$

where X_1 is photosynthetic rate; X_2 is electron transport rate; X_3 is stomatal conductance and X_4 is transpiration rate. Analysis of variance for PC_2 indicated that the PC_2 of Sicala 45 was slightly lower (P=0.054) than the PC_2 of Sicot 53 (Figure 5-5).

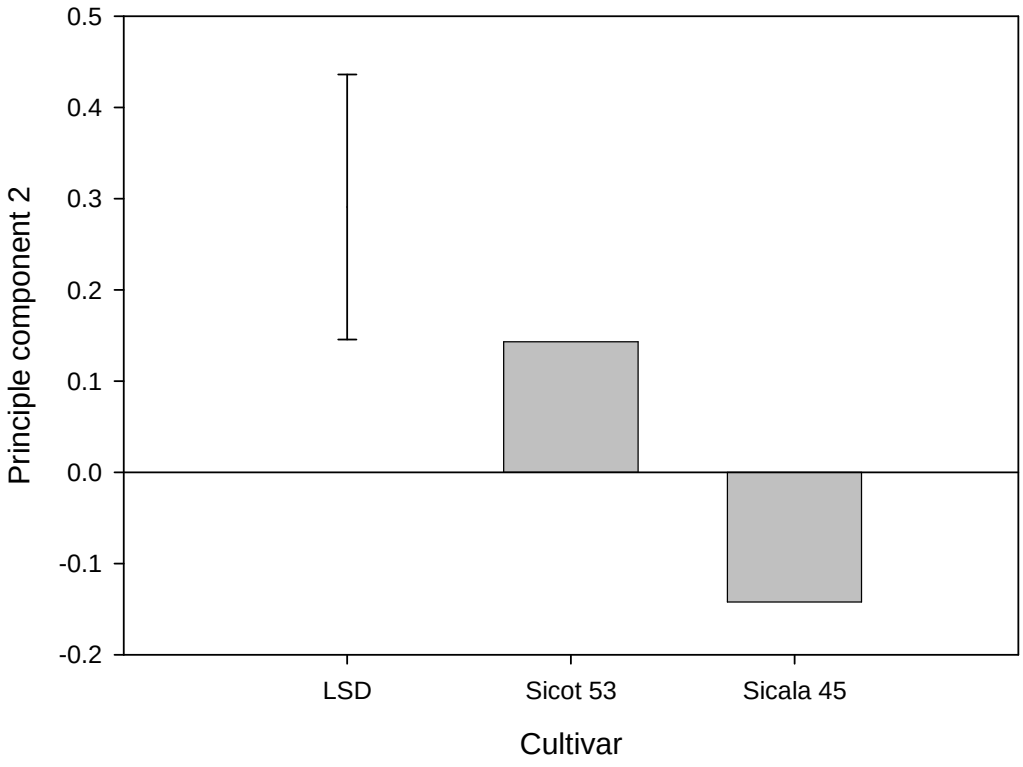

Figure 5-5 Principal component 2 for Sicot 53 and Sicala 45 plants. Data are pooled for plants measured under ambient (control) and high temperature (tent) regimes in the field and also for seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas. The vertical line represents the l.s.d. for cultivar main effects at the 95% confidence interval.

When PC_1 and PC_2 were plotted, the majority of control plots had a high PC_1, whilst the majority of tent plots had a low PC_1 (Figure 5-6). PC_1 of the control field regimes was higher (P<0.001) than that of the tent regime (Figure 5-5). There were no differences between seasons (P>0.05).

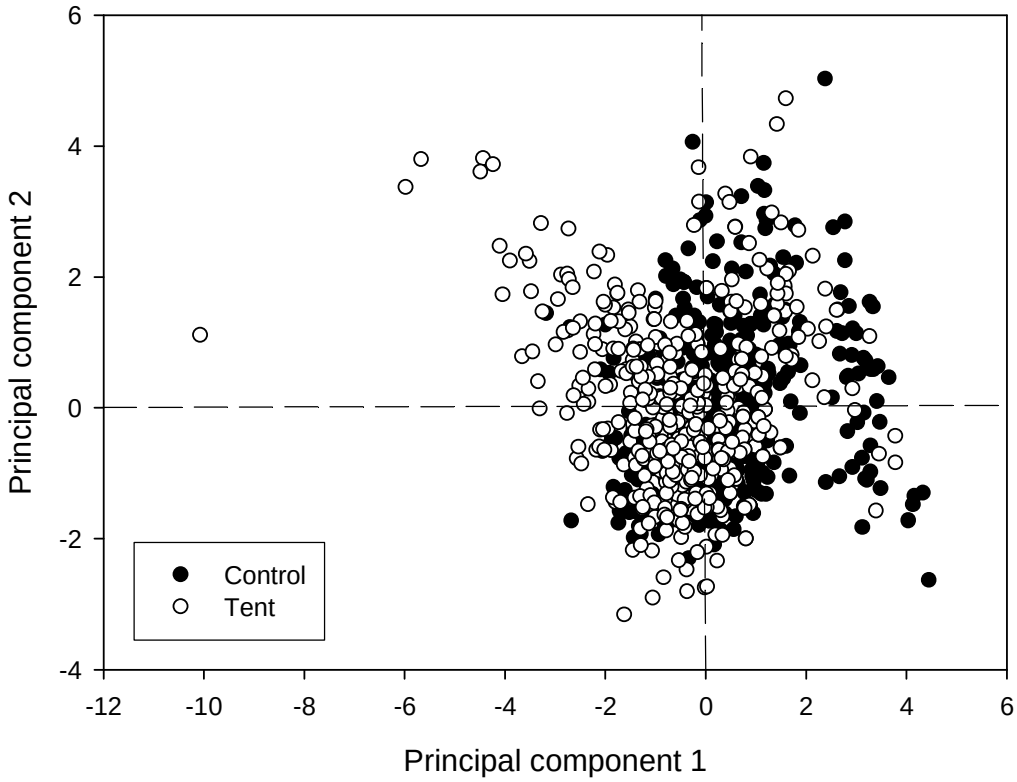

Figure 5-6 Plot of ambient (control) and high temperature (tent) regimes in the field for principal component 1 and principal component 2. Data are presented for cotton cultivars Sicot 53 and Sicala 45 and for season 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas.

Physiological response to heat stress, analysed separately for each season

The photosynthetic rate of cultivars Sicot 53 and Sicala 45 grown under ambient (control) and tent conditions in the field was higher (P<0.001) in season 3 compared with seasons 1 and 2, whilst electron transport rate was higher (P<0.001) for seasons 2 and 3 (Table 5-4). The stomatal conductance and transpiration rate was highest (P<0.001) in season 2 and lowest in season 1 (Table 5-4).

Table 5-4 Means and F test P values for photosynthesis, electron transport rate, stomatal conductance and transpiration rate, pooled for cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) and tent conditions in the field for seasons 1 (2006) and 3 (2007) at Narrabri and season 2 (2006) in Texas. Means followed by the same letter in the same row are not significantly at *P*=0.05.

Measurement	Season 1	Season 2	Season 3	Grand mean	Max LSD	*P* value
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	30.8 a	24.7 b	33.8 c	31.2	1.4	<0.001
Electron transport rate (μmol e^{-1} m^{-2} s^{-1})	182a	243b	255 b	238	14	<0.001
Stomatal conductance (mol H_2O m^{-2} s^{-1})	0.33 a	1.02 b	0.82 c	0.69	0.05	<0.001
Transpiration rate (mmol H_2O m^{-2} s^{-1})	5.3 a	15.7 b	12.1 c	10.4	0.5	<0.001

A summary of main effects and interactions for gas exchange and fluorescence for season 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas are presented in Table 5-5.

Table 5-5 Probability of cultivar and temperature treatment main effects and cultivar by treatment interaction for photosynthesis, electron transport rate, stomatal conductance and transpiration rate for individual seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas where n.s. represents F test P values that are not significant at *P*=0.05.

Measurement	Cultivar	Temperature treatment	Cultivar* temperature treatment
Season 1			
Photosynthesis	n.s.	n.s.	0.033
Electron transport rate	n.s.	<0.001	n.s.
Stomatal conductance	n.s.	<0.001	n.s.
Transpiration	n.s.	<0.001	n.s.
Season 2			
Photosynthesis	n.s.	<0.001	0.040
Electron transport rate	n.s.	<0.001	n.s.
Stomatal conductance	0.036	<0.001	n.s.
Transpiration	0.002	<0.001	0.028
Season 3			
Photosynthesis	<0.001	n.s.	0.033
Electron transport rate	<0.001	<0.001	0.031
Stomatal conductance	n.s.	<0.001	n.s.
Transpiration	n.s.	<0.001	n.s.

Photosynthesis

The photosynthetic rate of Sicot 53 was lower than Sicot 45 under ambient (control) conditions in all 3 seasons (Figure 5-7). However, the photosynthetic rate of Sicot 53 was higher than Sicala 45 (Figure 5-7) under the tents in seasons 1 and 2. For season 3, the photosynthetic rate of Sicala 45 was higher (*P*=0.033) under the tents compared with Sicot 53 and compared with both cultivars under ambient (control) conditions (Figure 5-7).

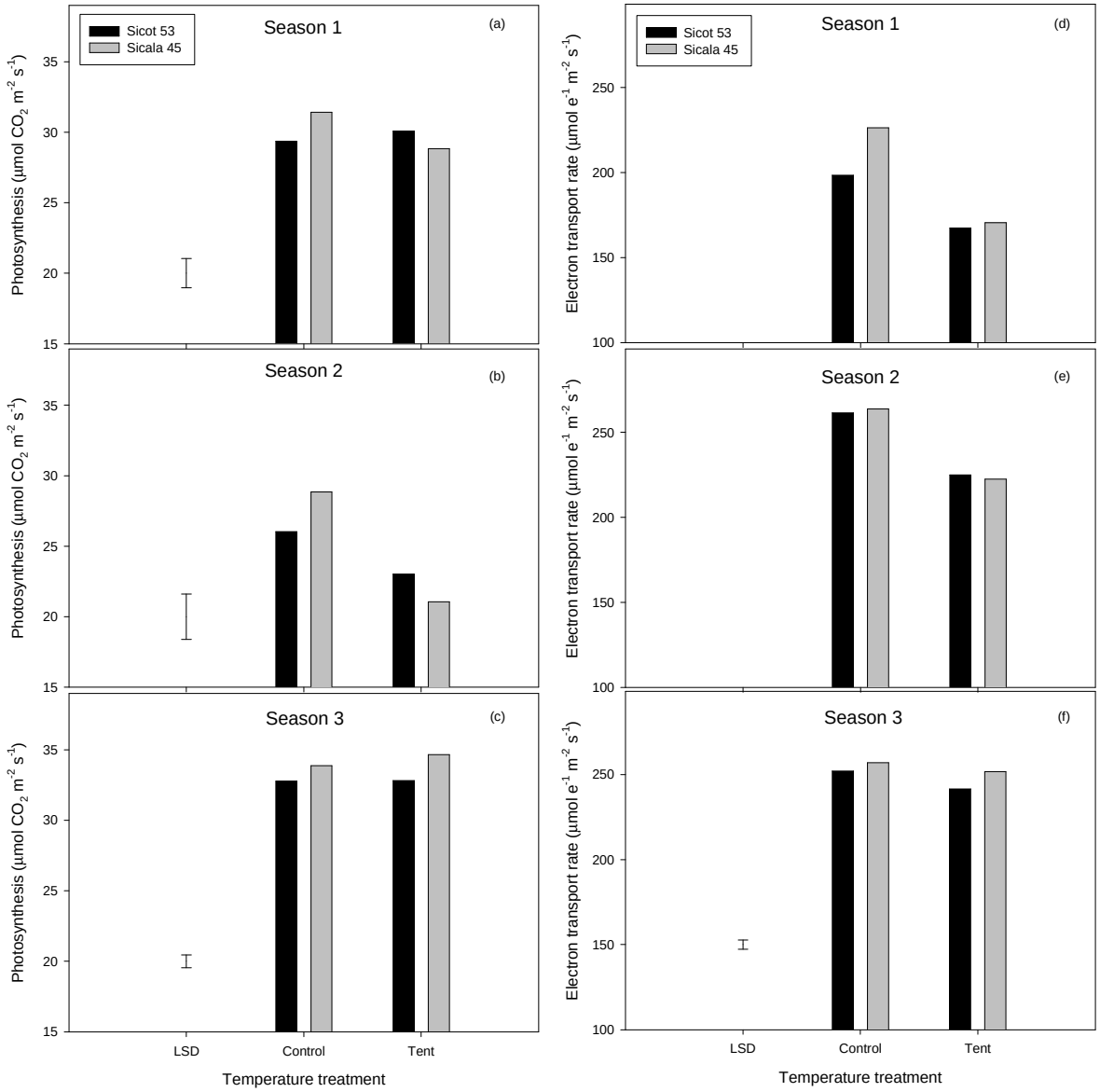

Figure 5-7 Mean (a, b, c) photosynthesis (µmol CO_2 m^{-2} s^{-1}) and (d, e, f) electron transport rate (µmol e^{-1} m^{-2} s^{-1}) of cotton cultivars Sicot 53 and Sicala 45 under ambient (control) tent (pooled for tent 1 and tent 2) field conditions during (a, d) seasons 1 (2006) and (c, f) season 3 (2007) at Narrabri and (b, e) season (2006) in Texas. The vertical lines in (a), (b), (c) and (f) represent the l.s.d. for temperature treatment by cultivar interaction at *P*=0.05.

Electron transport rate

The electron transport rate of plants grown under high temperature (tent) regimes was lower than under ambient (control) conditions for both seasons 1 and 2 ($P<0.001$). For season 3, the mean electron transport rate of Sicot 53 was lower under the tents compared with ambient (control) field conditions. There were no differences for electron transport rate of Sicala 45 plants under the tents compared with under control conditions (Figure 5-7).

Stomatal conductance and transpiration rate

Mean stomatal conductance and transpiration rate showed the same trends for season 1, 2 and 3. Mean stomatal conductance (Figure 5-8) and transpiration (data not presented) were higher under the high temperature (tent) regime compared with under ambient (control) conditions in the field for all seasons ($P<0.001$). The rate of stomatal conductance ($P=0.036$) and transpiration ($P=0.002$) were higher for Sicala 45 than Sicot 53 in season 2. There were no cultivar differences ($P>0.05$) in stomatal conductance (Figure 5-8) or transpiration in either season 1 or 3.

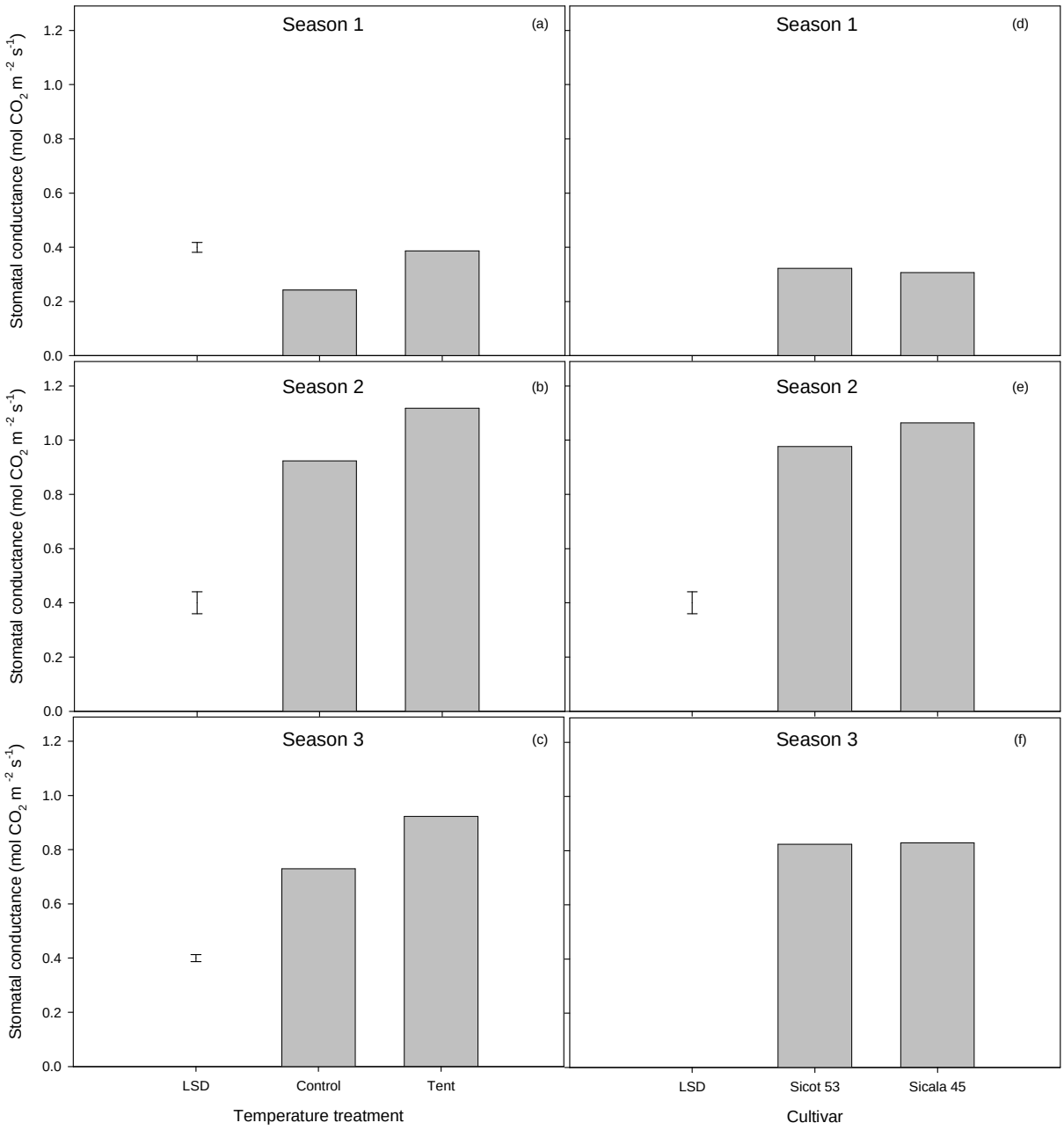

Figure 5-8 Mean stomatal conductance (µmol H_2O m^{-2} s^{-1}) under (a, b, c) ambient (control) and high temperature (tent) conditions, and for (d, e, f) cotton cultivars Sicot 53 and Sicala 45 in the field in (a, d) season 1 (2006) and (c, f) season 3 (2007) at the Narrabri field site and (b, e) season 3 (2006) at the Texas field site. The data presented in (a), (b) and (c) are temperature treatment main effect means and (d), (e) and (f) are cultivar main effect means. The vertical lines in (a), (b) and (c) indicate an l.s.d. value at *P*=0.05 for temperature treatment main effects and in (e), indicate l.s.d. values at *P*=0.05 for cultivar means.

A positive correlation exists between stomatal conductance and photosynthesis under ambient (control) conditions in all seasons, suggesting that conductance was not limiting to carbon assimilation (Table 5-6, Figure 5-9). A negative correlation between stomatal conductance and photosynthesis was found under the high temperature (tent) treatment in season 2 (Table 5-6, Figure 5-9). There was no relationship between stomatal conductance and photosynthesis under the tents in seasons 1 or 3 (Figure 5-9).

Table 5-6 Correlation between stomatal conductance and photosynthesis under ambient (control) and high temperature (tent) regimes in the field in season 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas, where n.s. represents F test P values where *P*<0.05.

Season	Temperature treatment	n	Adjusted R^2	Equation	F test P value
1	Control	134	0.08	y=24.82+25.16x	<0.001
2	Control	72	0.21	y=18.14+10.07x	<0.001
3	Control	360	0.35	y=23.90+13.90x	<0.001
1	Tent	134	n.s.	n.s.	n.s.
2	Tent	72	0.47	y=44.70-20.604x	<0.001
3	Tent	360	n.s.	n.s.	n.s.

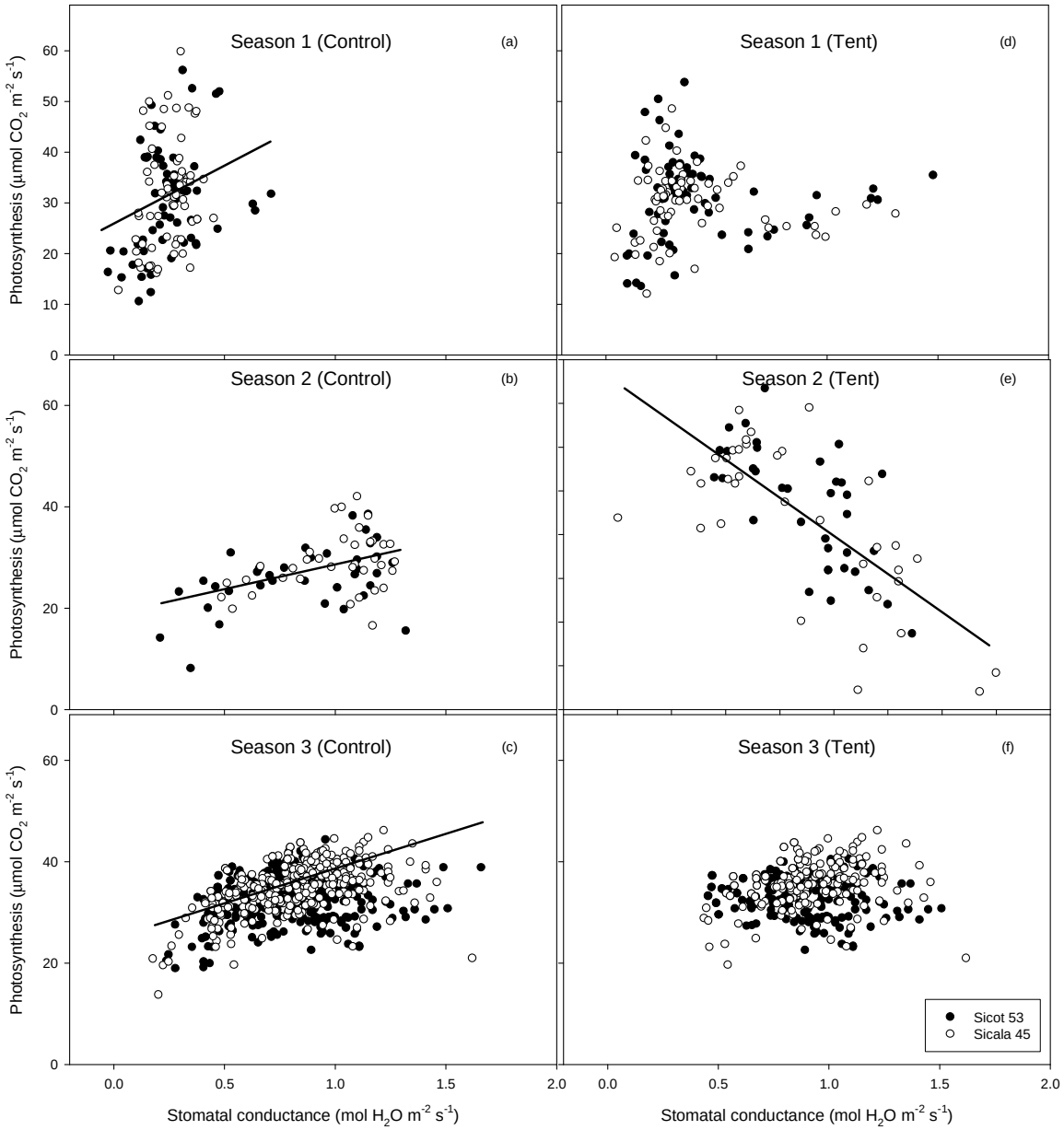

Figure 5-9 Relationship between stomatal conductance and photosynthesis for cotton cultivars Sicot 53 and Sicala 45 grown under (a, b, c) ambient (control) and (d, e, f) high temperature (tent) regimes in (a, d) season 1 (2006) and (c, f) season 3 (2007) at Narrabri and (b, e) season 2 (2006) at the Texas field site.

Physiological response to heat stress, analysed separately for each tent

A summary of main effects and interactions for gas exchange and fluorescence for each day under the tent or under ambient (control) regimes in the field for tent 1 in seasons 1 and 2 is presented in Table 5-7. Times at which significant cultivar differences were not determined under the tents were excluded from presentation (i.e. tent 2 in season 1 and tents 1 and 2 in season 3).

Table 5-7 F test P values for photosynthesis, electron transport rate, stomatal conductance and transpiration rate of cultivars Sicot 53 and Sicala 45 under ambient (control) and high temperature (tent 1) regimes in the field in season 1 (2006) in Narrabri and season 2 (2006) in Texas, where n.s. represents F test P values where *P*<0.05.

Measurement	Day	Cultivar	Temperature treatment	Cultivar * Temperature treatment
Season 1				
Photosynthesis	0	n.s.	0.006	n.s.
	2	n.s.	<0.001	0.007
	7	0.031	n.s.	n.s.
Electron transport rate	0	n.s.	0.009	n.s.
	2	n.s.	<0.001	0.036
	7	0.003	n.s.	n.s.
Stomatal conductance	0	n.s.	n.s.	n.s.
	2	n.s.	<0.001	n.s.
	7	n.s.	n.s.	n.s.
Transpiration rate	0	n.s.	n.s.	n.s.
	2	n.s.	<0.001	n.s.
	7	n.s.	n.s.	n.s.
Season 2				
Photosynthesis	0	n.s.	0.042	n.s.
	6	n.s.	<0.001	0.004
	8	n.s.	<0.001	n.s.
Electron transport rate	0	n.s.	n.s.	n.s.
	6	n.s.	<0.001	n.s.
	8	n.s.	<0.001	n.s.
Stomatal conductance	0	n.s.	<0.001	0.002
	6	0.049	0.002	n.s.
	8	n.s.	0.010	n.s.
Transpiration rate	0	n.s.	<0.001	0.003
	6	0.026	0.008	n.s.
	8	<0.001	n.s.	n.s.

Photosynthesis

For season 1, there was a general decrease in photosynthetic rate over time of measurement across all treatments (Figure 5-10). The photosynthetic rate was lower under the tents (day 2) compared with ambient (control) conditions and lowest for Sicala 45 compared with Sicot 53

(P=0.007). The photosynthetic rate of Sicot 53 was higher than Sicala 45 under optimal conditions (recovery), 7 days after initiation of the treatments (*P*=0.031) (Figure 5-10).

For season 2, the photosynthetic rate of plants grown under the tents (day 6) was lower than the control and was lower (*P*=0.042) for Sicot 53 than Sicala 45 under the tents (Figure 5-10).

Electron transport

The electron transport rate of plants under the tents (day 2) was lower compared with ambient (control) conditions (*P*=0.036) for season 1 (Figure 5-10). For season 2, the electron transport rate was decreased (*P*<0.001) under the tents (day 6) but this difference was not cultivar specific (Figure 5-10).

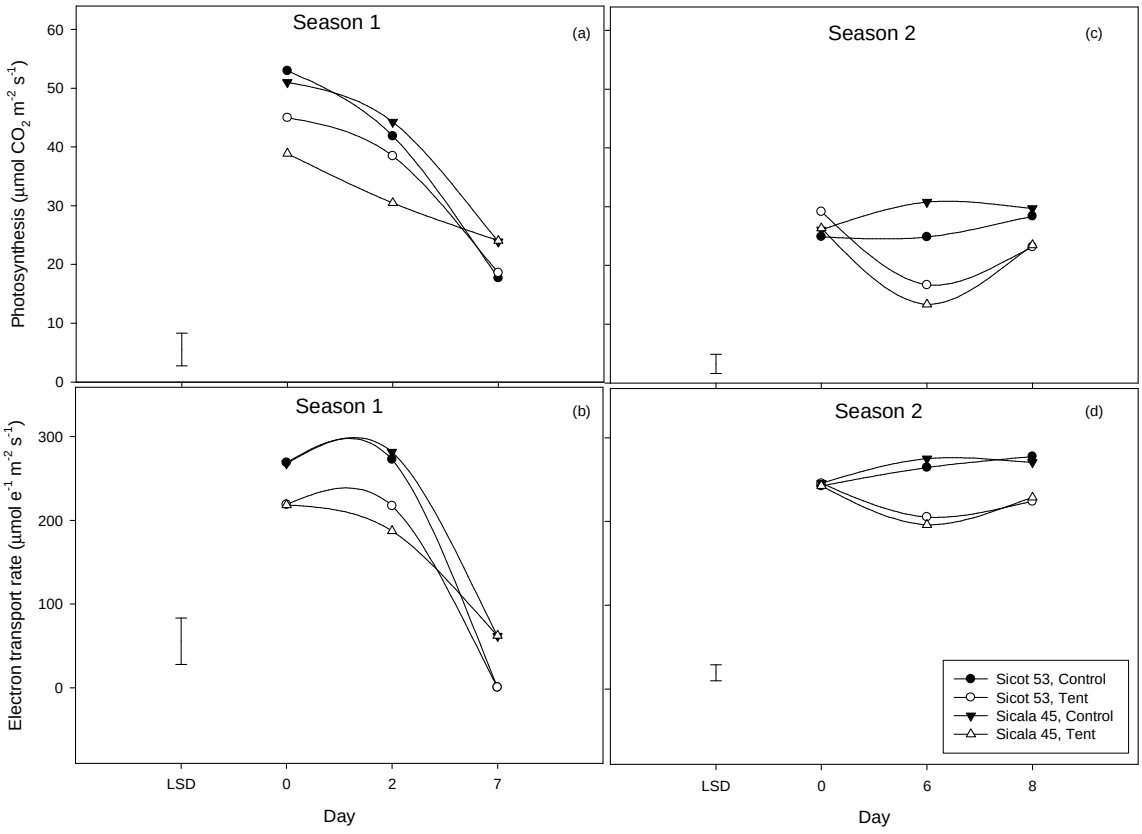

Figure 5-10 Mean (a, c) photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) and (b, d) electron transport rate (µmol e^{-1} m^{-2} s^{-1}) of cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) and high temperature (tent) regimes in the field under tent 1 in (a, b) season 1 (2006) at the Narrabri field site and (c, d) season 2 (2006) at the Texas field site. Measurements were taken prior to initiation of high temperature stress (day 0) and on various days post tent installation. Measurements were also taken after removal of the tents in season 1 (day 7) and season 2 (day 8). The vertical bar represents the l.s.d. for temperature treatment by cultivar interaction at *P*=0.05.

Stomatal conductance and transpiration

Stomatal conductance and transpiration rate decreased (P<0.001) under the tents for season 1 (Table 5-7). No cultivar differences were evident for stomatal conductance or transpiration in season 1. For season 2, stomatal conductance (P=0.002) and transpiration (P=0.008) was higher under the tents compared with ambient conditions (Table 5-7). Stomatal conductance (P=0.049) and transpiration rate (P=0.026) were higher for Sicala 45 than Sicot 53 in season 2 (Figure 5-10).

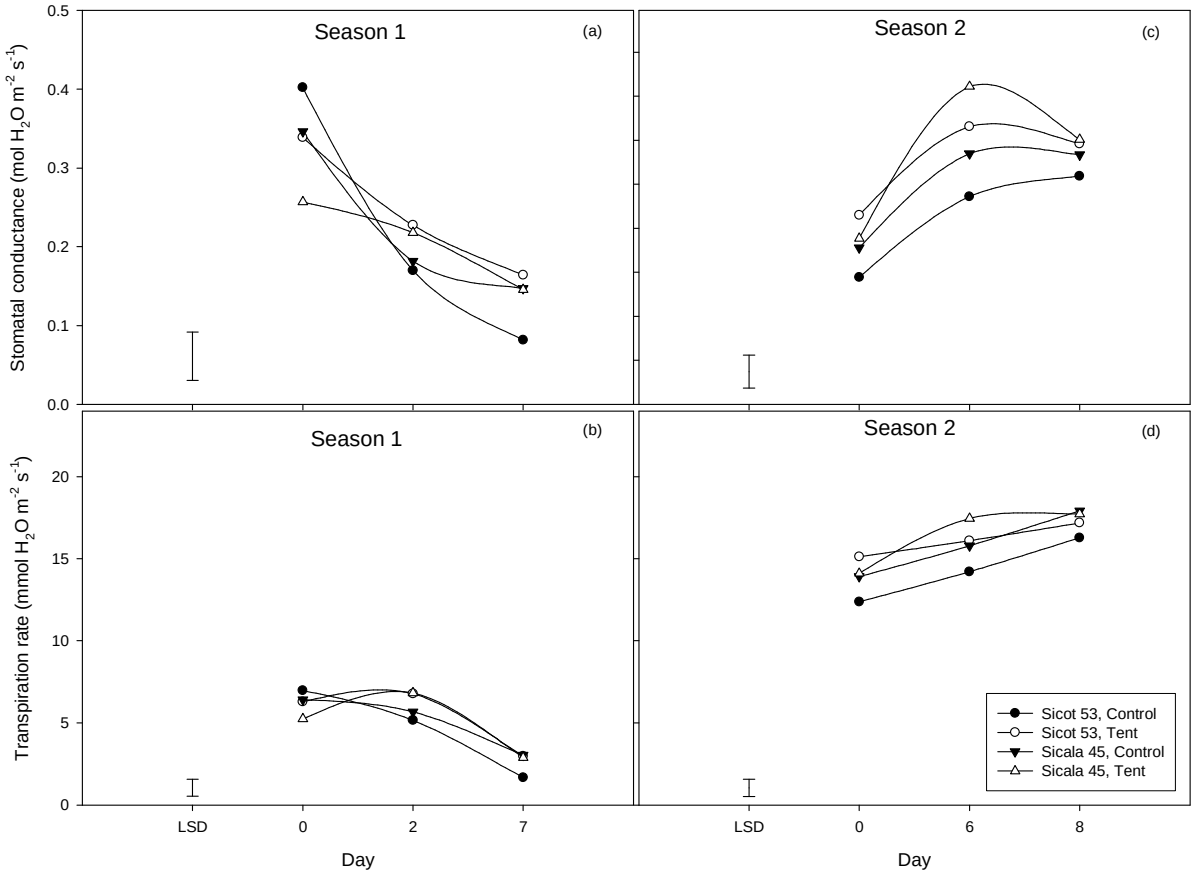

Figure 5-11 Mean (a, c) stomatal conductance (mol H_2O m^{-2} s^{-1}) and (b, d) transpiration rate (mmol H_2O m^{-2} s^{-1}) of cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) and high temperature (tent 1) regimes in the field in (a, b) season 1 (2006) at the Narrabri field site and (c, d) season 2 (2006) at the Texas field site. Measurements were taken prior to initiation of high temperature stress (day 0) and on various days post tent installation. Measurements were also taken after removal of the tents in season 1 (day y) and season 2 (day 8). The vertical lines represent the l.s.d. for temperature treatment by cultivar interaction at *P*=0.05.

Relationship between physiological measurements or indices and yield

Photosynthesis, electron transport rate, stomatal conductance, transpiration rate and principal components 1 and 2 were fitted to a linear regression to determine whether there was any correlation with seed cotton yield under field conditions. For tent 1 in season 1 a strong and (P<0.001) positive relationship was found between seed cotton yield and principal component 1 which accounted for 40 % of the variation, between seed cotton yield and electron transport rate which accounted for 36 % of the variation (Figure 5-12) and also between seed cotton yield and principal component 2 which accounted for 15 % of the variation (Table 5-8).

Although a positive relationship was found between seed cotton yield and photosynthesis, this relationship was not strong (P=0.036) and only accounted for 4 % of the total variation (Table 5-8). A negative relationship was found between seed cotton yield and stomatal conductance (P=0.002) which accounted for 14 % of the variation, and also for seed cotton yield and transpiration rate (P<0.001) which accounted for 21 % of the variation (Table 5-8). For tent 2 in season 3 a relationship (P<0.05) was determined for seed cotton yield and electron transport, stomatal conductance, transpiration rate or principal component 1 and the coefficients of variation accounted for less than 10 % of the variation (Table 5-8).

Table 5-8 Correlation between photosynthesis, electron transport rate, stomatal conductance, transpiration rate, principal component 1 or principal component 2 and seed cotton yield (g m^{-2}) under ambient (control) and high temperature (tent) regimes in the field for tent 1 in season 1 (2006) and/or tent 2 in season 3 (2007) in Narrabri, where n.s. represents F test P values where *P*<0.05.

Measurement	Season	n	Adjusted R^2	Equation	F test P value
Photosynthesis	1	85	0.04	y=286.1377+4.31x	0.033
	3	192	-	-	n.s.
Electron transport rate	1	85	0.36	y=-99.77+2.24x	<0.001
	3	192	0.03	y=297.22+0.83x	0.008
Stomatal conductance	1	85	0.14	y=584.75-518.10x	<0.001
	3	192	0.05	y=610.02-107.49x	<0.001
Transpiration rate	1	85	0.21	y=695.83-42.04x	<0.001
	3	192	0.05	y=688.73-13.71x	0.001
Principal component 1	1	85	0.4	y=161.29+114.37x	<0.001
	3	192	0.07	y=517.52+31.21x	<0.001
Principal component 2	1	85	0.15	y=487.09-45.45x	<0.001
	3	192	-	-	n.s.

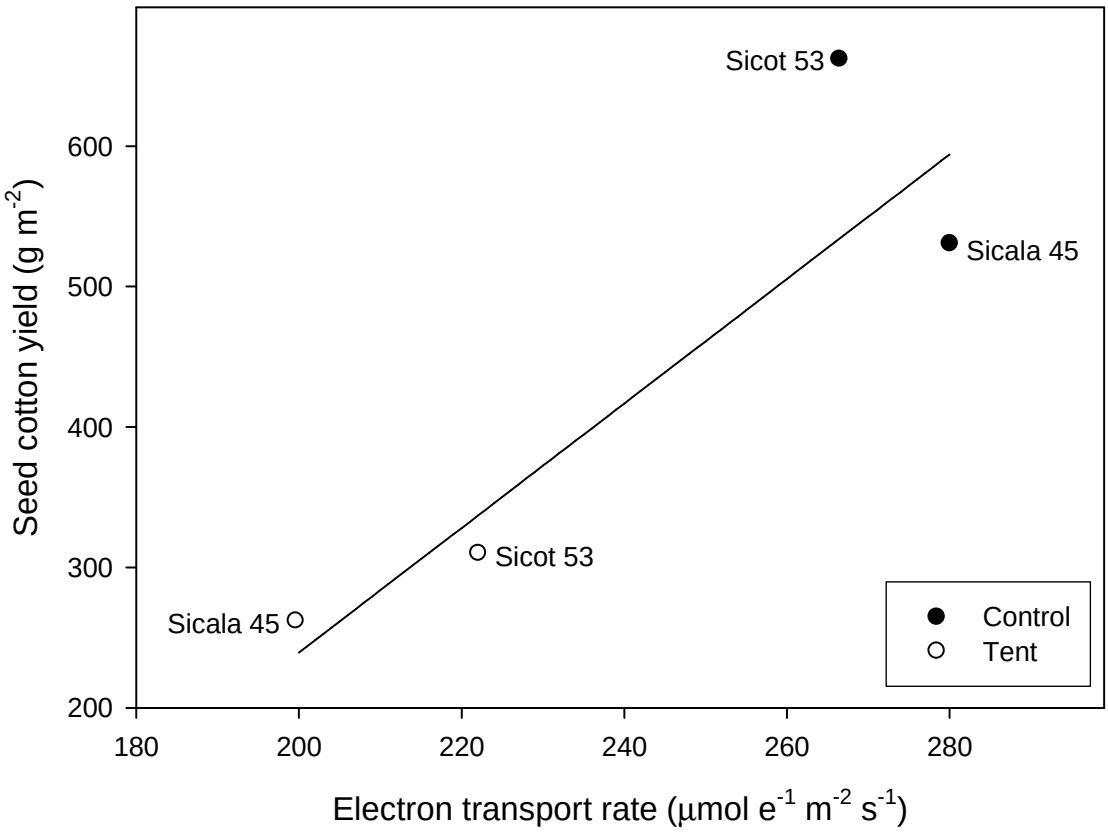

Figure 5-12 Correlation between electron transport rate and seed cotton yield for cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) field conditions and under the tents in season 1 (2006) at Narrabri. Each data point represents the treatment mean of 4 replicates.

5.3.2 **Experiment 2**

Photosynthetic rate and electron transport was plotted against irradiance for cotton cultivars Sicot 53 and Sicala 45, grown under optimal (32 ^{o}C) and high (42 ^{o}C) temperature conditions in the growth cabinet. The data were fitted to a waiting-in-line curve to generate a light saturation curve for both temperature regimes and cultivars. A summary for cabinet temperature and cultivar main effects interaction for light saturation curves fitted by a waiting-in-line distribution curve is summarised in Table 5-9.

Table 5-9 F test P values for coefficients of the responses using the waiting-in-line model of irradiance and photosynthesis or electron transport rate for cultivars Sicot 53 and Sicala 45 grown under control (32 ^{o}C) or high (42 ^{o}C) temperature regimes in the growth cabinet at Narrabri, NSW, where n.s. represents F test P values where *P*<0.05.

Parameter	Cultivar	Cabinet temperature	Cabinet temperature * Cultivar
Photosynthesis			
α0	n.s.	<0.001	n.s.
Optimum E	n.s.	<0.001	n.s.
Pmax	n.s.	<0.001	n.s.
Electron transport rate			
α 0	n.s.	<0.001	n.s.
Optimum E	n.s.	<0.001	n.s.
Pmax	n.s.	<0.001	n.s.

Photosynthetic efficiency (α0), optimum E and Pmax were lower (*P*<0.001) at 42 ^{o}C than at 32 ^{o}C however no cultivar differences were found for α0, optimum E or Pmax (Table 5-9) and hence a single waiting-in-line regression was fitted to the data for the optimal (32 ^{o}C) and high (42 ^{o}C) temperature growth cabinets (Figure 5-13).

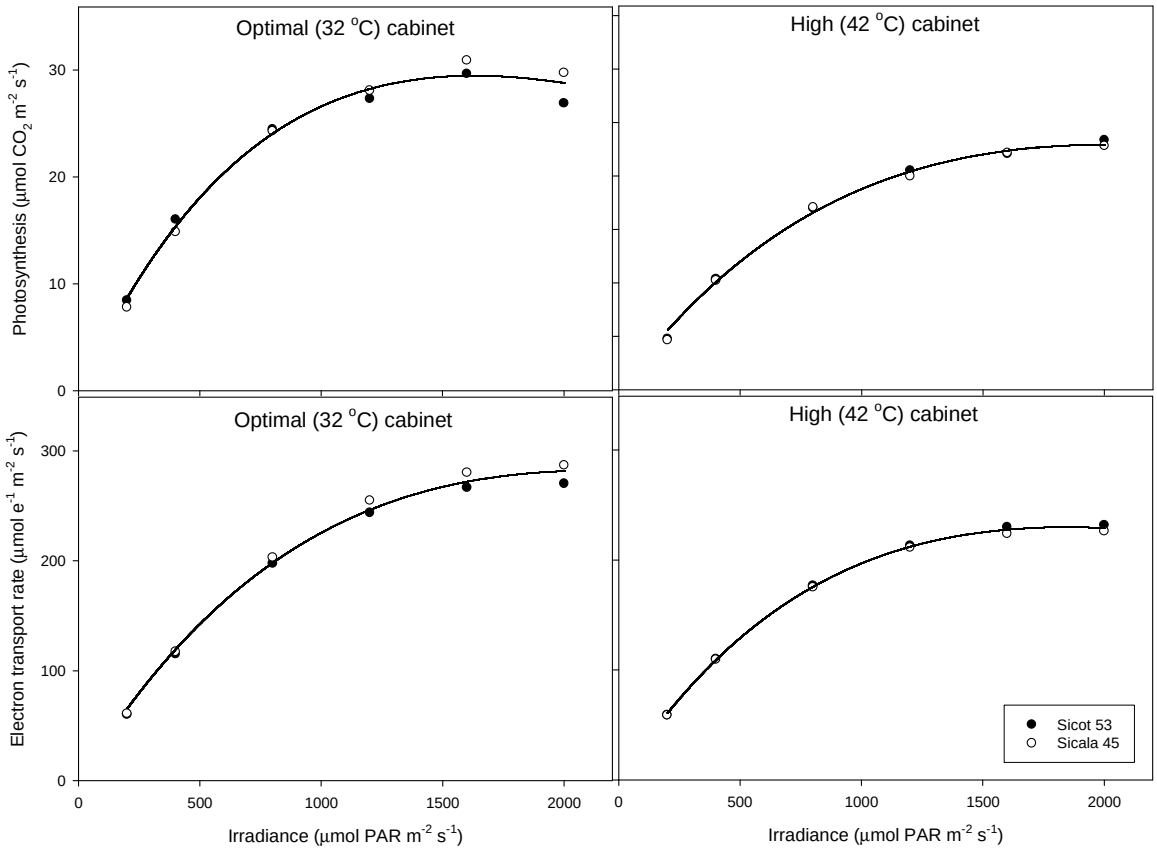

Figure 5-13 (a, c) Photosynthetic rate and (b, d) electron transport rate for a fitted waiting-in-line model for cotton cultivars Sicot 53 and Sicala 45, grown under (a, b) optimal (32 °C) and (c, d) high (42 °C) temperature regimes in a growth cabinet at Narrabri, NSW. Each data point represents the mean of 4 replicates.

The photosynthesis and electron transport rate light response curves were a good fit (adjusted $R^2 > 0.89$) and highly significant ($P<0.001$) (Table 5-10).

Table 5-10 Relationships for values fitted using the waiting-in-line model of irradiance and photosynthesis or electron transport rate and the linear model of irradiance and stomatal conductance or transpiration rate for cultivars Sicot 53 and Sicala 45 grown under control (32 ^{o}C) or high (42 ^{o}C) temperature regimes in the growth cabinet at Narrabri, NSW, where n.s. represents F-test values where *P*>0.05. The dashed line represents treatments for which *P*>0.05.

Cabinet (^{o}C)	n	Adjusted R^2	Equation	F test P value
Photosynthesis				
32	282	0.89	y=775.82*2.54e-5*x*exp(2.54e-5*x)	<0.001
42	232	0.92	y=632.88*2.32e-5*x*exp(2.32e-5*x)	<0.001
Electron transport rate				
32	287	0.98	y=766.55*4.72e-4*x*exp(4.72e-4*x)	<0.001
42	232	0.98	y=624.42*5.43e-4*x*exp(5.43e-4*x)	<0.001
Stomatal conductance				
32	282	-	-	n.s.
42	232	-	-	n.s.
Transpiration rate				
32	282	0.21	y=3.93+0.0011*x	<0.001
42	232	0.05	y=5.79+0.0015*x	<0.001

Stomatal conductance and transpiration rate were plotted against irradiance for cultivars Sicot 53 and Sicala 45, grown under optimal (32 ^{o}C) and high (42 ^{o}C) temperature regimes in the growth cabinet. The data were fitted to a linear regression to generate a light response curve for both temperature regimes and cultivars. There was no correlation between irradiance and stomatal conductance (Table 5-10). However, a positive correlation exists between irradiance and transpiration rate (Figure 5-14) under optimal and ambient conditions in the growth cabinet and explained 21 % and 5 % of the variation in the data respectively (Table 5-10). The y-intercept (P=0.009) and slope (P=0.022) of the regression differed for plants under optimal (32 ^{o}C) and high (42 ^{o}C) temperature regimes in the growth cabinet but no cultivar differences were evident for these parameters.

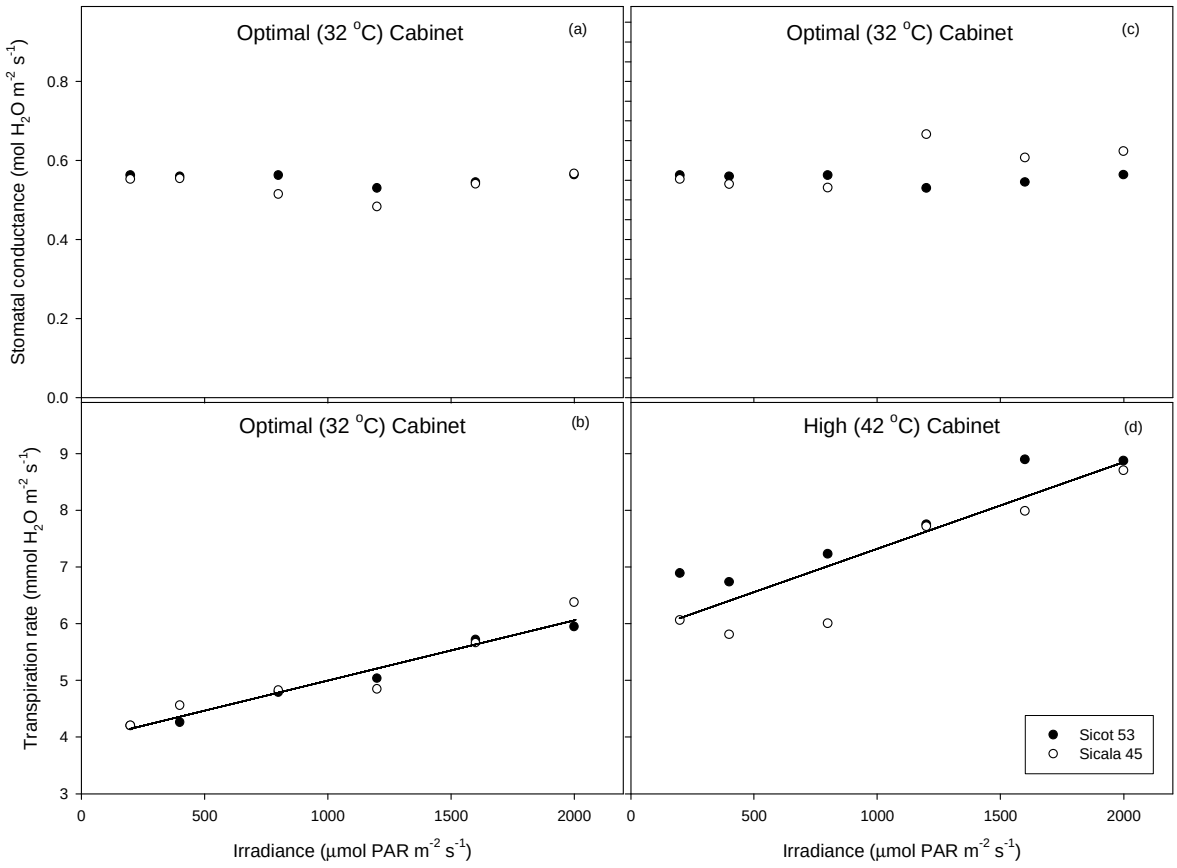

Figure 5-14 (a, c) Stomatal conductance and (b, d) transpiration rate for a fitted linear model for cotton cultivars Sicot 53 and Sicala 45, grown under (a, b) optimal (32 °C) and (c, d) high (42 °C) temperature regimes in a growth cabinet at Narrabri, NSW

A linear mixed model (REML) was run to determine cultivar differences in photosynthesis, electron transport rate, stomatal conductance and transpiration rate at various rates of irradiance that were not distinguishable using the model-fitting analysis. Photosynthesis and electron transport rate decreased (P<0.001) whilst stomatal conductance (P=0.012) and transpiration rate (P<0.001) increased under high temperatures in the growth cabinet (Table 5-11). Furthermore, the decrease in electron transport rate for Sicala 45 under high (42 °C) temperatures in the cabinet was greater compared with Sicot 53 at high levels of irradiance (P<0.001) (Table 5-11). No cultivar differences were evident for photosynthesis, stomatal conductance or transpiration rate (Table 5-11).

Table 5-11 F test P values for photosynthesis, electron transport rate, stomatal conductance and transpiration rate of cultivars Sicot 53 and Sicala 45 under control (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet and at various levels of irradiance (200, 400, 800, 1200, 1600, 200 µmol PAR m^{-2} s^{-1}), where n.s. represents F test P values where *P*<0.05.

Measurement	Cultivar	Cabinet temperature	Irradiance	Cultivar * Cabinet temperature	Cultivar * Irradiance	Cabinet temperature * Irradiance	Cultivar * Cabinet temperature * Irradiance
Photosynthesis	n.s.	<0.001	<0.001	n.s.	n.s.	<0.001	n.s.
Electron transport rate	n.s.	<0.001	<0.001	0.007	n.s.	n.s.	<0.001
Stomatal conductance	n.s.	0.012	n.s.	n.s.	n.s.	n.s.	n.s.
Transpiration rate	n.s.	<0.001	<0.001	n.s.	n.s.	n.s.	n.s.

There was an interaction (P<0.001) between cultivar and cabinet temperature for leaf temperature (Figure 5-15). Leaf temperature was higher for leaves exposed to high (42 ^{o}C) air temperatures in the growth cabinet compared with optimal (32 ^{o}C) air temperatures. Under optimal (32 ^{o}C) air temperatures, leaf temperature was higher for Sicala 45 compared with Sicot 53. However, there was no cultivar differentiation for leaf temperature in the high (42 ^{o}C) air temperature cabinet.

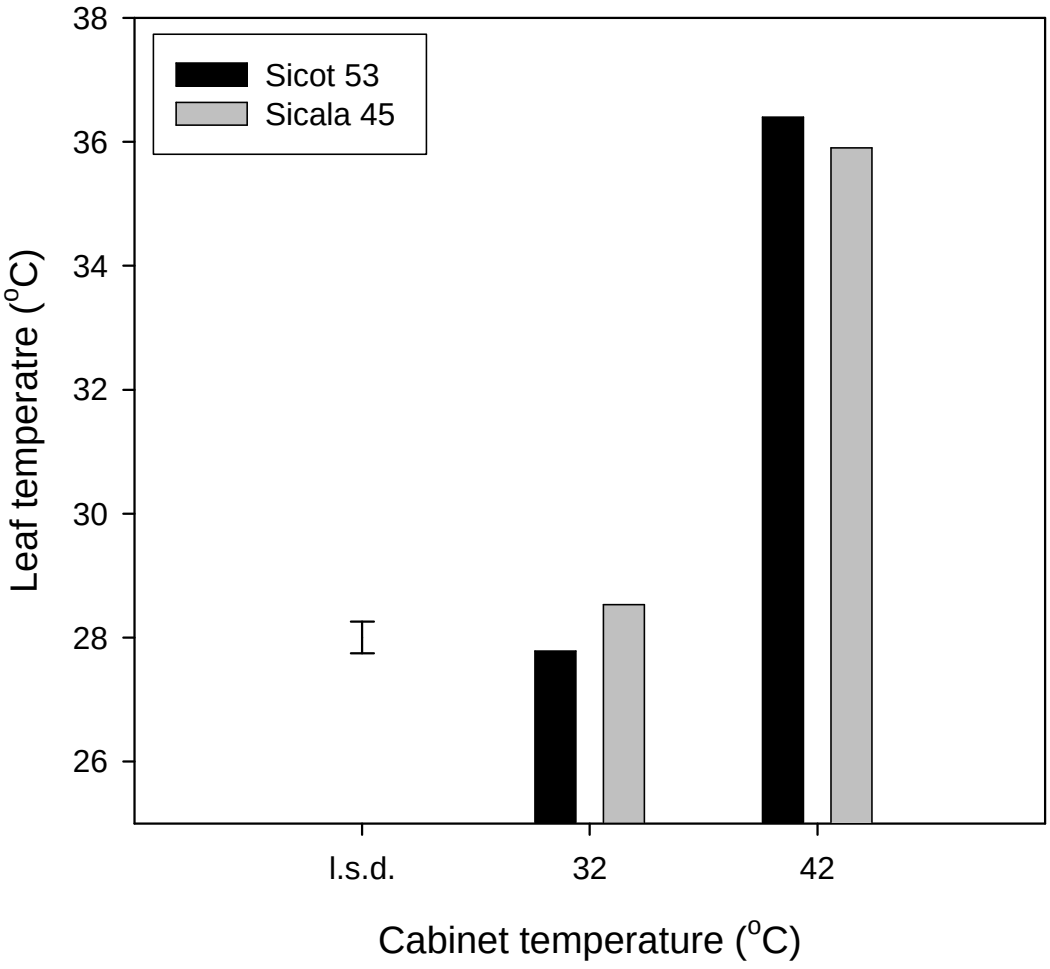

Figure 5-15 Mean leaf temperature (^{o}C) for cultivars Sicot 53 and Sicala 45 grown under control (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet. The vertical line indicates an l.s.d. value at P=0.05 for a cabinet temperature by leaf temperature interaction.

5.4 **Discussion**

Leaf level measurements of electron transport rate and photosynthesis successfully detected differences in the response of cotton cultivars to high temperature stress in the field but not in a growth cabinet. Measurements of stomatal conductance and transpiration were however, unsuccessful in detecting cultivar differences in both environments.

Electron transport rate and photosynthesis were higher for the heat tolerant cultivar Sicot 53 under high temperatures in the field. For seasons 1 and 2, the photosynthetic and electron transport rate of Sicot 53 plants was slightly higher under the tents in the field compared with Sicala 45 (Figure 5-7). Conversely, stomatal conductance and transpiration were higher for Sicala 45 compared with Sicot 53 (Figure 5-8) under the tents in season 2. However, no cultivar differences were evident in physiological function for measurements taken under tent 2 in season 1, or under any tent in season 3, thereby suggesting that cultivar specificity for physiological function under high temperature stress in the field is highly variable.

To account for differences in seasonal weather and field sites, physiological measurements were pooled within each season. Furthermore, pooling of physiological data across the 3 seasons provided a simple indication of cultivar specific physiological capacity under high temperatures in the field, regardless of specific season variability. These two analyses showed that photosynthesis and electron transport rate were reduced whilst stomatal conductance and transpiration rate were increased under the tents (Figure 5-1, Figure 5-2). Pooled analysis within and across 3 seasons was able to resolve cultivar differences in photosynthesis and Sicala 45 was lower compared with Sicot 53 under the tents (Figure 5-1). Analysis of individual tents at various times throughout the growing seasons was sufficiently sensitive to determine cultivar differences in photosynthesis for tent 1 in seasons 1 and 2 and for electron transport rate for tent 1 in season 1 which corresponds

with prolonged exposure to high (45 ^{o}C) temperatures under the tents (Figure 5-10). Hence, pooled analysis may provide a simple indication of gross changes in plant physiology in response to abiotic stress and accounts for periods whereby cultivar differences were not evident due to external variables. However, analysis of data taken on individual measurement days is necessary to confidently and consistently identify cultivar specific tolerance to high temperature stress in the field. This method of analysis may also be employed to identify cultivar differences in heat tolerance at various developmental stages, as well as under varying weather conditions thereby providing a more comprehensive indication of stress tolerance.

At high levels of irradiance, the decrease in electron transport rate under high temperatures in the growth cabinet was greater for Sicala 45 compared with Sicot 53 (Table 5-11) but no associated cultivar differences were determined for leaf temperature (Figure 5-15). Cultivar specificity for electron transport rate under high temperatures in the growth cabinet has been previously reported (Bibi *et al.* 2008) and agrees with the current field experiments indicating a greater decrease for electron transport rate of Sicala 45 under the tents compared with Sicot 53.

No cultivar discrimination was evident for photosynthesis at various irradiance levels and at high temperatures in the growth cabinet (Table 5-11) and agrees with previous research indicating no cultivar specificity for photosynthesis under elevated temperatures in a growth cabinet (Bednarz and van Iersel 2001; Bibi *et al.* 2008). Similarly, no cultivar differentiation was evident for stomatal conductance or transpiration under high temperatures in the growth cabinet (Figure 5-14). This contradicts previous research indicating cultivar specificity for stomatal conductance of cotton under high temperatures in the glasshouse (Rahman 2005).

Fitting a light response curve for photosynthesis, electron transport rate, stomatal conductance or transpiration rate did not improve the resolution of the measurements for detection of cultivar differences in physiological function under high temperatures in the growth cabinet (Table 5-9). Hence, evaluation of the irradiance response curve of cotton under high temperatures in the growth cabinet was not an effective method of determining cultivar differences in physiology under heat stress.

Photosynthesis was consistently decreased when leaf material was subjected to high temperature stress under tents in the field when data was considered under individual tents, pooled within a season or pooled across 3 seasons. This is consistent with previous work indicating a decline in carbon assimilation increasing air temperature (Bednarz and van Iersel 2001). Furthermore, Sicala 45 had lower photosynthetic rates than Sicot 53 under the tents (Figure 5-1). This is consistent with previous work indicating cultivar differences for carbon assimilation under moisture deficit in the field (Ullah *et al.* 2008) and may be attributed to genotypic differences in leaf temperature (Quinsenberry *et al.* 1994), but this cultivar specificity has not been reported for photosynthesis under *in-situ* high temperature stress in the field.

Photosynthesis is a highly complex process that is variably affected by local environmental conditions in the field. Pooled analysis of photosynthesis across multiple seasons may suggest evidence of genotypic heat tolerance in a long term cotton production system, but analysis for each individual season indicated that tent-induced decreases in photosynthesis were cultivar specific for all seasons whilst cultivar differences were only evident for electron transport rate in season 3 (Figure 5-7). This suggests that cultivar specificity for photosynthesis of plants under prolonged exposure to high (45 ^{o}C) temperatures (Table 3-6) in hot seasons (seasons 1 and 2) is more likely to be primarily

attributed to Rubisco activase activity (Crafts-Brandner and Salvucci 2004; DeRidder and Salvucci 2007; Law and Crafts-Brandner 1999; Long and Bernacchi 2003). Analysis of six cotton cultivars by Pettigrew and Turley (1998) found no cultivar specificity for Rubisco activity in the absence of stress, but there may be potential for identification of cultivar specific Rubisco activity for cotton grown under abiotic stress conditions (Bose and Ghosh 1995). There was no correlation between photosynthesis and yield, thereby suggesting that although Rubisco activation may be the primary limitation to photosynthesis, it may not be the primary limitation to yield under field conditions.

Similar decreases were noted for photosynthesis and electron transport under the tents indicating that these physiological processes are inhibited to a similar degree under high temperature stress (Figure 5-10). This is consistent with comparable reductions in photosynthesis and electron transport rate under growth cabinet conditions in this study (Figure 5-13), thus suggesting that electron transport rate may be the limitation to photosynthesis under the tents and under high temperature stress in the growth cabinet (cf. Wise *et al.* 2004) and indicating that rapid determination of electron transport rate may be used to predict photosynthesis under field conditions (Earl and Tollenaar 1999).

Electron transport rate was positively correlated with yield for seasons 1 (Table 5-8). This is probably the first report of a strong relationship between ETR and yield which suggests potential development of fluorescence measurements as a tool for screening a broad genetic range of cultivars for thermotolerance under field conditions. The reliability of ETR measurements for heat tolerance determination may be attributed to high repeatability of fluorescence measurements, compared to high environmental sensitivity and complexity associated with photosynthesis measurements. Genotypic differences for water and carbon dioxide flux may influence photosynthetic capacity under stress. Season

specific analysis showed that a negative relationship existed between photosynthesis and conductance in season 2 (Figure 5-9), which is consistent with the work of Lu *et al*. (1997) who showed that photosynthesis reached a plateau at air temperatures of above 30 ^{o}C, whilst stomatal conductance continued to increase under well-watered conditions. The data under the tents are different from previous field and glasshouse studies suggesting that stomatal conductance decreased and was positively correlated with photosynthesis under high temperatures (Rahman 2005) and water stress conditions (El-Sharkawy and Hesketh 1964; Leidi *et al.* 1993; Pettigrew 2004; Ullah *et al.* 2008). High stomatal conductance and transpiration under the tents in this study may be due to high relative humidity (Barbour and Farquhar 2000) and an absence of water deficit stress, thus providing evidence for different physiological stress responses to heat stress for plants grown under well watered, compared with drought conditions.

Overall, stomatal conductance and transpiration rate were higher for Sicala 45 compared with Sicot 53 whilst photosynthesis and electron transport were higher for Sicot 53. This cultivar specificity indicates that Sicala 45 has a higher potential for heat avoidance under high temperature stress in the field, associated with a decline in photosynthesis (Lu *et al.* 1997) and yield potential which was not evident under high temperature stress in the growth cabinet. This may be attributed to a higher level of stress in Sicala 45 compared with Sicot 53 under field conditions and hence, a greater demand for heat dissipation mechanisms to maintain leaf temperature. Selection for enhanced evaporative cooling under high temperature stress increases the susceptibility of the plant to water stress (Lu *et al.* 1994), thus increasing the water input requirement of the system. As future farming systems may be operating under water limiting conditions, breeding programs need to target selection of water-use efficient heat tolerant genotypes, rather than heat avoiding genotypes.

Furthermore, stomatal conductance and transpiration were negatively correlated with yield in seasons 1 and 3 (Table 5-8) however, it is likely that this is a coincidence due to an unsuccessful heat avoidance mechanism where increased stomatal conductance and transpiration under the tents did not prevent yield reduction (Lu *et al.* 1994). The correlation coefficient (R^2) for stomatal conductance and transpiration with yield were higher in season 1 than season 3 suggesting that genes for thermotolerance are more strongly up-regulated only under warmer conditions or at later growth stages, thereby highlighting the importance of screening for heat stress under hot conditions in the field (Lopez *et al.* 2003b; Rahman 2005). On the basis of conductance and yield data, Sicala 45 appears to have relatively lower tolerance to high temperatures and thus would be preferentially excluded from breeding programs for heat tolerance.

A single index for plant physiological function under abiotic stress was created and provided a broader picture of gross physiological changes in response to high temperature stress than analysis of single physiological measurements in isolation. In this study, principal component analysis was used to describe changes in plant physiological function under the tents compared with ambient (control) field conditions, pooled across three growing seasons and two locations. The plant physiological capacity index (PC_1) provided an indication of the overall changes in photosynthesis, electron transport rate, stomatal conductance and transpiration in response to temperature stress whilst PC_2 suggested subtle differences between cotton cultivars under field conditions. Principal component analysis of the physiological capacity of Sicot 53 and Sicala 45 under ambient (control) and tent regimes in the field suggested that photosynthesis and electron transport rate are decreased under the tents, whilst stomatal conductance and transpiration rate are increased under the tents. Cultivar differences have similarly been determined for quality and yield in broccoli (Tan *et al.* 1999b) and barley (Rajala *et al.* 2007) and physiological traits and

yield in cotton under water-deficit stress in the field (Ullah *et al.* 2008). These findings are consistent to that of analysis of each individual physiological process, pooled across the 3 seasons and hence the principal component analysis is an effective method of presenting a large array of data in a simple index. This index may be further extended to incorporate yield and fibre quality parameters to effectively identify heat tolerant cultivars under field conditions

Although the plant physiological index provides a broad-picture of cotton cultivar responses to high temperature stress in the field these physiological parameters varied between seasons for which, environmental variables largely overshadowed genotypic differences in physiological function (Pettigrew and Turley 1998). For example, the Australian cotton cultivars exhibited lower photosynthesis and higher stomatal conductance in season 2 at the Texas field site (Figure 5-7), thereby highlighting the importance of screening for stress tolerance in local environments. Although pooling photosynthesis, electron transport rate, stomatal conductance and transpiration rate across the seasons did not increase the resolution for detection of cultivar differences in heat tolerance, the physiological index may provide a simplistic tool for identification of key physiological functions influenced by abiotic stress over a number of seasons and across multiple locations.

5.5 **Conclusion**

Plant physiological measurements provide a valuable resource in describing the basis of stress-responsive yield under field conditions, provided that the variable nature of field experiments is considered. Sicot 53 was consistently shown to have a high capacity for photosynthesis and stomatal conductance at all levels of measurement in the field thus indicating relatively high temperature tolerance. Conversely, Sicala 45 was consistently

shown to have higher stomatal conductance and transpiration under high temperature stress in the field, indicating a high capacity for heat avoidance, but not necessarily tolerance.

Pooled measurements of physiological parameters provide a simple index for determining cultivar differences in physiological function within an entire growing season, or across multiple seasons. Pooled measurements and the physiological index consistently showed that Sicot 53 had a higher photosynthetic rate than Sicala 45. However, consideration of measurements on individual days under the tents indicate specific circumstances such as weather conditions or growth stages where cultivar differences in photosynthesis and electron transport rate occur. Hence, it would be beneficial to use both broad-spectrum and specific analyses of physiological function to effectively determine which cultivars have a relatively higher level of thermotolerance and which conditions may be targeted to minimise cultivar specific heat sensitivity.

Electron transport rate has the greatest potential for development of a rapid and reliable diagnostic tool for heat tolerance determination. However, inconsistencies under field conditions indicate the importance of evaluation over a number of seasons and at a number of time periods throughout each season. Recommendations for genotypes with superior stress tolerance may then be based on measurements taken on days where the stress is evident. These recommendations may be further strengthened by the supplementation of physiologically based methods for stress screening, by cost-effective, rapid and reliable laboratory-based screening assays for thermotolerance (Bibi *et al.* 2008; Burke 2007). This may be achieved by targeting the underlying biochemical processes that contribute to differences in plant physiology under abiotic stress in the field.

Chapter 6 Screening for Cultivar Specific Thermotolerance at the Cellular Level

6.1 **Introduction**

Reductions in photosynthesis above the thermal kinetic window for cotton (32 ^{o}C) (Burke *et al.* 1988) can be partially attributed to decreased membrane integrity and reduced selectivity of cytoplasmic and plasma membranes associated with protein denaturation and loss of enzyme functionality (Gupta 2007). Breeding through selection of biochemical fitness under environmental stress may result in the development of stress tolerant genotypes. Traditional stress tolerance screening assays using biochemical measurements have focused on sampling of glasshouse grown plants exposed to highly regulated temperature treatments.

Membrane permeability has been extensively employed for crop thermotolerance determination, particularly in glasshouse-grown wheat (Assad and Paulsen 2002; Bajji *et al.* 2002; Blum and Ebercon 1981; Saadalla *et al.* 1990a; Shanahan *et al.* 1990), pasture species (Schaff *et al.* 1987) sorghum (Sullivan 1971), *Brassica spp.* (Hossain *et al.* 1995), cowpeas (Ismail and Hall 1999) soybean (Martineau *et al.* 1979a; Sethar *et al.* 1997) and cotton (Ashraf *et al.* 1994; Bibi *et al.* 2008). However, these assays use high temperatures generated through growth cabinet or water bath regulation to generate stress and few studies have evaluated these techniques under field conditions.

Indirect selection for membrane integrity using plant physiological measurements and assays can be further developed through the use of molecular techniques. Targets for such breeding programs include reducing levels of unsaturated fatty acids to counteract membrane fluidity (Murakami 2000), increased osmolyte accumulation (Alia *et al.* 1998)

and increased heat shock protein synthesis (Lee *et al.* 1995), particularly with respect to sensitive physiological stages such as flowering (Ismail and Hall 1999).

Similarly, quantification of enzymatic viability under temperature stress in the laboratory has been reported for wheat (Porter *et al.* 1995), pasture species (Schaff *et al.* 1987) and cotton (Burke 2007; de Ronde and van der Mescht 1997; McDowell *et al.* 2007; McMichael and Burke 1994; Tan *et al.* 1999a) grown under glasshouse conditions. These methods have successfully determined some crops, growth stages or cultivars to have a high relative thermotolerance under glasshouse and growth cabinet conditions.

Laboratory assays for biochemical screening for thermotolerance rely on small tissue samples under concentrated heat stress. However, there is little extension of this knowledge to actual field conditions; hence the assumption that these methods can be used to select for superior cultivars under high temperature stress in the field has not been validated. Although the variable nature of the field makes it difficult to isolate a specific stress and the specific impacts of this stress on the plants, it is essential to establish whether cultivar differences found under growth cabinet conditions actually translate to field systems and hence determine the validity of biochemical assays for stress tolerance screening in breeding programs (Marcum 1998).

A series of experiments was conducted to determine the effectiveness of laboratory based screening for cultivar specific thermotolerance using the membrane leakage and enzymatic viability assays, and to assess whether these differences are reflected under field conditions, thus validating the use of rapid and reliable laboratory screening mechanisms for the identification of thermotolerance in cotton cultivars under field conditions. The aim of this series of experiments was to determine genotypic specific thermotolerance of

cotton cultivars Sicot 53 and Sicala 45 using the cellular membrane integrity and enzyme viability assays for;

(a) field-grown plant material by evaluation of a temperature response curve (25, 35, 40, 45, 50, 55, 65 ^{o}C) via incubation in a thermally regulated water bath (experiment 1);

(b) field-grown plant material under ambient (control) or high (tent) temperatures and correlated to yield (experiment 2); and

(c) growth cabinet grown plant material under optimal (32 ^{o}C) or high (42 ^{o}C) temperature regimes (experiment 3).

Specifically, the hypothesis is that there are no cultivar specific differences in tolerance to high temperature stress that are distinguishable by application of the membrane integrity and enzyme viability assays under growth cabinet and field conditions.

6.2 **Materials and methods**

6.2.1 **Site description**

Field experiment 1 was conducted at The Australian Cotton Research Institute, Narrabri during season 1 (2006). Experiment 2 was conducted in the field during seasons 1 (2006) and 3 (2007) in Narrabri and season 2 (2006) in Texas. Experiment 3 was a controlled environment study conducted at the Australian Cotton Research Institute, Narrabri (Table 6-1). Details of each experiment are presented in Chapter 3.

Table 6-1 Description of experiments 1, 2 and 3 used for assessing membrane integrity and enzyme viability under field and growth cabinet conditions

Experiment	Site	Season	Year	Location
1	Field	1	2006	Narrabri
2	Field	1	2006	Narrabri
2	Field	2	2006	Texas
2	Field	3	2007	Narrabri
3	Growth cabinet	-	2007	Narrabri

6.2.2 **Treatments**

Experiment 1

Cotton genotypes Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2 were grown in a randomised block design with four replicates, blocked down the field. Plants were sampled under ambient conditions between 1300 and 1430 h then immediately transported back to the laboratory. The third youngest fully expanded leaf was collected from 4 plants per treatment. Leaf discs of 10 mm diameter were cut from the interveinal portion of the leaf and incubated at various temperatures (25, 35, 40, 45, 50, 55, 60 ^{o}C) for 2 h in a water bath. The leaf tissue was analysed for membrane integrity and enzyme viability.

Experiment 2

Leaves from cotton cultivars Sicot 53 and Sicala 45 were sampled under ambient (control) conditions and under tents between 1300 and 1430 h then immediately transported to the laboratory. Leaf discs (10 mm diameter) were sampled from the interveinal portion of the leaf and then incubated at control (25 ^{o}C) and high (45 ^{o}C) temperatures in a water bath. The leaf tissue was simultaneously analysed for membrane integrity and enzyme viability. Whole plants were harvested and processed for yield as previously described (Chapter 4).

Experiment 3

Pots containing Sicot 53 or Sicala 45 plants were transferred from the glasshouse to the growth cabinet as previously described (Chapter 3). Plants were sampled for membrane

integrity and enzyme viability at 1230 h (Time 0) and subsequently sampled every 24 h for 3 d. Leaf discs were punched from the interveinal area of the 4th youngest fully expanded leaf with 4 leaves per treatment. Discs were analysed for cell membrane integrity and enzyme viability after a 2 h incubation at control (25 ^{o}C), moderately high (45 ^{o}C) and killing (90 ^{o}C) temperatures in a water bath.

6.2.3 **Measurements**

Cell membrane integrity assay

Five discs from each leaf were triple rinsed with distilled water to remove exogenous electrolytes and placed in 25 mL sealed glass vials containing 10 mL distilled water. The vials were incubated in a controlled temperature water bath at various temperatures (specific for each experiment) for 2 h at a specified incubation temperature (t). Samples incubated at high water bath temperatures (>25 ^{o}C) were left to cool 25 ^{o}C. Initial electrical conductivity (IEC_t), a measure of membrane leakage, was determined using a low range (0 to 1990 μS/cm), waterproof ECTestr calibrated conductivity meter (Oakton Instruments, Vernon Hills, IL, USA). Discs were then autoclaved at 121 ^{o}C and 103 kPa for 15 mins then cooled to 25 ^{o}C. Final electrical conductivity (FEC_t) of the solution was measured with the calibrated conductivity meter. Relative electrical conductivity (REC_t) was then determined (Equation 6-1) and an increasing REC_t shows a decreasing membrane integrity (Flint *et al.* 1967; McDowell *et al.* 2007).

$$REC_t = \left(\frac{IEC_t}{FEC_t}\right) * 100 \qquad \text{(Equation 6-1)}$$

Relative cellular injury (RCI_t) was also determined (Equation 6-2) to evaluate membrane integrity under high air temperatures as well as localised heat stress in a temperature controlled water bath. For this measurement, the IEC_t and FEC_t were determined for leaf

discs incubated at a treatment temperature (t) or a control temperature (c) for 2 h in a temperature controlled water bath. RCI_t similarly increases with decreasing membrane integrity (Rahman *et al.* 2004; Sullivan 1971).

$$RCI_t = \left(1 - \frac{1 - \left(\frac{IEC_t}{FEC_t}\right)}{1 - \left(\frac{IEC_c}{FEC_c}\right)}\right) * 100 \qquad \text{(Equation 6-2)}$$

Enzyme viability assay

Discs were triple rinsed with distilled water to remove exogenous residues and 2 discs were placed in a 25 mL sealable glass vial containing 0.5 mL distilled water (Steponkus and Lanphear 1967). The vials were incubated at different temperatures (t) (specified for each experiment) for 2 h in a controlled temperature water bath. A phosphate buffer solution containing 0.01 M phosphate buffered saline (0.138 M NaCl; 0.0027 M KCl with TWEEN® 20 (0.05 % v/v), pH 7.4, at 25 °C) and 0.8 % w/v 2,3-5, triphenyltetrazolium chloride (TTC) (Merck) was prepared and 8 mL was added to each vial. The leaf was vacuum infiltrated at -33 kPa for 15 mins to ensure TTC uptake into the leaf and left to incubate at 25 ^{o}C in the dark for 24 h. Discs were triple rinsed with distilled water, submerged in 2 mL of 95% (v/v) ethanol and incubated for 24 h in the dark. Enzyme viability was measured spectrophotometrically at 530 nm using 95 % ethanol as a reference.

A high absorbance at 530 nm (Abs_t) indicates strong reduction of the TTC salt to a red coloured triphenyl formazan due to the dehydrogenase activity of the mitochondrial respiratory chain and hence indicates either low stress conditions or high inherent plant-based tolerance to stress (de Ronde *et al.* 1995; McDowell *et al.* 2007; Steponkus and Lanphear 1967). Low absorbance is indicative of impaired dehydrogenase activity and decreased capacity for reduction of the TTC salt to a red formazan product. This indicates

a high level of damage to the respiratory enzymes, and hence low enzyme viability, most likely attributed to abiotic stress imposed by the high temperature treatments.

Acquired high temperature tolerance ($AHTT_t$) was determined (Equation 6-3) as an index for enzyme viability under high temperature stress and this value increases with increasing viability of respiratory enzymes in the mitochondria. $AHTT_t$ evaluates enzyme viability under high air temperatures as well as localised heat stress in a temperature controlled water bath. To calculate this measurement the absorbance at 530 nm for leaf discs incubated for 2 h at a control (Abs_c) and high (Abs_t) temperature and for leaf discs held for 10 minutes at a killing temperature (Abs_k) in a temperature controlled water bath were used (Porter *et al.* 1995).

$$AHTT_t = \left(\frac{Abs_t - Abs_k}{Abs_c - Abs_k}\right) * 100 \qquad \text{(Equation 6-3)}$$

For experiment 3, the spectrophotometer was unavailable and a micro plate manager (Bio-rad Laboratories) was used to measure sample absorbance at 530 nm. All samples were incubated in tetrazolium buffer and ethanol as described above. After 24 h incubation in ethanol, 150 μL of each sample was transferred from the vial into a 96 well plate. Absorbance of an average of eight, 95 % v/v ethanol samples was subtracted from the absorbance of each sample at 530 nm using a Bio-Rad micro plate reader. Samples with high absorbance in the spectrophotometer were underestimated on the micro plate reader. All data presented are calibration of the absorbance at 530 nm from the micro plate reader (x), to the spectrophotometer (Abs_t) (Equation 6-4), to enable effective comparison between experiments 1, 2 and 3.

$$Abs_t = 0.0382 + 2.0964x \quad \text{(Equation 6-4)}$$

Where Abs_t = absorbance at 530 nm calibrated for a spectrophotometer and x = absorbance at 530 nm using a microplate reader

6.2.4 **Data analysis**

Experiment 1

REC_t was plotted against water bath incubation temperature (^oC) and a 4 parameter Gompertz model (Equation 6-5) was fitted to each cultivar using regression analysis in SigmaPlot 9.0. One-way ANOVA (water bath incubation temperature) was used to determine cultivar differences for REC at various levels of water bath incubation temperature. The water bath temperature at which 50% REC_t occurred (T_{50}) was calculated for each replicate as the water bath incubation temperature for which REC_t is equal to 50% (Equation 6-5). Cultivar differences for T_{50} were compared using one-way ANOVA (cultivar).

$$REC_t = y_0 + a * e^{-e\left(\frac{-(x-x_0)}{b}\right)} \quad \text{(Equation 6-5)}$$

Where x = water bath temperature, y_0 = Asymptotic REC as temperature decreases indefinitely (i.e. initial REC_t), a = asymptotic increase in REC_t that occurs as x approaches infinity, x_0 = temperature (^oC) at which the absolute growth rate is maximal and b = relative growth rate at x_0.

Abs_t was plotted against water bath incubation temperature (^oC) and a 3 parameter Gompertz model (Equation 6-6) was fitted for each cultivar. One-way ANOVA (cultivar) was used to determine cultivar differences for Abs_t at various levels of water bath incubation temperature.

$$Abs_t = a*e^{-(\frac{-(x-x0)}{b})}$$ (Equation 6-6)

Where x = water bath temperature, a = asymptotic decrease in Abs_t that occurs as x approaches infinity, x_0 = temperature (^oC) at which the absolute growth rate is maximal and b = relative growth rate at x_0.

Experiment 2

Two-way ANOVA (cultivar*temperature treatment) was conducted for REC_t or Abs_t to determine cultivar differences under ambient (control) or tent regimes in the field. Analysis was firstly conducted for the pooled averages for season 1, 2 and 3 combined and then separately for each season individually.

A linear regression was fitted to correlate REC_t or Abs_t with seed cotton yield under ambient or tent regimes in the field. Analyses were performed for Sicot 53 and Sicala 45 under ambient (control) conditions or under the tents for the seasons 1 and 3 combined and then for each season separately. Season 2 was excluded from analysis as yield data from under the tents was misplaced.

Experiment 3

Two-way ANOVA (cultivar*cabinet time) was conducted for REC_t or Abs_t under control (32 ^oC) or high (42 ^oC) temperature regimes in the growth cabinet and also various water bath temperatures (25 or 45 ^oC) for the interaction between cabinet time (d) and cultivar (Sicot 53 and Sicala 45). Data for the control (32 ^oC) and high (42 ^oC) growth cabinet were analysed separately as the 2 growth cabinet treatment temperatures were imposed successively and not simultaneously. Two-way ANOVA (cultivar*cabinet time) was conducted for RCI_t or $AHTT_t$ under control (32 ^oC) or high (42 ^oC) growth cabinet temperature regimes for the interaction between growth cabinet time (d) and cotton cultivar.

6.3 **Results**

6.3.1 **Experiment 1**

Membrane leakage increased (Table 6-2) sigmoidally with increasing temperatures for all cultivars (P<0.001) (Figure 6-1). The mean REC_t of Sicala 45 was higher than the mean REC_t for the other three cotton genotypes at water bath incubation temperatures of 40 and 45 ^oC. The extent of membrane leakage was not different between cultivars in the control (25 ^oC) or high temperature (50 and 65 ^oC) treatments. The temperature at which 50 % REC_t (T_{50}) occurred was lower for Sicala 45 compared with Sicot 53, CSX 99209-376 and Sicala V2 (Figure 6-2).

Table 6-2 Fitted equations to the relationships between relative electrical conductivity (4-parameter Gompertz model) or absorbance at 530 nm (3-parameter Gompertz model) with increasing water bath temperatures for cotton cultivars Sicot 53, Sicala 45, CSX 99209-376 and Sicala V-2 grown under ambient field conditions in season 1 (2006) in Narrabri. Y represents REC_t and x represents water bath temperature (^oC), where n.s. represents F test values where P>0.05.

Genotype	n	R^2	Equation	F test P value
Relative electrical conductivity				
Sicot 53	24	0.94	y=11.15+62.04*e(-e(-(x-42.17)/2.89))	<0.001
Sicala 45	24	0.93	y=13.89+64.30*e(-e(-(x-37.77)/2.02))	<0.001
CSX 99209-376	24	0.96	y=11.83+71.09*e(-e(-(x-42.05)/2.79))	<0.001
Sicala V-2	24	0.97	y=11.39+64.10*e(-e(-(x-44.82)/0.25))	<0.001
Absorbance at 530 nm				
Sicot 53	24	0.82	y=1.07*e(-e(-(x-42.61)/-3.16))	<0.001
Sicala 45	24	0.63	y=1.04*e(-e(-(x-41.82)/-10.45))	<0.001
CSX 99209-376	24	0.78	y=177.41*e(-e(-(x+130.98)/-95.28))	<0.001
Sicala V-2	24	0.58	y=0.80*e(-e(-(x-43.94)/-8.95))	<0.001

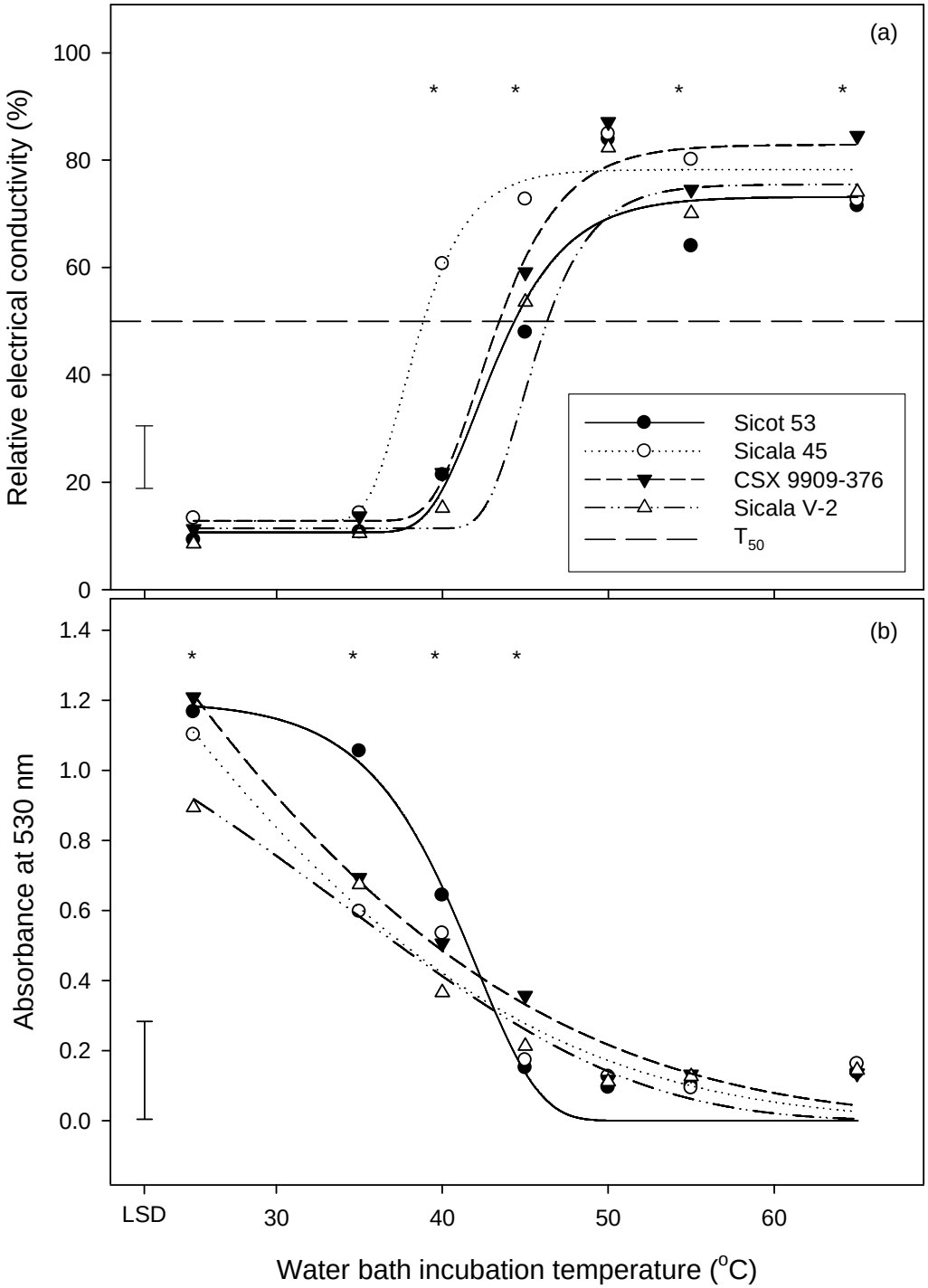

Figure 6-1 (a) Mean relative electrical conductivity (%) and (b) mean absorbance at 530 nm of cotton leaf tissue of cotton cultivars Sicot 53, Sicala 45, Sicala V-2 and breeding line CSX 99209-376, grown under field conditions during season 1 (2006) at Narrabri. The regression lines were fitted for a (a) 4-parameter and (b) 3-parameter Gompertz model for each cultivar. The dashed horizontal lines represent the time to 50% (a) relative electrical conductivity. Asterisks (*) represent a water bath incubation temperature at which there is a significant difference between cultivars for (a) relative electrical conductivity or (b) absorbance at the 95% confidence interval. The vertical lines represent the l.s.d. for temperature by cultivar interaction at *P*=0.05.

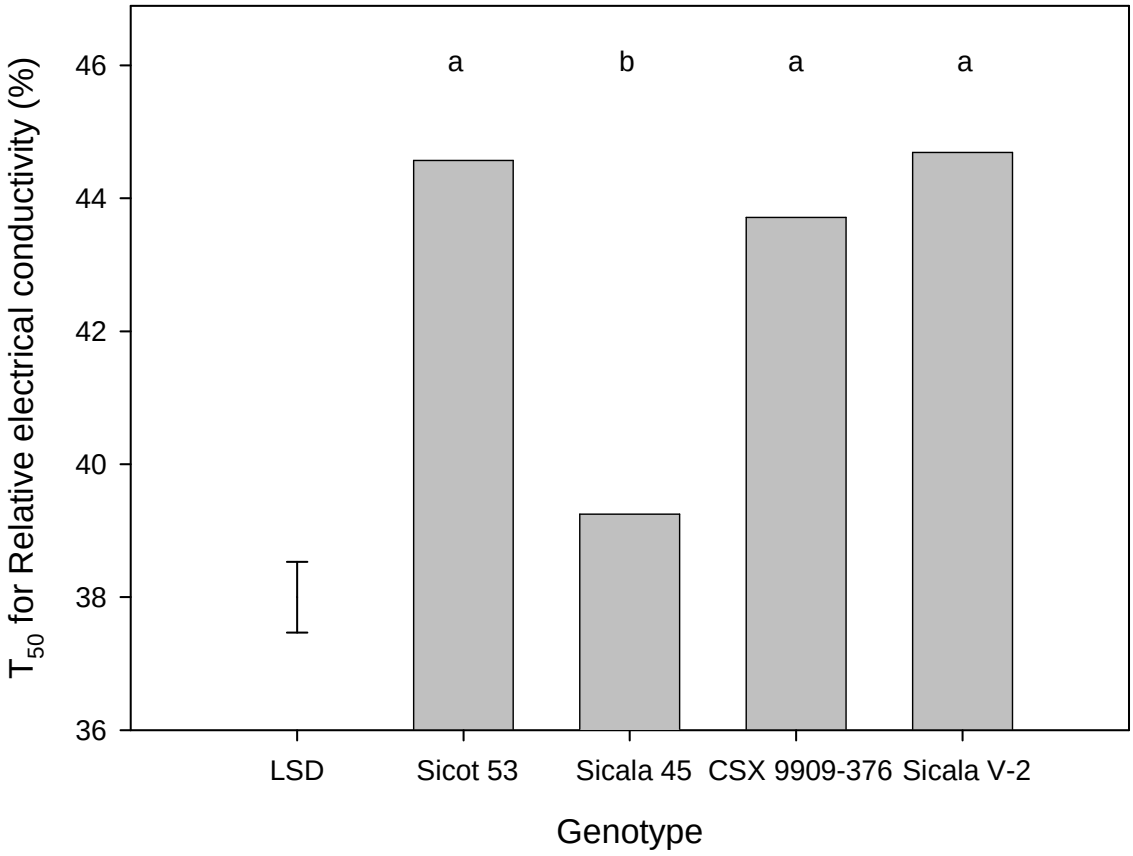

Figure 6-2 Temperature (^{o}C) at which 50 % relative electrical conductivity occurred for cotton cultivars Sicot 53, Sicala 45, Sicala V-2 and breeding line CSX 99209-376 grown under ambient field conditions in season 1 (2006) in Narrabri. Bars represented with the same letter are not different at *P*=0.05. The vertical line represents the l.s.d. for cultivar at *P*=0.05 for relative electrical conductivity T_{50}.

A 3-parameter Gompertz model was fitted for each cultivar for the Abs_t of cotton tissue incubated for 2 h at various temperatures in a temperature controlled water bath (Table 6-2). Abs_t decreased with increasing temperature (P<0.001) (Figure 6-1). A cultivar specific response to water bath temperature was identified (P=0.026) and the Abs_t of Sicot 53 was higher than the other three genotypes at both 35 and 40 ^{o}C (Figure 6-1). There was no cultivar differentiation at temperatures exceeding 50 ^{o}C.

6.3.2 **Experiment 2**

Biochemical response to heat stress in the field, pooled over 3 seasons

The REC_t of cotton cultivars Sicot 53 and Sicala 45 was determined under ambient (control) and tent conditions for seasons 1, 2 and 3 as these two cultivars exhibited the greatest differences for REC_t and Abs_t in experiment 1. The REC_t of leaves under the tents was higher (*P*=0.038) than under ambient (control) field conditions, but was no cultivar differentiation for conductivity for either treatment. The Abs_t was higher (*P*=0.025) for Sicot 53 than Sicala 45 plants but there were no differences between the tents and ambient (control) field conditions (Figure 6-3). No interaction was determined.

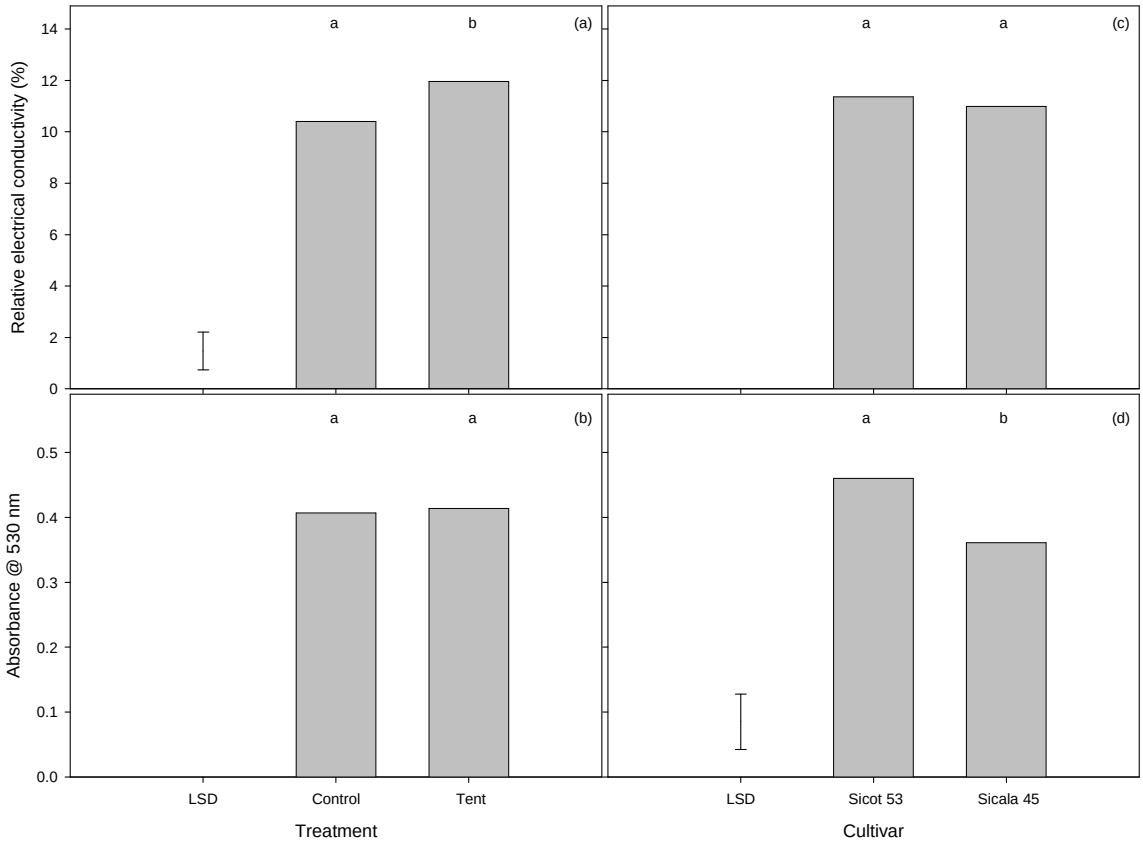

Figure 6-3 Mean (a, c) relative electrical conductivity (%) and (b, d) absorbance at 530 nm for (a, b) ambient (control) and high temperature (tent) conditions, and for (c, d) cotton cultivars Sicot 53 and Sicala 45 in the field, pooled for seasons 1 (2006) and 3 (2007) at the Narrabri field site and season 2 (2006) at the Texas field site. The vertical line in (a) indicates the l.s.d. value at *P*=0.05 for temperature treatment main effects and in (d), indicates the l.s.d. value at *P*=0.05 for cultivar means.

Biochemical response to heat stress, for each season

REC_t and Abs_t were analysed separately for each tent event in each season. The REC_t under the tents was higher than under ambient field conditions in season 1 (P=0.014) (Table 6-3), but there no cultivar differences were found (Figure 6-4). For season 3, REC_t of Sicala 45 was higher (P=0.042) (Table 6-3) under the tents compared with Sicot 53 under the tents and all plants under ambient (control) regimes in the field (Figure 6-4). There were no temperature treatments or cultivar differences for REC_t in season 2. There were no differences between treatment regimes or cultivars for Abs_t for season 1 (2006) or season 3 (2007) at the Narrabri field site (Table 6-3).

Table 6-3 Probability of cultivar and temperature treatment main effects (control, tents) and cultivar by treatment interaction for relative electrical conductivity (%) and absorbance at 530 nm for cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) conditions and under the tents during seasons 1 (2006) and 3 (2007) at the Narrabri field site and in season 2 (2006) at the Texas field site, where n.s. represents *P*>0.05 and – represents times for which measurements were not taken.

Season	Temperature treatment	Cultivar	Temperature treatment * Cultivar
Relative electrical conductivity (%)			
1	0.014	n.s	n.s
2	n.s	n.s	n.s
3	n.s	n.s	0.042
Relative cellular injury (%)			
1	-	-	-
2	n.s.	n.s.	n.s.
3	n.s.	n.s.	n.s.
Absorbance at 530 nm			
1	n.s.	n.s.	n.s.
2	-	-	-
3	n.s	n.s	n.s
Acquired high temperature tolerance			
1	-	-	-
2	-	-	-
3	n.s	n.s	n.s

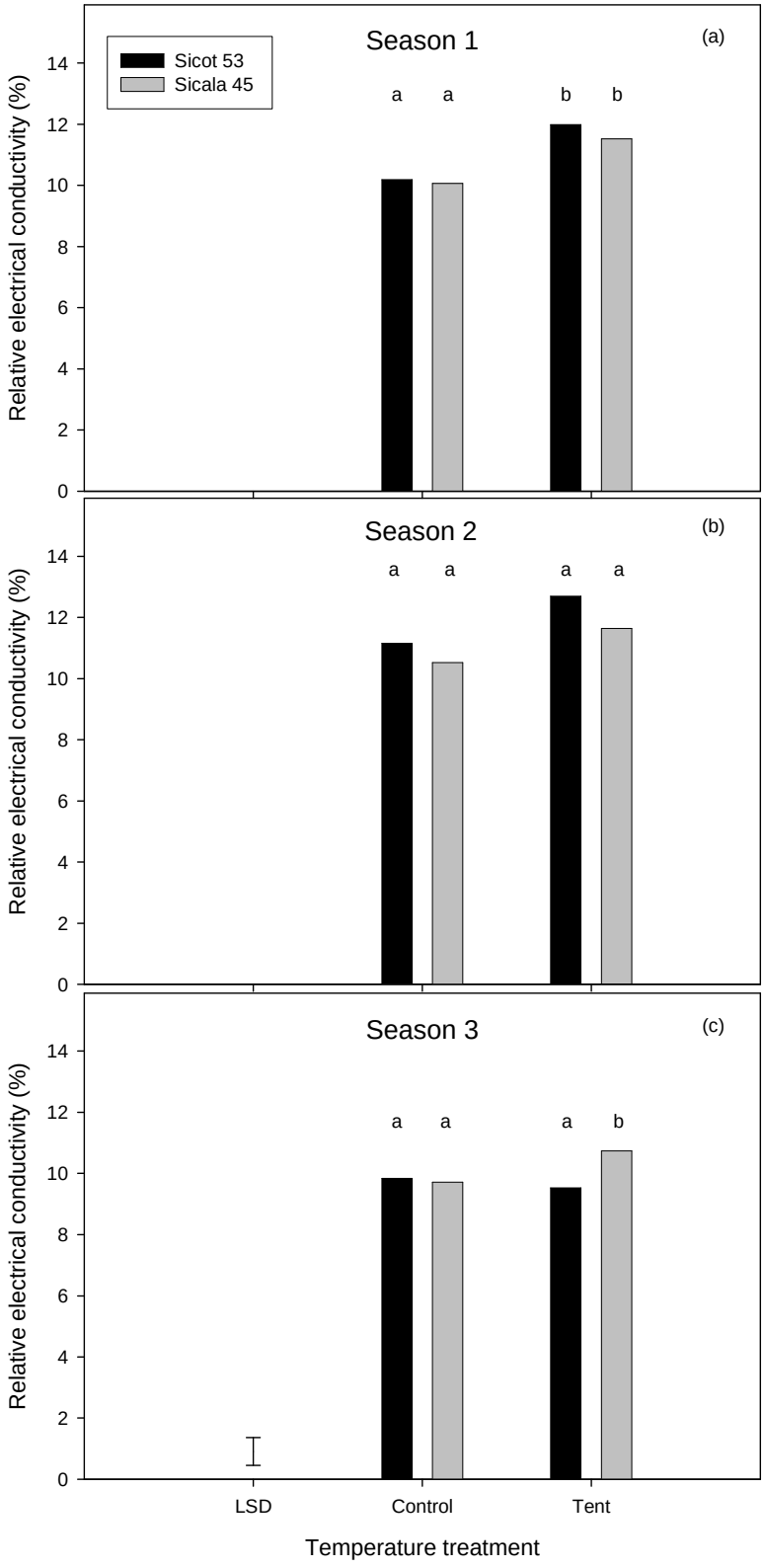

Figure 6-4 Mean relative electrical conductivity (%) for cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) and high temperature (tent) conditions in the field in (a) season 1 (2006) and (c) season 3 (2007) at the Narrabri field site and (b) season 2 (2006) at the Texas field site. Vertical bars represented with the same letter are not different at *P*=0.05. The (c) vertical line indicates the l.s.d. value at *P*=0.05 for temperature treatment by cultivar interaction *P*=0.05.

Relationship between REC_t or Abs_t and yield

REC_t and Abs_t means for 4 replicates of cultivars Sicot 53 and Sicala 45 under ambient (control) and tent regimes in the field during seasons 1 (2006) and 3 (2007) in Narrabri were fitted to a linear regression to determine whether there was any correlation with seed cotton yield under field conditions. A negative (P=0.033) (Table 6-4) relationship was found between REC_t and yield for season 1 (Figure 6-5) and accounted for 90 % of the variation (Table 6-4). No relationship was found between REC_t and yield for season 3 (Table 6-4). There was no relationship between Abs_t and yield in seasons 1 or 3 (Table 6-4). A relationship could not be determined for season 2 as seed cotton yield data from under the tents was misplaced.

Table 6-4 Correlation between relative electrical conductivity (%) or absorbance at 530 nm and seed cotton yield (g m^{-2}) under ambient (control) and high temperature (tent) regimes in the field in season 1 (2006) and/or 3 (2007) in Narrabri, where n.s. represents P>0.05 in the F-tests.

Season	n	Adjusted R^2	Equation	F test P value
Relative electrical conductivity (%)				
1	16	0.90	y=1269.24-78.36x	0.033
3	16	n.s.	n.s.	n.s.
Absorbance at 530 nm				
1	16	n.s.	n.s.	n.s.
3	16	n.s.	n.s.	n.s.

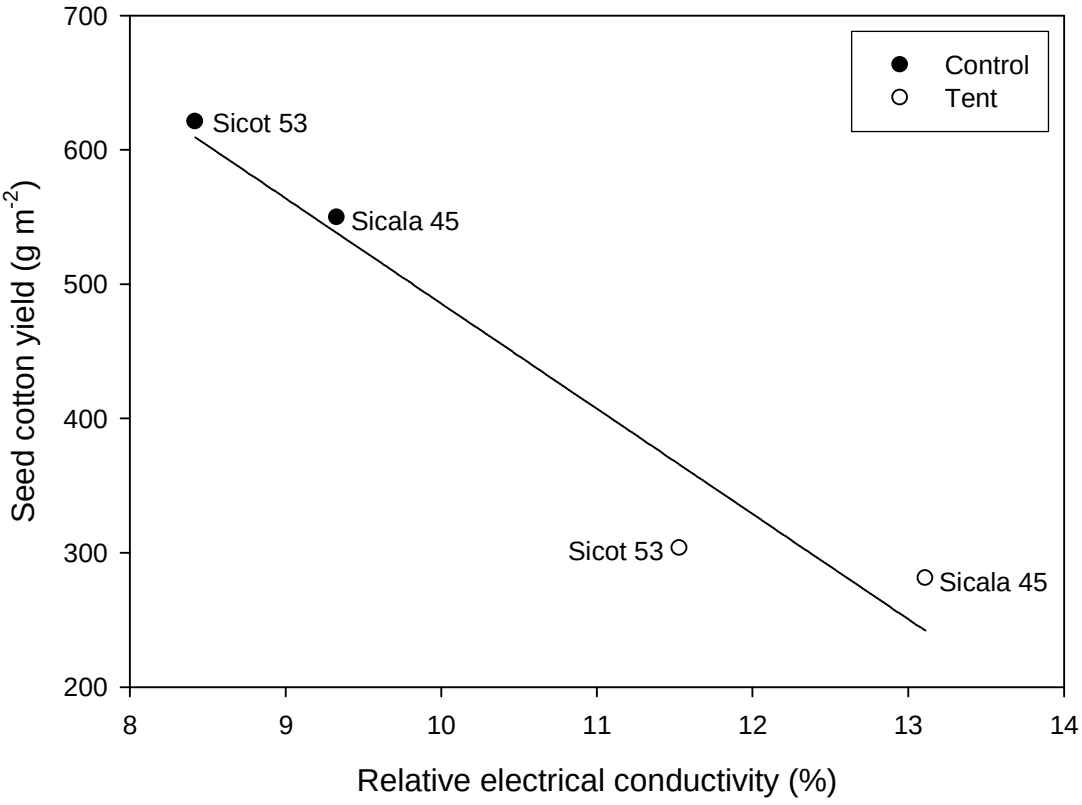

Figure 6-5 Correlation between relative electrical conductivity (%) and seed cotton yield cotton cultivars Sicot 53 and Sicala 45 grown under ambient (control) and tent regimes in season 1 (2006) and at Narrabri. Data presented are the treatment means of 4 replicates.

6.3.3 **Experiment 3**

A summary of main effects and interactions for REC_t, RCI_t, Abs_t and $AHTT_t$ for Sicot 53 and Sicala 45 cotton plants at control (32 ^oC) or high (42 ^oC) temperatures in the growth cabinet and pooled for various sampling times (0, 1, 2, and 3 d) during the incubation is presented in Table 6-5.

Table 6-5 Probability of cultivar and cabinet incubation time (h) main effects and cultivar by incubation time interaction for relative electrical conductivity (%) and absorbance at 530 nm for cotton cultivars Sicot 53 and Sicala 45 grown under optimal (32 ^{o}C) or high (42 ^{o}C) temperature regimes in a temperature controlled growth cabinet at Narrabri, 2006 and subsequently incubated for 2 h at optimal (25 ^{o}C), high (45 ^{o}C) or killing (90 ^{o}C) temperatures in a temperature controlled water bath, where n.s. represents F values where *P*>0.05.

Assay	Water bath temperature (^{o}C)	Cabinet Time (d)	Cultivar	Cabinet Time * Cultivar
Control (32 ^{o}C) temperature growth cabinet				
REC_t	25	n.s.	n.s.	n.s.
REC_t	45	<0.001	n.s.	n.s.
RCI_t	-	<0.001	n.s.	n.s.
Abs_t	25	n.s.	0.022	n.s.
Abs_t	45	0.014	n.s.	n.s.
$AHTT_t$	-	n.s.	n.s.	n.s.
High (42 ^{o}C) temperature growth cabinet				
REC_t	25	0.002	n.s.	n.s.
REC_t	45	0.006	0.018	0.019
RCI_t	-	n.s.	n.s.	0.023
Abs_t	25	0.030	n.s.	n.s.
Abs_t	45	0.001	<0.001	0.044
$AHTT_t$	-	<0.001	0.050	n.s.

There was no cultivar differentiation for REC_t of Sicot 53 and Sicala 45 leaf discs grown at optimum (32 ^{o}C) temperature regimes in the growth cabinet (Table 6-5). Subsequently, there were no cultivar differences for RCI_t of Sicot 53 and Sicala 45 plants grown under optimal (32 ^{o}C) temperature regimes in the growth cabinet (Table 6-5).

There was no cultivar differentiation for REC_t of plants grown under high (42 ^{o}C) temperatures in the growth cabinet and in the absence of supplementary heat treatment in the water bath (Figure 6-6). The REC_t of Sicala 45 plants was higher (*P*=0.019) (Table 6-5) after both 2 and 3 days incubation at 42 ^{o}C in the growth cabinet and after 2 h incubation at high (45 ^{o}C) temperatures in the water bath (Figure 6-6). Similarly, the RCI_t of Sicala 45 was also higher compared with Sicot 53 grown for 3 d at high (42 ^{o}C) temperatures in the growth cabinet (*P*=0.023) (Figure 6-6).

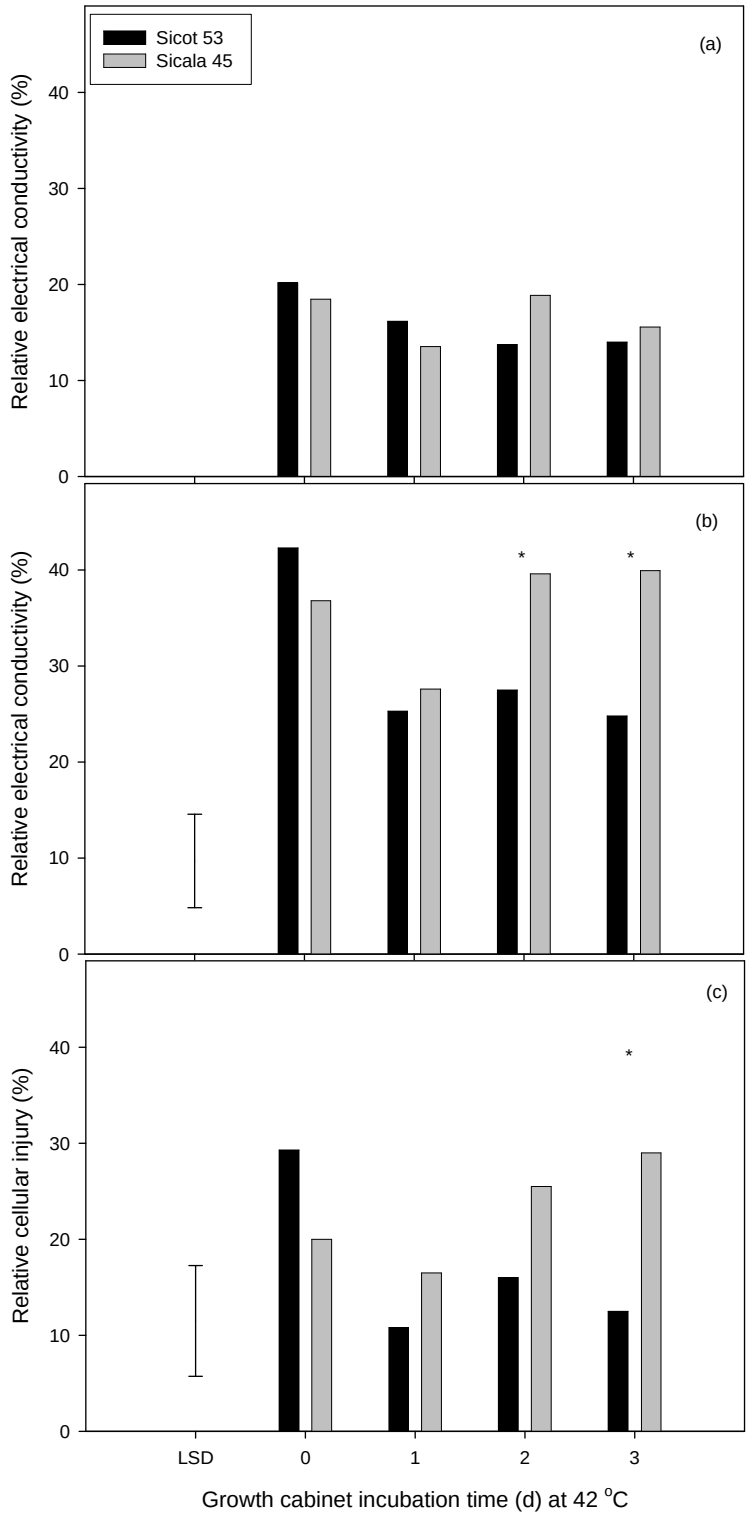

Figure 6-6 (a, b) Relative electrical conductivity (%) and (c) relative cellular injury (%) of cotton cultivars Sicot 53 and Sicala 45, grown at high (42 °C) temperatures in the growth cabinet and subsequently incubated for 2 h at (a) optimal (25 °C) and (b) high (45 °C) temperatures in a thermally controlled water bath. The (b, c) vertical lines represent the l.s.d. for growth cabinet incubation time by cultivar interaction at *P*=0.05. The asterisks (*) represent growth cabinet incubation times for which the mean relative electrical conductivity differs between the two cultivars at *P*<0.05.

The Abs_t for Sicala 45 was higher than Sicot 53 (P=0.022) (Table 6-5) grown at optimal (32 ^oC) temperatures in the growth cabinet (Figure 6-7). Supplementary incubation of leaf tissue for 2 h at 45 ^oC in a temperature controlled water bath did not generate cultivar differences in Abs_t and no cultivar differences were evident for the RCI under optimal (32 ^oC) growth cabinet conditions (Table 6-5).

For plants grown at high (42 ^oC) temperatures in the growth cabinet and incubated at 25 ^oC in the water bath, Abs_t decreased (P=0.030) (Table 6-5) across the measurement period (Figure 6-7). For plants grown under high (42 ^oC) temperatures in the growth cabinet and incubated at 45 ^oC for 2 h in a temperature controlled water bath, Abs_t was higher (P=0.044) (Table 6-5) for Sicala 45 compared with Sicot 53 on days 1, 2 and 3 (Figure 6-7). Subsequently, the $AHTT_t$ of Sicala 45 was higher (P=0.050) (Table 6-5) compared with Sicot 53 grown at high (42 ^oC) temperatures in the growth cabinet (Figure 6-7). The $AHTT_t$ of cotton plants grown at high (42 ^oC) temperatures in the growth cabinet increased (P<0.001) with increasing exposure to high temperatures (Figure 6-7).

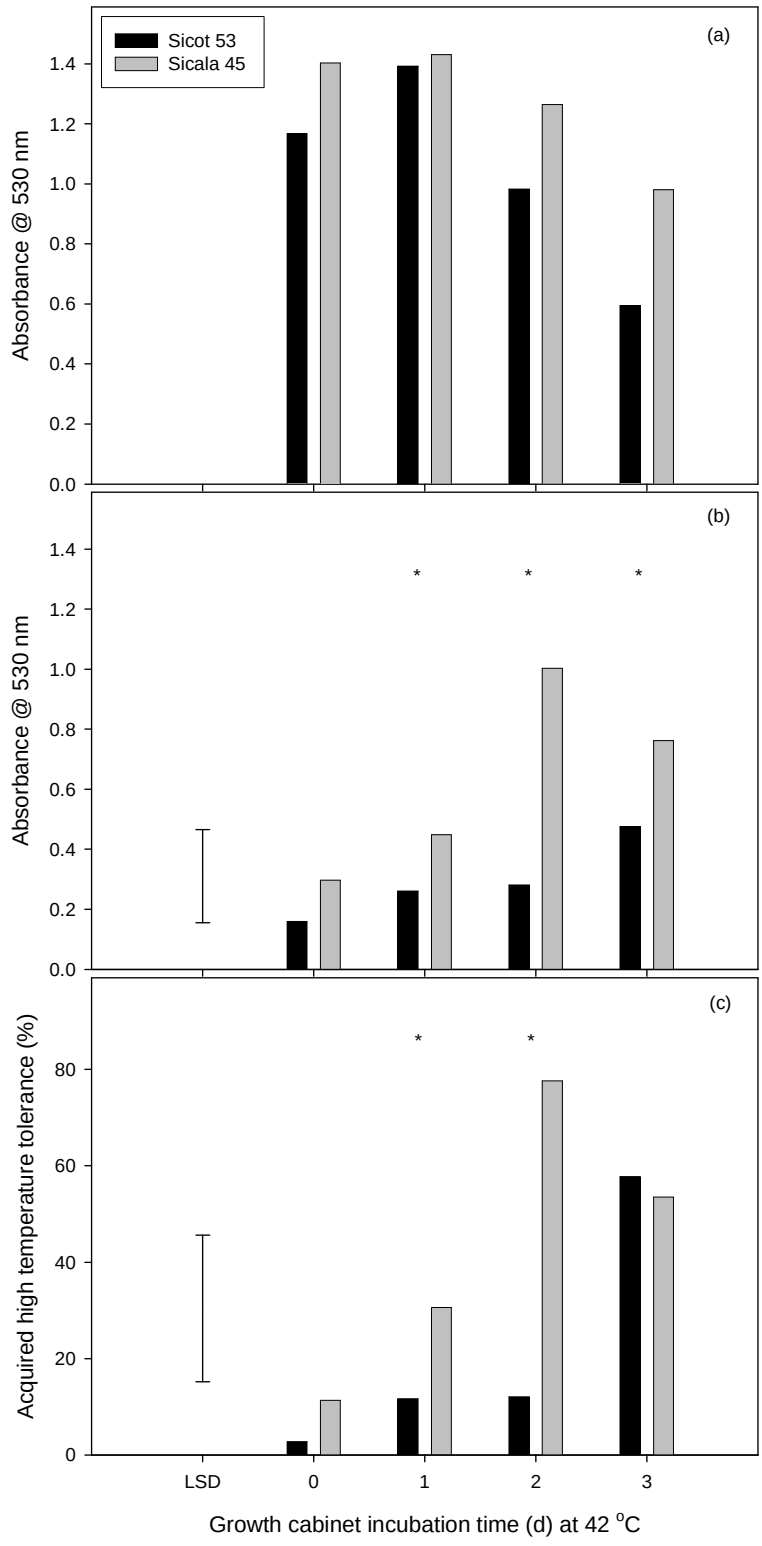

Figure 6-7 (a, b) Absorbance at 530 nm and (c) acquired high temperature tolerance (AHTT) in leaf tissue of cotton cultivars Sicot 53 and Sicala 45, grown at high (42 °C) temperatures in the growth cabinet and subsequently incubated for 2 h at (a) optimal (25 °C) and (b) high (45 °C) temperatures in a thermally controlled water bath. The (b, c) vertical lines represent the l.s.d. for growth cabinet incubation time by cultivar interaction at *P*=0.05. The asterisks (*) represent growth cabinet incubation times for which the mean absorbance differs between the two cultivars at *P*<0.05.

6.4 **Discussion**

The relative electrical conductivity (REC_t) and 2, 3, 5-triphenyltetrazolium chloride (Abs_t) assays were able to detect cultivar specific responses to high temperature stress and were able to distinguish between a relatively heat tolerant and non-tolerant genotype. Cultivar differences in membrane integrity (REC_t) were consistent for leaf material exposed to high temperature stresses derived under laboratory, growth cabinet and field conditions although cultivar differences in enzyme viability (Abs_t) were inconsistent across these environments.

Utilising the REC_t assay Sicot 53 had consistently lower conductivities than Sicala 45 when leaf material was subjected to high temperature stress in water baths, in the growth cabinet and field. When temperature differences were generated with water baths in the laboratory, REC_t of field-grown leaf material increased with exposure to increasing temperatures and the rate of change was cultivar specific (Figure 6-1). Thermally dependent and sigmoidal increases in membrane leakage have been reported for sorghum (Sullivan 1971) and cowpea (Ismail and Hall 1999). The temperature at which 50 % leakage (T_{50}) occurred was 5.5 ^{o}C lower for Sicala 45 compared to Sicot 53 (Figure 6-2) thereby indicating that Sicot 53 has a relatively higher level of heat tolerance. Cultivar specific differences in membrane leakage based on laboratory imposition of high temperature stress have been reported cotton exposed to cold (McDowell *et al.* 2007) and heat (Ashraf *et al.* 1994; Bibi *et al.* 2008) stress but have not used the T_{50} calculation based on incubation temperature to quantify these cultivar differences effectively.

Similarly, REC_t increased for Sicala 45 leaf material incubated at high (42 ^{o}C) temperatures in the growth cabinet for a 2 or 3 d incubation period whereas the REC_t of Sicot 53 leaf material remained relatively constant (Figure 6-6). Cultivar differences in

REC_t have been determined for cotton (Bibi *et al.* 2008) and cowpea (Ismail and Hall 1999) leaf material under high air temperatures in the growth cabinet.

In attempting to improve the resolution of the REC_t assay determination of relative cellular injury (RCI_t) using temperature controlled water baths, cultivar differences in membrane integrity occurred at 3 d growth cabinet incubation time (Figure 6-6). Cultivar differences for RCI_t have been determined for cotton (Ashraf *et al.* 1994; Rahman *et al.* 2004), *Brassica sp.* (Hossain *et al.* 1995) and Kentucky bluegrass (Marcum 1998).

The REC_t assay was also conducted on material subjected to high temperature stress in the field to assess the resolution of the assay in identifying heat tolerance under field conditions. Exposure of leaf material to *in-situ* high temperature stress under tents increased electrolyte leakage from field grown leaf material (Figure 6-3). However, analysis of individual seasons indicated that REC_t was only increased under the tents in seasons 1 (2006) and 3 (2007) in Narrabri and electrolyte leakage was higher for Sicala 45 compared with Sicot 53 in season 3 only (Figure 6-4). Cultivar specificity for membrane integrity under high temperature stress in the field has been described (Ismail and Hall 1999; Rahman *et al.* 2004) but not under *in-situ* high temperature stress or in the absence of supplementary high temperature treatment in the water bath. Furthermore, temperature treatment and cultivar differences were not detected for RCI_t (Table 6-3), suggesting that this method was not as sensitive as REC_t in this study.

When REC_t of leaf material grown under ambient (control) and tent field conditions was compared to yield, a strong negative relationship was determined between REC_t and yield was determined only for season 1 (Figure 6-5). A relationship exists between REC_t and yield in wheat (Blum and Ebercon 1981), beans (Schaff *et al.* 1987), sorghum (Sullivan 1971) and cotton (Rahman *et al.* 2004) but only occurs under stressed conditions (Blum

and Ebercon 1981; Chen *et al.* 1982; Rahman *et al.* 2004). This is the first study to show a relationship between REC_t and yield under *in-situ* high temperature stress in the field as well as in the absence of supplementary heat stress in the laboratory.

No relationship between REC_t and yield was found in season 3 (Figure 6-5) which is similar to work on wheat (Shanahan *et al.* 1990) and soybeans (Martineau *et al.* 1979a). This suggests that the 7 h maximum exposure of plants to extreme high temperature (45 ^oC) stress under the tents in season 3 (Table 3-6) may not have been sufficiently severe to induce changes in cell membrane permeability or that whole plant compensation to short term high temperature stress was sufficient to permit a recovery period during mild mornings or evenings, thus contributing to acquired thermotolerance (Larkindale *et al.* 2005). This may be attributed to whole plant compensatory mechanisms such as mobilisation of protective heat shock proteins (Burke *et al.* 1985) or increased capacity for heat dissipation through transpiration (Taiz and Zeiger 2006) thus contributing to acquired thermotolerance (Larkindale *et al.* 2005).

Unlike the REC_t assay, cultivar differences for enzyme viability were not consistent across environments. Sicot 53 showed relatively high enzyme viability under high temperatures in the water bath whereas Sicala 45 had higher enzyme viability under high temperature stress and no cultivar differences were found under tents in the field.

Dissimilar to REC_t, Abs_t decreased with exposure to increasing temperatures in the water bath (Figure 6-1). Decreased enzyme viability in response to increasing exposure to high temperatures has been reported for cotton root tissue (McMichael and Burke 1994) and correlated to electrolyte leakage and membrane integrity (Schaff *et al.* 1987). Sicot 53 had a higher capacity for enzyme function under mild heat stress and Abs_t was 57 % higher at 35 ^oC compared with Sicala 45 (Figure 6-1). Cultivar specificity for enzyme viability

using the Abs_t assay has been reported in cotton for prolonged exposure to simultaneous water deficit and heat stress (de Ronde and van der Mescht 1997) but not heat stress as an individual entity. However, the success in enzyme viability tests for detecting high temperature tolerance in wheat (Porter *et al.* 1995) and *Phaseolus sp.* (Schaff *et al.* 1987) and cold stress in cotton (McDowell *et al.* 2007) confer potential for development of this method for identifying heat tolerance in cotton cultivars.

Genotypic differences in enzyme viability were also determined for plants grown under high temperature regimes in the growth cabinet which was similar to other research (Porter *et al.* 1995; Schaff *et al.* 1987). However, under high temperatures (42 ^{o}C) in the growth cabinet, Sicala 45 had higher absorbance and subsequently, higher $AHTT_t$ than Sicot 53 (Figure 6-7), thus suggesting higher thermotolerance. This is the reverse of findings for REC_t results indicating that Sicot 53 has a higher level of thermotolerance compared with Sicala 45. The inability of Sicot 53 to maintain enzyme viability under high temperature stress in the growth cabinet may indicate the importance of an acclimation period for the development of superior stress tolerance by increased respiratory capacity and continuation of ATP production for plant growth and development under field conditions (cf. Atkin and Tjoelker 2003). Acclimation to high temperatures is evident in the growth cabinet study, as the acquired high temperature tolerance of Sicala 45 and Sicot 53 increased with increasing exposure (days) to high temperature stress. Furthermore, acclimation to heat stress in the field is often complicated by simultaneous exposure to radiation and drought stress, thus influencing cultivar rankings for stress tolerance (de Ronde and van der Mescht 1997).

Temperature treatment and cultivar differences for enzyme viability were not detected using the Abs_t or AHTT assays for field grown leaf material (Table 6-3). Although

genotypic specificity has been widely reported for enzyme viability under high temperature stress in the water bath or growth cabinet (de Ronde and van der Mescht 1997; Porter *et al.* 1995; Schaff *et al.* 1987), no reports have evaluated use of the Abs_t or AHTT assays for heat tolerance determination under field conditions. Furthermore, Abs_t did not correlate with yield under field conditions which is consistent with similar work on glasshouse grown beans (Schaff *et al.* 1987).

Overall, the REC_t assay generated sufficient resolution to identify consistent differences in genotypic heat tolerance in response to high temperature stress. The REC_t assay has consistently shown that Sicot 53 is relatively more thermotolerant than Sicala 45. This trend is evident across a range of environments and water bath incubation temperatures and is consistent with data from field-based and growth cabinet experiments. This indicates that laboratory and growth cabinet based screening experiments may be employed to identify potential thermotolerance of cotton cultivars in the field. There is also a correlation between relative electrical conductivity and yield under hot field conditions (season 1). The reliability of this assay indicates potential for use as a screening tool for thermotolerance in the field. Hence, this assay may be employed to screen a large number of cotton cultivars for thermotolerance, provided that an adequate stress is used but this temperature is sufficiently low to distinguish between cultivars.

Conversely, no consistent effects of temperature treatment or cultivar were evident for enzyme viability under high temperature stress using the Abs_t or AHTT assays. This indicates that the use of laboratory or growth cabinet screening programs for thermotolerance determination based on these assays should be approached with trepidation and requires substantial field validation before recommendations may be proposed for superior stress tolerant cultivars. Further research is required to develop this

assay as a screening tool for identification of in-field heat tolerance and the resolution of this method may be improved by incorporation of a larger number of genotypes and further validation of the assay under a greater range of field sites and over seasons. Alternative methods of generation of in-field heat stress may also aid in increasing the sensitivity of this method and approaches such as infra-red heat may be explored to deliver a constant stress (Nijs *et al.* 1996).

The specific nature of single enzyme assays for heat tolerance determination may provide insufficient resolution for identification of subtle genotypic differences in response to high temperature stress and hence, a holistic approach may be more suitability for heat tolerance determination. Broad-spectrum screening assays such as REC_t identify the symptoms of a range of biochemical and physical changes in the leaf tissue and hence, the decreased specificity of this assay may strengthen the resolution cultivar discrimination for heat tolerance. Conversely, identification of the underlying processes contribution to heat tolerance using gene profiling may highlight candidate genes for further development.

6.5 **Conclusion**

The REC_t assay was able to identify Sicot 53 as relatively thermotolerant compared with Sicala 45 whereas the Abs_t assay was unable to determine cultivar resolution under high temperature stress in laboratory, growth cabinet and field environments consistently. Hence, the REC_t assay has the most potential for development of a rapid and reliable assay for heat tolerance identification. However, inconsistencies in these assays highlight the importance of a holistic approach to stress tolerance determination. Assays for heat tolerance determination should be used in conjunction with gene-level measurements to determine the underlying basis of this tolerance and whole plant and leaf level measurements to determine whether the data from these assays are applicable to local

field-based production systems. These cultivars can then be incorporated into current breeding programs to ensure that cotton yields are maintained under high temperature stress.

Chapter 7 Screening for Cultivar Specific Thermotolerance at the Gene Level

7.1 Introduction

Molecular techniques provide insight as to the genetic and biochemical basis of plant functionality. Molecular tools such as microarray and quantitative real time polymerase chain reaction (qRT-PCR) can be used to identify the genes involved in both abiotic and biotic stresses.

DNA microarray is a powerful tool for surveying the expression patterns of thousands of genes simultaneously. This enables rapid determination of differential gene expression between two RNA populations, thus providing a global and integrated analysis of biological processes in response to stress. Quantitative RT-PCR may be used for time course validation of gene expression under high temperature stress. Determination of cultivar specificity for specific genes or gene families involved in the heat stress response may provide a platform for directed breeding for increased crop stress tolerance (Ishitani *et al.* 2004; Zhang and Blumwald 2001).

High temperature stress and acclimation to high temperatures induces a cascade of differential gene regulation in plants (Busch *et al.* 2005) and molecular techniques can be employed to determine the response of individual genes or general pathways to abiotic stress (Busch *et al.* 2005; Klok *et al.* 2002) particularly when no obvious phenotype is attributed to the stress response (Kennedy and Wilson 2004). While several genes contributing to thermotolerance have been identified in *Arabidopsis* (Alia *et al.* 1998; Gao *et al.* 2008; Larkindale *et al.* 2005; Lee *et al.* 1995; Lohmann *et al.* 2004; Schramm *et al.* 2006), there is little specific research on gene functionality in cotton (Dowd *et al.* 2004) under high temperature stress.

Interactions between plants and heat stress have been well characterised on a whole plant, physiological and biochemical level. Heat shock proteins play a primary role in the heat stress response and contribution to acquired thermotolerance (Busch *et al.* 2005), however this response is often insufficient to protect plant functionality completely. Inhibition of cell metabolism may limit physiological processes such as photosynthesis and respiration completely and hence, energy availability is unable to meet demand for growth and development under such conditions (Kant *et al.* 2008). The underlying genetic basis of these processes however, remains largely unknown.

Molecular studies have shown that regulation of genes associated with Rubisco activity underlies photosynthetic performance (DeRidder and Salvucci 2007). The activation state of Rubisco is the primary limitation to photosynthesis in cotton under stress (Salvucci and Crafts-Brandner 2004a) and is exacerbated by destabilisation of Rubisco activase, a chaperone for Rubisco, thus reducing yield potential under high temperature stress (DeRidder and Salvucci 2007; Salvucci and Crafts-Brandner 2004c). Breeding programs targeting the genotype specific protection of Rubisco from deactivation and increasing the thermal stability of Rubisco activase under prolonged periods of stress may achieve superior thermotolerance and hence yield under field conditions in hot seasons (Salvucci 2008).

An experiment was conducted to determine the effectiveness of molecular techniques for the determination of cultivar differences in gene expression under high temperature stress in the growth cabinet. The aim of this experiment was to identify cultivar specific thermotolerance using microarray technology and to quantify these differences using real-time polymerase chain reaction time course analysis under a controlled environment (growth cabinet). The hypothesis tested was that there are cultivar specific differences in gene expression under high temperature stress.

7.2 **Materials and methods**

7.2.1 **Temperature treatments**

Plants were established in the glasshouse and transferred to the growth cabinet for temperature treatments as previously described in Chapter 3.

7.2.2 **Plant sampling**

The third youngest fully expanded leaf of plants at the first square physiological age was sampled for all experiments. Four leaf tissue samples per treatment were collected at 0, 0.5, 1, 2, 3, 4 and 7 h after initiation of the high temperature stress. Time 0 samples were collected at 3 h into the photoperiod. For RNA preparations, whole leaves were excised at the junction of the lamina and petiole and immediately snap frozen in liquid nitrogen. Leaves were stored at -80 ^{o}C. To lyse cells and liberate ribonucleic acid (RNA), leaves were ground to a fine powder in liquid nitrogen in a pre-frozen mortar and pestle to maintain RNA integrity and a 0.1 g sub-sample was taken for small-scale RNA extraction in accordance with the protocol developed by Wan and Wilkins (1994).

7.2.3 **Quantitative real time-PCR**

The relative expression of the gene encoding 1,5-biphosphate carboxylase/oxygenase activase (Rubisco) alpha2 (GhRCAα2) was quantified by qRT-PCR analysis over a 7 h period after the imposition of high temperature stress in the growth cabinet. This particular gene was chosen for real-time analysis as it has been described for cotton as a circadian, yet heat responsive gene (DeRidder and Salvucci 2007) but has not been determined for cultivar specificity. A difference in Rubisco activase expression between the optimal (32 ^{o}C) and high (42 ^{o}C) temperature growth cabinet is interpreted as the occurrence of heat stress.

Microarray analysis is relatively expensive and provides only a point in time determination of gene expression (Kennedy and Wilson 2004). Therefore, qRT-PCR was used to determine the optimal time point for comprehensive gene profiling using microarray analysis. This point was determined as the time point for which a heat stress by cultivar interaction was at a maximum. The process for mRNA extraction and gene expression determination using qRT-PCR is described below.

Tissue homogenisation

Cotton plants typically have a high level of secondary metabolites, phenolics, terpenes and polysaccharides that may either have similar properties to, or interfere with, nucleic acids, thereby severely limiting potential recovery of high quality RNA. Hence, total cellular RNA was selectively precipitated using a small scale, hot borate method specifically designed for extraction from cotton tissue to reduce interference from these compounds during the homogenization stage (Wan and Wilkins 1994). Although phenols are largely precipitated by polyvinylpryolidone (PVP) in the borate buffer, the alkaline conditions in conjunction with the presence of borate and dithiothreitol protect RNA from residual polyphenolic interference. For RNA isolation, all buffers and chemical solutions were diluted with 0.1% (v/v) diethylpyrocarbonate (DEPC) in distilled water and autoclaved before use to inactivate both protein and non-protein based ribonucleases. To supplement grinding, detergents (SDS and sodium deoxycholate) were included in the borate buffer solution to liberate cytoplasm and RNA through cell lysis and dissolution of membranes as well as broad scale protein denaturation.

To aid the recovery of high quality RNA, 10 μL of proteinase K (25 mg/mL) and 10 μL dithiothreitol (DTT) (154 mg/mL) were added to 1 mL of preheated (80 ^{o}C) borate buffer solution (Table 7-1) in a 2.0 mL microcentrifuge tube and mixed for 6 seconds

on a vortex mixer. The proteinase K facilitated degradation of endogenous enzymes during homogenisation, thereby limiting the activity of ribonucleases on RNA and subsequent formation of protein-phenolic compounds. DTT was added to reduce oxidation of phenolic compounds and inhibit ribonucleases (Wilkins and Smart 1996).

Table 7-1 Borate buffer solution

Borate buffer chemicals	Concentration	Manufacturer	Chemical formula
Borax: Disodium tetraborate	200 mM	Chem-supply Pty Ltd, Gillman, SA, Australia	$Na_2B_4O_7.10H_2O$
Ethylene glycol-bix (2-aminoethyl-ester)-N,N,N',N'-tetraacetic acid (EGTA)	30 mM	Sigma, St Louis, MO, USA	$C_{14}H_{24}N_2O_{10}$
Sodium dodecyl sulfate (SDS)	1% (w/v)	Amresco, Solon, Ohio, USA	$C_{12}H_{25}NaO_4S$
Sodium deoxycholate	1% (w/v)	Sigma Aldrich	$C_{24}H_{39}NaO_4$
Polyvinyl-pyrrolidone (PVP 40 000)	2% (w/v)	Sigma	
Diethyl pyrocarbonate (DEPC)	0.1% (v/v)		$C_6H_{10}O_5$

The hot buffer solution was transferred into the 2 mL microcentrifuge tube containing 0.1 g frozen cotton leaf tissue and vortex mixed until the tissue was held in suspension. The tubes were stored at 42 ^{o}C in a water bath until all samples were prepared and then kept in mild agitation for 90 min at 42 ^{o}C on a G24 environmental incubator shaker (New Brunswick Scientific Co Inc, New Brunswick, NJ, USA) to facilitate carbohydrate removal and inhibition of polyphenolic interference (Wan and Wilkins 1994).

RNA isolation

To precipitate proteins, 200 µL cold 1M potassium chloride was added to the homogenised samples and kept for 1 h on ice. The tubes were centrifuged at 13000 *g* for 20 min at 4 ^{o}C in a 5415D centrifuge (Eppendorf). The supernatant was extracted and transferred to a new 2 mL microcentrifuge tube and RNA was precipitated

overnight (approximately 12 h) in an equivalent volume of 4M lithium chloride at 4 ^{o}C for selective precipitation of RNA.

The sample tubes were centrifuged at 13000 *g* for 20 min in the cold room and the pellet was washed 3 times with 1 mL cold 2M lithium chloride until the supernatant was clear. The pellet was resuspended with 450 µL DEPC water. Polysaccharides, residual proteins and pigments were precipitated with 50 µL cold 2M potassium acetate (pH 5.5) for 5 min on ice, thereby removing salt-insoluble detergents and polysaccharides. The tubes were centrifuged at 13000 *g* for 20 min and the supernatant was transferred to a new 2 mL microcentrifuge tube. Nucleic acids were precipitated for 10 min on ice in 1500 µL 95% ethanol. The tubes were centrifuged at 13000 *g*, the supernatant discarded and the pellet left to air dry for 5 min. The pellet was re-suspended in 100 µL of 0.1% DEPC water and stored at -20 ^{o}C for further analysis.

RNA concentration and quality analysis

RNA concentration and protein contamination was measured on a on a Bio-lab ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) at wavelengths of 260, 280 and 230 nm against a 95% ethanol blank. For all samples, the 260/230 nm and 280/230 nm ratios were above 2.0, indicating a minimal and acceptable level of protein and organics contamination (Wilkins and Smart 1996).

The products of the total cellular RNA extraction were subjected to electrophoresis in a horizontal 1% agarose gel in a 50% tris-acetate (TAE) / distilled water buffer at 160 volts for 20 minutes. For visualisation of migration, 1 µL loading dye was added to 5 µL RNA extraction solutions and set in a 24 well comb. To fluoresce RNA under ultra-violet light, ethidium bromide (0.00001%) was added to the agarose gel. RNA

migration through the gel was compared to a Gene Ruler TM 1kb DNA ladder (0.5 μg/μL) for effective comparison between samples.

A sub-sample of extractions for microarray were denatured to remove any secondary structure and further analysed for quality on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA). For this analysis, a 1 μL RNA samples (200 to 500 ng RNA/μL) was loaded onto a pressurised RNA nano chip, containing 5 mL RNA 6000 nano marker and run against a comparative volume of ladder solution. A RNA integrity number (RIN) score of 5.5 to 8.5 was observed for all tissue samples, indicating that all samples were of sufficient quality for microarray processing.

Reverse transcription of RNA

Ribulose 1,5-biphosphate carboxylase/oxygenase activase alpha2 (G*h*RCAα2) (DeRidder and Salvucci 2007) cDNA was obtained using the SuperScript III reverse transcriptase system as described by the manufacturer (Invitrogen Life Technologies). RNA 5 μg was diluted into 20 μL DEPC water in a 2.0 mL click cap microtube. Oligo-dT 3 μL (2 μg/μl, 23 mer dT with C/G/A at 3 end) was added and the tubes were vortexed and centrifuged in a butterfly centrifuge. To denature the RNA and facilitate binding to the oligo-dT, the sample tubes were incubated at 70 ^{o}C for 10 min in a PC-960C cooled thermal cycler (Corbett Research), and then transferred immediately to ice to prevent re-annealing. A reverse transcriptase master mix solution was prepared (Table 7-2) and 17 μL was added to each microtube (Table 7-2). The tubes were incubated at 42 ^{o}C for 1 h in a cooled thermal cycler. The samples were stored at -20 ^{o}C until analysis.

Table 7-2 Reverse transcriptase solution

Master mix solution	Vol / PCR tube (μL)
5 x 1st strand synthesis buffer	8
0.1M DTT	4
Deoxyribonucleotide triphosphate (dNTP) mix (containing 5nM each of deoxyadenosine triphosphate (dATP), deoxycytodine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP))	4
Superscript TM II reverse transcriptase enzyme (200 U/ml)	1

Quantitative Real-time Polymerase Chain Reaction

A gene expression time series was developed using qRT-PCR. The qRT-PCR was performed on each sample with 3 technical replicates for both a 10 μM β-tubulin forward (5'-GAACATGATGTGGTGCTGC) and reverse (5'-AGCTGTGAACTGCTCACTC) primer as a control to account for constitutive RNA expression in the leaf), and a G*h*RCAα1 forward (5'-TGACGAAGTGAGGAAATGGAT) and reverse (5'-TCAGCAACAAGCATGTTTCCA) gene of interest primer (Sigma-Genosys) (DeRidder and Salvucci 2007). For each sample, 15 μL cDNA (4 ng/μL) was diluted in 450 μL distilled water (autoclaved) and transferred to a 0.1 mL DNA and RNA free tube (Corbett Research) containing 15 μL buffer solution (Table 7-3). All samples were run in triplicate for 40 cycles which included a denaturing phase (95 ^{o}C held for 15 secs), an annealing phase (60 ^{o}C held for 15 secs) and an elongation phase (72 ^{o}C held for 20 secs) in a Rotor-Gene 2000 real-time cycler (Corbett Research). For all measurements, expression of G*h*RCAα2 is relative to expression of the β-tubulin housekeeping gene (control), which should only be dependent on the amount of cDNA added to each reaction. Relative levels of gene expression for G*h*RCAα2 and β-tubulin were determined using comparative quantification to a standard solution.

Table 7-3 Buffer solution for qRT-PCR

Reagent	Volume (μL)
10 x polymerase chain reaction buffer	2
50 nM magnesium chloride	1.2
5 mM deoxyribonucleotide triphosphate mix	0.8
10 x SYBR ® green (in DMSO)	1
10 μM forward primer	1
10 μM reverse primer	1
Platinum® Taq DNA polymerase	0.08
Distilled H_2O (autoclaved)	7.92

7.2.4 **Microarray**

Cotton tissues sampled at 1 h after the onset of high temperature stress and at a comparative time in the optimal growth cabinet was analysed for broad-spectrum gene expression. Leaf tissue was stored at -80 ^{o}C and mRNA extraction, quality testing and cDNA generation was performed according to the above procedure. Array processing was performed by The Walter and Eliza Hall Institute of Medical Research at the Australian Genome Research Facility (AGRF), Parkville, Victoria, Australia. RNA quality was determined on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA, USA). Relative expression for 24,132 genes was determined on an Affymetrix GeneChip system with scanner 3000 7G and autoloader.

7.2.5 **Data analysis**

Quantitative RT-PCR

Two-way analysis of variance (ANOVA) (cultivar*cabinet time) was conducted for a time course analysis of relative expression of G*h*RCAα2 for cultivars Sicot 53 and Sicala 45 at various incubation times in the growth cabinet. Analysis was run separately for plants grown under optimal (32 ^{o}C, control) and high (42 ^{o}C) temperatures in the growth cabinet.

Two-way ANOVA (cultivar*cabinet temperature) was conducted to determine the relative expression of G*h*RCAα2 of Sicot 53 and Sicala 45 plants grown under optimal (32 ^{o}C) or high (42 ^{o}C) temperatures in the growth cabinet for each individual cabinet incubation time point.

Microarray

For all heat stress comparisons, relative expression refers to the expression of genes under high (42 ^{o}C) temperatures relative to expression under optimal (32 ^{o}C) temperatures in the growth cabinet, pooled for cultivars Sicot 53 and Sicala 45. An adjusted P value was generated to determine genes that were up or down regulated under high temperatures in the growth cabinet at $P<0.05$ (Smyth 2005).

For all cultivar comparisons, the expression of genes for cultivar Sicala 45 or Sicot 53 under high (42 ^{o}C) temperatures compared with expression under optimal (32 ^{o}C) temperatures in the growth cabinet for $P<0.05$ (Smyth 2005). Hence, relative expression refers to the expression of genes under high (42 ^{o}C) temperatures relative to expression under optimal (32 ^{o}C) temperatures in the growth cabinet for either Sicot 53 or Sicala 45.

For visual representation of gene expression using MapMan software, a value of 1 on a $\log_2$ scale (2-fold) induction/repression limit was used to identify genes that were significantly induced or repressed by the temperature treatments at $P<0.05$. All figures are presented on a $\log_2$ scale, where the red colour represents a decrease and the blue colour represents an increase in relative gene expression between 2 and -2 on a $\log_2$ scale. This represents a 4-fold difference between the temperature treatments or cultivars on a normal scale.

Transcript expression was graphed using MapMan 2.2.0 (Max-Planck-Institute for Molecular Plant Physiology). Due to limited information availability for the functionality of cotton genes, gene descriptions of *Arabidopsis thaliana* (The Arabidopsis Information Resource 2008) were used to supplement results for *Gossypium hirsutum* expression. For all *Arabidopsis thaliana* gene comparisons, the sequence of identical base pairs had a high degree of similarity with *Gossypium* genes, indicated by an E-value of <0.001 using a blast nucleotide comparison. This assumes that a similar base pair configuration indicates similar gene function in different plant species.

7.3 **Results**

7.3.1 **Rubisco activase expression using qRT-PCR**

The relative expression of a gene associated with Rubisco activase (G*h*RCAα2) was determined for cultivars Sicot 53 and Sicala 45 under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet using qRT-PCR. The expression of G*h*RCAα2 followed a diurnal decrease (P<0.001) (Table 7-4) under optimal (32 ^{o}C) and high (42 ^{o}C) temperature regimes (Figure 7-1). The maximum steady-state transcription levels occurred at 0.5 h after the onset of the treatment period and subsequently decreased to a minimum within 3 h of initiation of the photoperiod (Figure 7-1).

At optimal temperatures the relative expression of G*h*RCAα2 was higher (P=0.015) for Sicot 53 compared with Sicala 45 (Table 7-4) across the entire time course. Maximum differentiation between temperature treatments and cultivars occurred at 1.0 h after initiation of the treatment period (Figure 7-1). At this time point, the relative expression of G*h*RCAα2 was higher (P=0.002) for Sicot 53 compared with Sicala 45 under high (42 ^{o}C) temperature regimes (Figure 7-1). Subsequently, samples taken from both cultivars at 1.0 h into the treatment period were taken for further

microarray analysis, on the basis of maximum cultivar differentiation under the high temperature treatment.

Table 7-4 Probability of incubation time and cultivar treatment main effects and incubation time by cultivar interactions for relative expression of G*h*RCA*α*2 for cotton cultivars Sicot 53 and Sicala 45 grown under optimal (32 ^{o}C) and high (42 ^{o}C) temperature regimes in the growth cabinet at Narrabri, where n.s. represents not significant F test P values for *P*=0.05.

Growth cabinet incubation temperature (^{o}C)	Incubation time	Cultivar	Cultivar * Incubation time
32	<0.001	0.015	n.s.
42	<0.001	n.s	<0.001

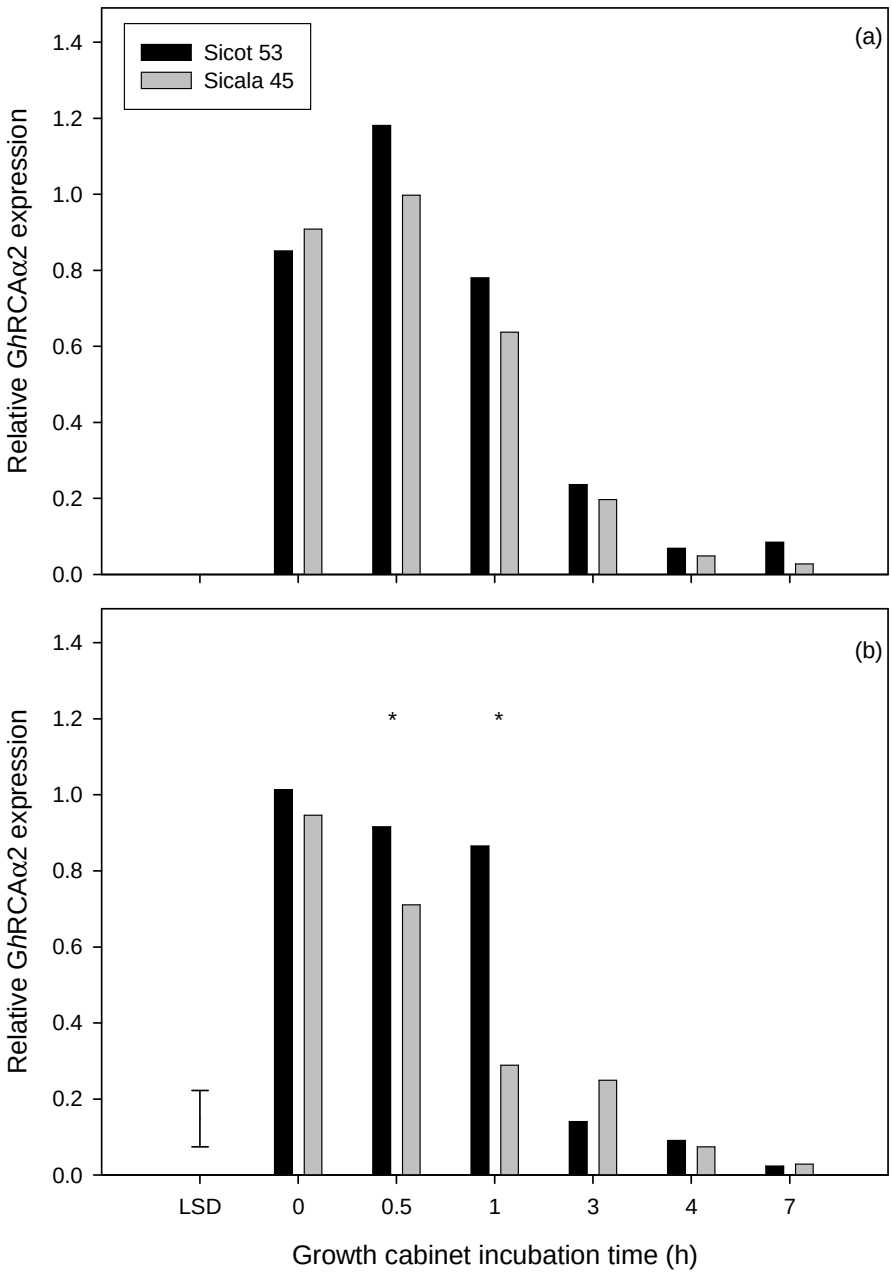

Figure 7-1 Mean relative expression of G*h*RCA*α*2 for Sicot 53 and Sicala 45 grown in the growth cabinet at temperatures of (a) 32 ^{o}C or (b) 42 ^{o}C during the photoperiod. The (b) asterisks (*) represent growth cabinet incubation times for which the difference between the cultivar means exceeds the l.s.d. The (b) vertical line represents the l.s.d. for growth cabinet incubation time by cultivar interaction at *P*<0.05.

7.3.2 **Microarray**

Gene expression under high temperature stress

The relative expression of 24133 genes was determined for cultivars Sicot 53 and Sicala 45 grown under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth

cabinet using the Affymetrix gene chip. Individual genes were then assigned a gene group and sub-group according to function (Table 7-5). Data presented are pooled for cultivars Sicot 53 and Sicala 45 and indicate the relative expression of a particular gene under high (42 ^{o}C) temperatures and compared with expression at optimal (32 ^{o}C) temperatures.

Table 7-5 Gene groups comprising MapMan determined gene sub-groups that are up- or down-regulated under high (42 ^{o}C) and compared with optimal (32 ^{o}C) temperatures in the growth cabinet

Gene group	Gene sub-group	No. genes
Electron flow and ATP production		90
	Photosynthesis	15
	Glycolysis	15
	Oxidative pentose phosphate pathway	4
	TCA cycle/ organic acid transformations	22
	Mitochondrial electron transport/ ATP synthesis	5
	Redox regulation	25
	Chloride metabolism	4
Carbohydrate metabolism		74
	Major carbohydrate metabolism	15
	Minor carbohydrate metabolism	19
	Cell wall	40
Lipid Metabolism		52
Protein metabolism		612
	RNA	189
	DNA	59
	Nucleotide metabolism	19
	Nitrogen metabolism	3
	Amino acid metabolism	50
	Sulfur metabolism	8
	Protein	284
Secondary metabolism		96
	Secondary metabolism	30
	Hormone metabolism	63
	Polyamine metabolism	2
Stress		98
	Biotic stress	12
	Heat stress	57
	Light stress	1
	Cold stress	3
	Drought stress	12
	Touch/wounding stress	2
	Unspecified stress	11
Signaling & transport		186
Cell & Development		128
Not assigned/Miscellaneous		652

High (42 ^{o}C) temperatures induced a 2-fold up-regulation of 325 individual genes and down-regulation of 249 individual genes, compared to optimal (32 ^{o}C) temperatures. Although the majority of these genes had no assigned function (33%), protein metabolism (31%) comprised the largest proportion of gene groups affected by high (42 ^{o}C) temperatures in the growth cabinet (Figure 7-2).

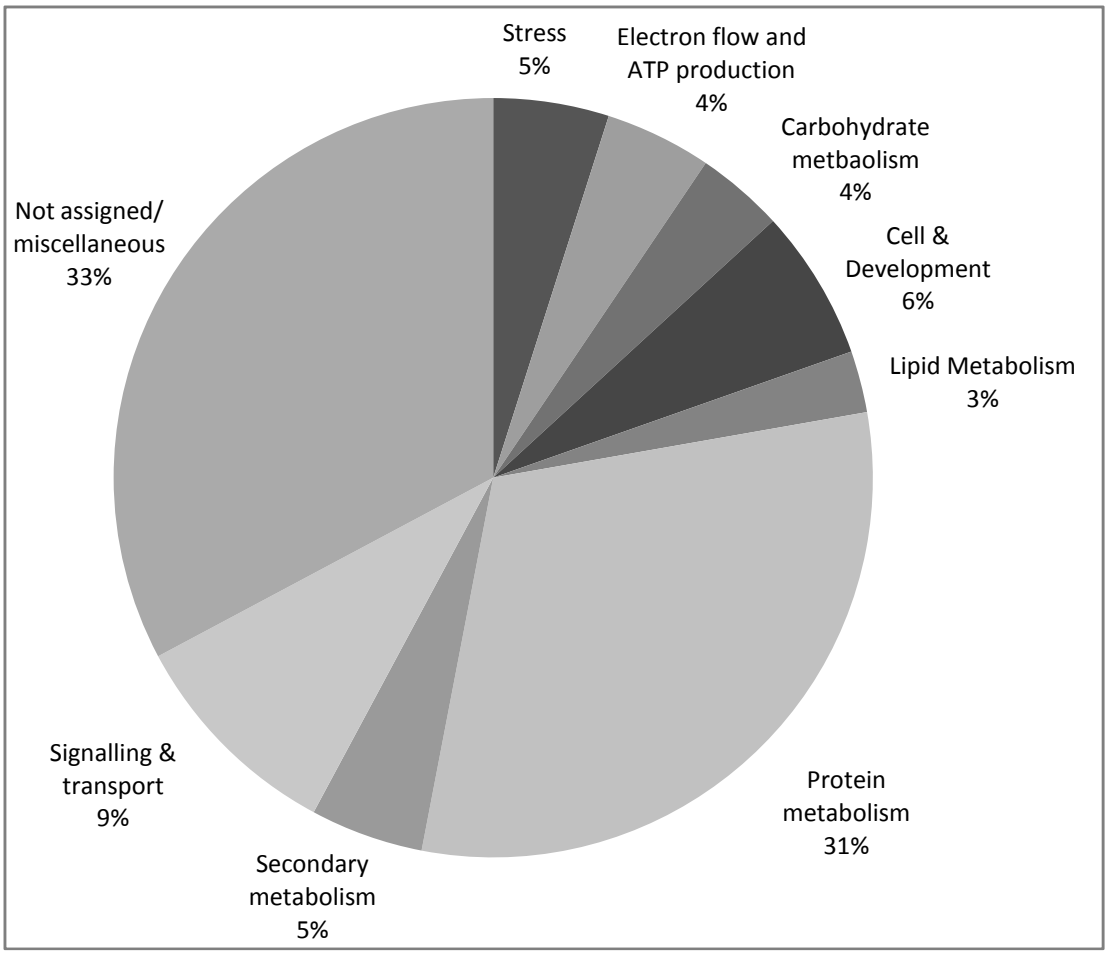

Figure 7-2 Gene groups that are up- or down-regulated under high (42 ^{o}C) and compared with optimal (32 ^{o}C) temperatures in the growth cabinet.

Stress genes contributed 5% of the overall heat response (Figure 7-2) and a summary of the genes related to abiotic and biotic stress are summarised in Figure 7-3. Heat stress contributed about 61% of total stress genes affected by the treatment (Figure 7-3), primarily in the form of genes encoding heat shock proteins (Table 7-6), thus

indicating the mobilisation of a heat stress response in the plant tissue. Several individual genes with assigned functions associated with cold, drought and salt stress were differentially expressed under high (42 ^{o}C) temperature treatment in the growth cabinet (Figure 7-3).

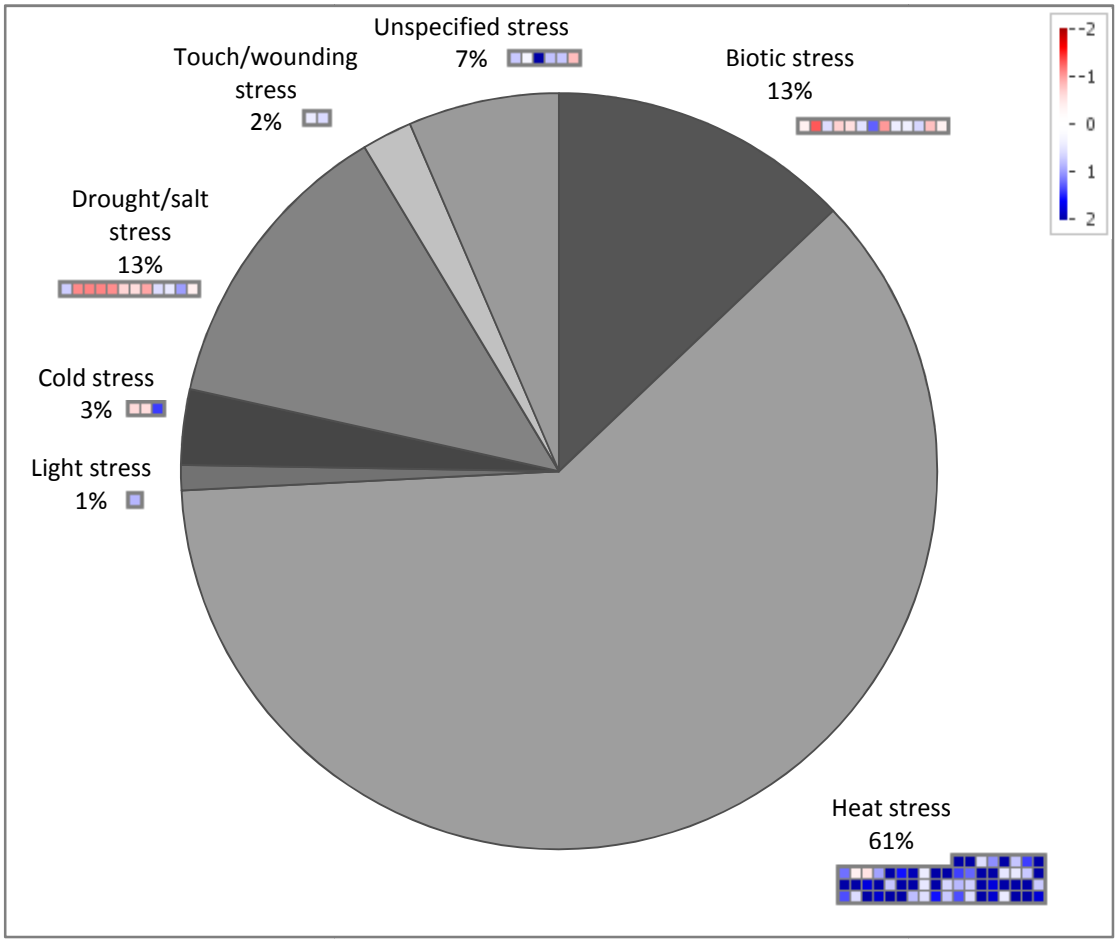

Figure 7-3 Distribution of biotic and abiotic stress genes, and relative expression of these genes for cultivars Sicot 53 and Sicala 45 grown under high (42 ^{o}C) compared with optimal (32 ^{o}C) temperatures in the growth cabinet. Red squares represent genes for which gene expression decreases, whilst blue squares represent genes for which expression is up-regulated on a $\log_2$ scale for plants grown under high (42 ^{o}C) temperatures compared with plants grown under optimal (32 ^{o}C) temperatures in the growth cabinet.

Table 7-6 Relative expression of selected stress genes up-regulated for cultivars Sicot 53 and Sicala 45 under high (42 °C) and optimal (32 °C) temperatures in the growth cabinet.

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Drought/salt stress	DT455898	1.643	1.33E-02	At4g16390.1	chloroplastic RNA-binding protein P67, putative	2.15E-140
Heat stress	CO070151	1.77	1.46E-02	At5g62020.1	heat shock transcription factor 6 (HSF6)	1.16E-75
Heat stress	CO125371	1.805	2.31E-02	At4g24280.1	heat shock protein cpHsc70-1	2.63E-107
Heat stress	AW186892	2.635	2.71E-03	At4g24190.1	shepherd (SHD)	1.14E-107
Heat stress	CA992849	2.956	1.28E-03	At3g44110.1	DnaJ protein AtJ3	1.54E-170
Heat stress	DT545357	3.428	3.47E-04	At4g25200.1	mitochondrion-localized small heat shock protein	4.99E-62
Heat stress	DW496991.1	4.792	6.07E-03	At1g54050.1	heat shock hsp20 protein family	2.77E-33
Heat stress	CO132723	4.901	1.25E-05	At4g11660.1	heat shock factor protein 7 (HSF7)	1.05E-57
Heat stress	DT050385	4.981	6.49E-06	At3g23990.1	chaperonin (CPN60/HSP60)	1.35E-10
Heat stress	DT456116	8.919	5.54E-04	At2g26150.1	heat shock transcription factor family	2.66E-72
Heat stress	DT467180	9.761	2.25E-05	At5g12020.1	class II heat shock protein	1.12E-35
Heat stress	DW513189.1	15.343	1.80E-04	At4g25200.1	mitochondrion-localized small heat shock protein	2.47E-59
Heat stress	DT049773	22.439	1.38E-05	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Heat stress	DW503063.1	28.87	1.17E-04	At5g52640.1	heat shock protein 81-1	2.42E-112
Heat stress	DR455451	30.145	2.24E-03	At4g27670.1	small heat shock protein, chloroplast precursor (HSP21)	8.71E-64
Heat stress	DW517704.1	35.33	1.84E-04	At4g10250.1	endomembrane-localized small heat shock protein	2.11E-15
Heat stress	CA992719	38.989	1.19E-04	At4g10250.1	endomembrane-localized small heat shock protein	3.92E-31
Heat stress	DW503697.1	83.99	7.78E-07	At1g07400.1	heat shock protein	1.40E-49
Light stress	DW505008.1	1.817	2.22E-02	At5g11580.1	expressed protein	8.90E-80

Changes in genes involved in metabolism

Overall metabolism was up-regulated following 1 h heat stress at 42 ^{o}C in the growth cabinet (Figure 7-4). Genes involved in the metabolism of starch and sucrose, lipids and amino acids and subsequently cell wall synthesis related genes were strongly down-regulated (Table 7-8). Genes involved in electron transport from the tricarboxylic acid (TCA) cycle and through the mitochondria were down-regulated (Table 7-8), whilst genes involved in Rubisco expression were up-regulated under heat stress (Table 7-7).

Several genes involved in mitochondrial electron transport were affected by high temperature stress in the growth cabinet (Figure 7-4). Although genes encoding transport proteins at complex I and II were induced, the majority of genes affected were down-regulated in response to heat stress (Table 7-8). Several genes involved in protein-mediated metabolite transport were down-regulated under heat stress (Table 7-8). Multiple genes encoding electron transfer from complex III to complex IV, via cytochrome C were down-regulated under high temperatures (Table 7-8). Genes associated with uncoupling proteins, responsible for the movement of H+ across the membrane were also down-regulated (Figure 7-4).

Multiple genes involved in the photosynthetic pathway were differentially regulated in response to high temperature stress in the growth cabinet (Figure 7-4). Several genes involved in the regulation and expression of Rubisco were strongly up-regulated (P<0.001) under high temperature stress in the growth cabinet as well as a gene involved in calcium ion binding (Table 7-7). A glyceraldehyde-3-phosphate dehydrogenase gene was down-regulated (P<0.001) under high temperature stress in the growth cabinet (Table 7-8).

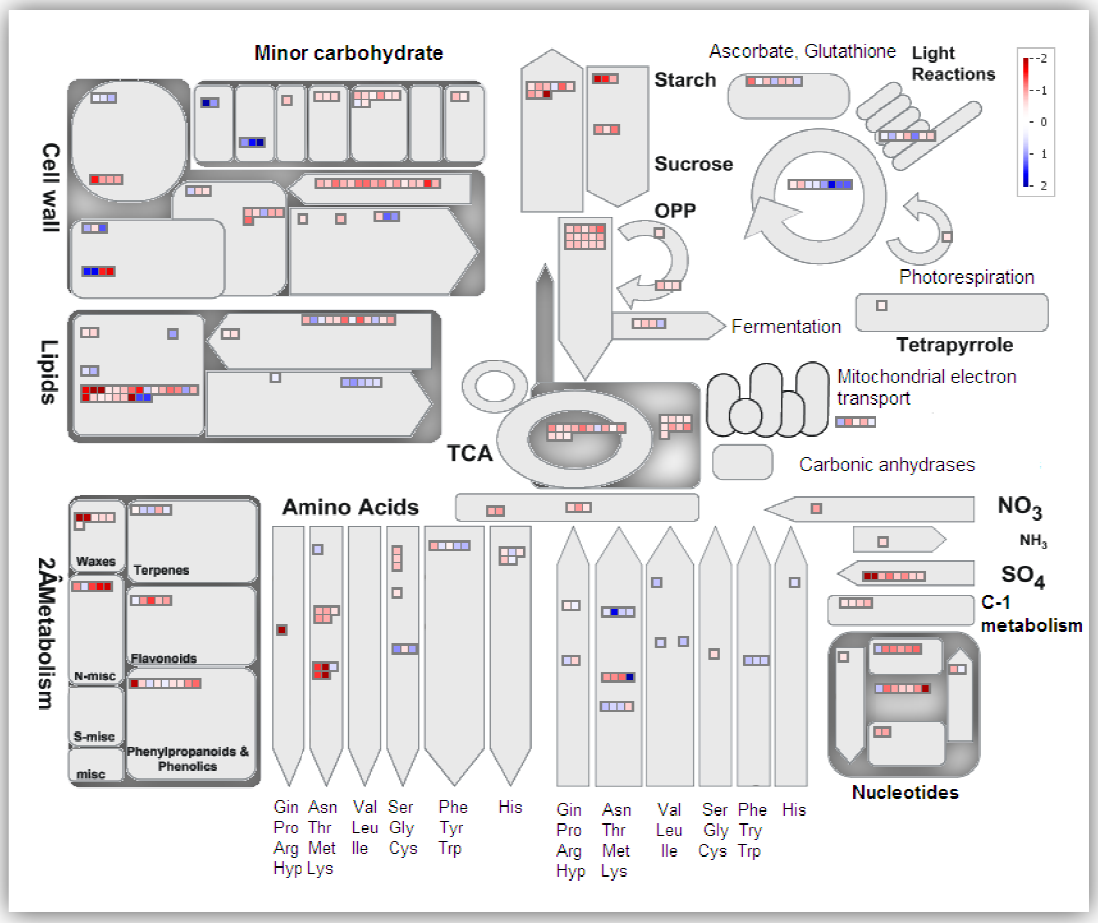

Figure 7-4 Relative expression of metabolism genes for cultivars Sicot 53 and Sicala 45 grown under optimal (32 °C) and high (42 °C) temperatures in the growth cabinet. Red squares represent genes for which gene expression decreased, whilst blue squares represent genes for which expression was up-regulated on a $\log_2$ scale for plants grown under high (42 °C) temperatures, compared with plants grown under optimal (32 °C) temperatures in the growth cabinet.

Table 7-7 Relative expression of metabolism genes involved in energy generation and transfer, up-regulated under high (42 °C) and compared with optimal (32 °C) temperatures in the growth cabinet for cultivars Sicot 53 and Sicala 45.

Representative public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
CO072814	2.449	3.50E-03	At2g28000.1	RuBisCO subunit binding-protein alpha subunit/60 kDa chaperonin alpha subunit	1.67E-154
CO091076	1.558	2.83E-02	At5g17400.1	mitochondrial ADP, ATP carrier protein	1.85E-34
CO121719	2.455	1.37E-02	At2g28000.1	RuBisCO subunit binding-protein alpha subunit/60 kDa chaperonin alpha subunit	3.75E-107
DN800322	2.237	1.20E-02	At1g06680.1	Oxygen-evolving enhancer protein 2; calcium ion binding	5.11E-85
DN800322	2.237	1.20E-02	At1g06680.1	photosystem II oxygen-evolving complex 23 (OEC23)	5.11E-85
DN817738	1.4	4.75E-02	At4g20130.1	expressed protein	4.62E-49
DR458096	2.003	4.73E-04	At2g28000.1	ATP binding protein binding	0.00
DT051416	1.599	2.29E-02	At1g07890.2	ascorbate peroxidase, putative (APX)	2.26E-101
DT456151	1.823	2.53E-02	At2g43400.1	electron transfer flavo protein ubiquinone oxidoreductase -related	5.68E-66
DV849478	3.338	2.27E-04	At2g28000.1	RuBisCO subunit binding-protein alpha subunit/60 kDa chaperonin alpha subunit	8.87E-23
DW226042.1	1.543	9.27E-03	At5g14590.1	isocitrate dehydrogenase [NADP+]	2.94E-33
DW497212.1	1.798	3.46E-02	At1g03600.1	photosystem II protein family	2.14E-43
DW508175.1	1.742	6.83E-03	At1g75270.1	dehydroascorbate reductase	1.75E-32
DW509246.1	1.241	4.12E-02	At1g63460.1	glutathione peroxidase	7.62E-70

Table 7-8 Relative expression of genes involved in energy generation and transfer, down-regulated under high (42 °C) and compared with optimal (32 °C) temperatures in the growth cabinet for cultivars Sicot 53 and Sicala 45.

Representative public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
AI729300	0.244	3.35E-03	At1g02190.1	CER1 protein	6.14E-67
AI730914	0.555	3.99E-02	At2g21250.1	mannose 6-phosphate reductase (NADPH-dependent)	7.52E-81
AI731438	0.475	1.89E-03	At5g58970.1	uncoupling protein (AtUCP2)	1.58E-101
BQ412199	0.631	9.94E-03	At5g19760.1	mitochondrial 2-oxoglutarate/malate translocator	2.42E-125
CA992949	0.546	1.08E-02	At2g22500.1	mitochondrial carrier protein family	3.32E-113
CO127818	0.481	3.02E-02	At5g14040.1	mitochondrial phosphate transporter	1.69E-105
DT047184	0.492	2.68E-02	At3g59480.1	fructokinase	1.84E-58
DT463008	0.197	1.52E-02	At3g22890.1	ATP sulfurylase -related	3.97E-37
DT463094	0.508	2.25E-02	At5g53460.1	glutamate synthase [NADH], chloroplast	2.05E-169
DV848944	0.344	1.68E-01	At1g42970.1	glyceraldehyde-3-phosphate dehydrogenase B subunit	0.00
DW233179.1	0.675	2.32E-02	At3g62650.1	expressed protein putative mitochondrial carrier protein	9.79E-25
DW493894.1	0.491	1.26E-02	At5g53580.1	aldo/keto reductase family	5.76E-113
DW496260.1	0.28	2.62E-04	At2g40835.1	4-alpha-glucanotransferase -related	1.08E-24
DW500449.1	0.639	6.91E-02	At4g10040.1	cytochrome c	9.94E-56
DW510782.1	0.565	2.31E-02	At4g34200.1	D-3-phosphoglycerate dehydrogenase (3-PGDH)	6.22E-54
DW512717.1	0.641	6.70E-02	At2g29990.1	NADH dehydrogenase family	2.88E-91

Cultivar differences for overall gene expression

The relative expression of genes for Sicot 53 or Sicala 45 under high (42 ^{o}C) temperatures, compared with optimal (32 ^{o}C) temperatures in the growth cabinet are summarised for gene sub-groups (Table 7-5) and are listed individually for Sicot 53 (Figure 7-5) and for Sicala 45 (Figure 7-5). The total number of genes that were differentially expressed under the two temperature regimes was higher for Sicala 45 compared with Sicot 53. A number of heat-associated stress genes were up-regulated in both cultivars under high temperature stress in the growth cabinet. These genes consisted of predominantly heat shock proteins and were higher in number for Sicala 45 compared with Sicot 53. Multiple genes involved in carbohydrate, lipid and secondary metabolism and transport, as well as genes involved in RNA, DNA and nucleotide synthesis were strongly down-regulated for Sicala 45 under heat stress. Although these genes may indirectly influence the photosynthetic and mitochondrial electron transport pathways, no transcripts directly involved with these pathways were cultivar specific in response to high temperature stress.

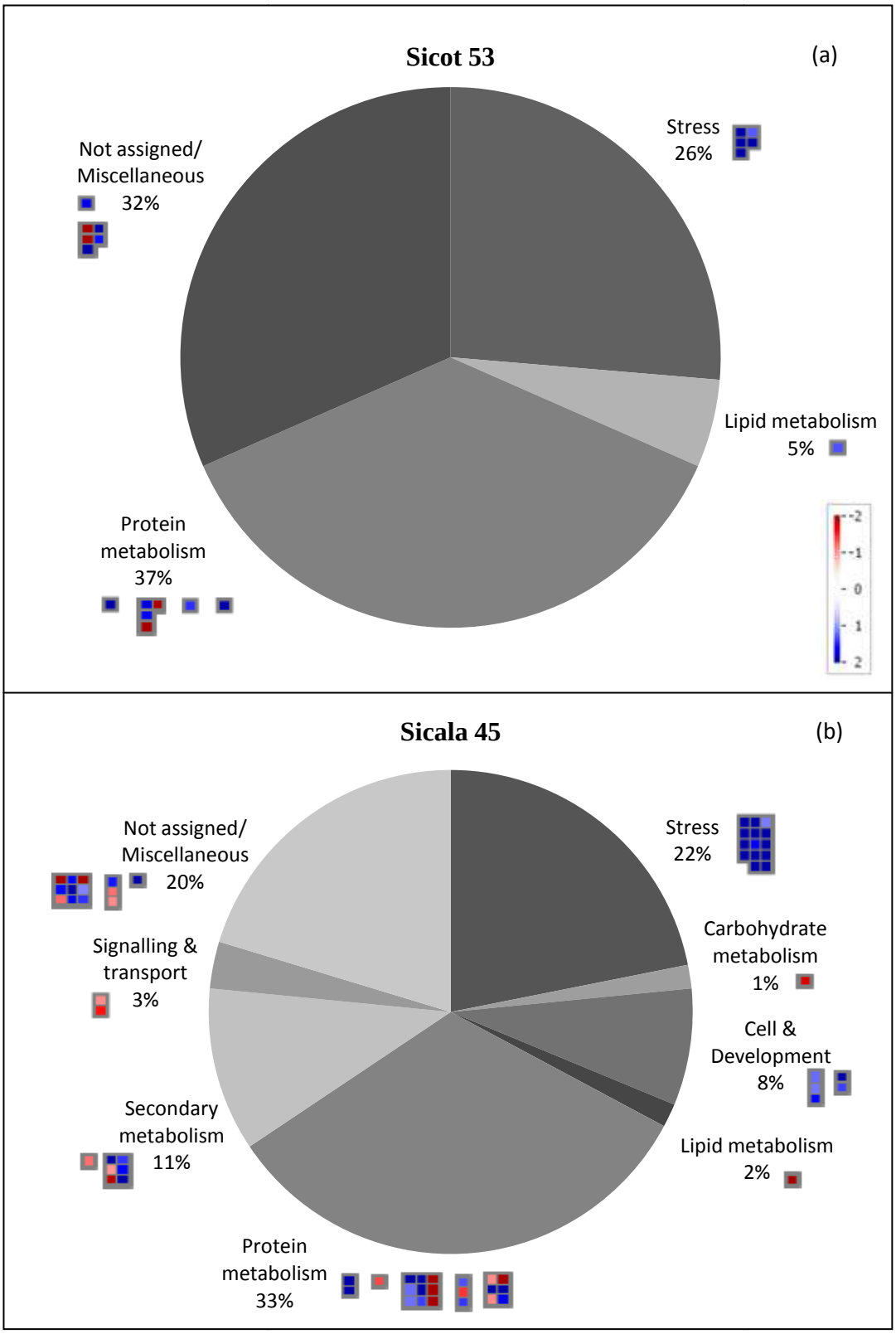

Figure 7-5 Relative expression of (a) Sicot 53 and (b) Sicala 45 genes under high (42 ^{o}C) temperatures in the growth cabinet. Red squares represent genes for which gene expression decreased, whilst blue squares represent genes for which expression increased on a $\log_2$ scale for plants grown under high (42 ^{o}C) temperatures, compared with plants grown under optimal (32 ^{o}C) temperatures in the growth cabinet

Table 7-9 Selected genes for Sicot 53 that are up-regulated or down-regulated in response to heat stress in the growth cabinet

Pathway	Public ID	Relative expression	P value	Arabidopsis hit	Description	E-Value
Up-regulated genes						
Stress	DT046994	4.81	4.69E-02	At3g16050.1	ethylene-inducible protein	5.09E-24
Stress	DT048069	2.52	4.69E-02	At2g41540.2	glycerol-3-phosphate dehydrogenase	6.12E-19
Stress	DW506829.1	3.41	4.45E-02	At5g65260.1	RNA recognition motif (RRM)	1.68E-90
Down-regulated genes						
Stress	DT054070	0.25	4.57E-02	At2g21660.2	glycine-rich RNA-binding protein	3.74E-38
Stress	DT461768	0.26	4.45E-02	At2g21660.2	glycine-rich RNA-binding protein	2.52E-36
Protein metabolism	CA993457	0.06	4.57E-02	At5g54770.1	thiazole biosynthetic enzyme precursor	3.59E-142

Table 7-10 Selected genes for Sicala 45 that are up-regulated or down-regulated under high temperature stress in the growth cabinet

Pathways	Public ID	Relative expression	P value	Arabidopsis Hit	Description	E-Value
Up-regulated genes						
Stress	DT047015	3.29	3.97E-02	At5g20720.2	chloroplast Cpn21 protein	1.00E-45
Stress	DT049773	25.71	4.23E-02	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Stress	DV850132	15.42	4.44E-02	At5g47220.1	ethylene responsive element binding factor 2	7.88E-43
Protein metabolism	DR454255	4.14	3.95E-02	At1g80160.1	glyoxalase family protein	1.02E-63
Protein metabolism	CA992712	7.12	3.32E-02	At5g52640.1	heat shock protein 81-1	8.99E-66
Protein metabolism	DW503697.1	96.75	1.54E-02	At1g07400.1	heat shock protein, putative	1.40E-49
Protein metabolism	DR458062	2.28	3.32E-02	At5g27620.1	cyclin family	5.01E-37
Protein metabolism	CO132723	5.02	1.99E-02	At4g11660.1	heat shock factor protein 7	1.05E-57
Secondary metabolism	CA992719	56.33	3.93E-02	At4g10250.1	endomembrane-localised small heat shock protein	3.92E-31
Secondary metabolism	DW505128.1	6.98	4.44E-02	At4g27670.1	small heat shock protein, chloroplast precursor	1.96E-25
Down-regulated genes						
Stress	CO076413	0.04	1.99E-02	At5g54770.1	thiazole biosynthetic enzyme precursor	2.81E-140
Stress	DT461768	0.23	4.05E-02	At2g21660.2	glycine-rich RNA-binding protein	2.52E-36
Protein metabolism	DW224339.1	0.24	4.03E-02	At5g27150.1	sodium proton exchanger (NHX1)	8.76E-15
Protein metabolism	DN799904	0.47	4.10E-02	At4g03230.1	receptor kinase -related	4.75E-101
Protein metabolism	DW225422.1	0.3	4.44E-02	At1g17345.1	auxin- (indole-3-acetic acid-) induced protein	1.60E-25
Cell & Development	DW485677.1	0.47	4.10E-02	At2g18960.1	ATPase 1, plasma membrane-type	0.00
Secondary metabolism	DW513352.1	0.49	4.44E-02	At1g50430.1	sterol delta-7 reductase	2.93E-45
Secondary metabolism	DW503233.1	0.47	4.03E-02	At3g02750.1	protein phosphatase 2C (PP2C)	2.62E-114
Signaling & transport	DT048308	0.29	4.33E-02	At2g40840.1	glycosyl hydrolase family 77	2.73E-20

7.4 **Discussion**

Cultivar differences in gene expression were detected under high temperatures in the growth cabinet using microarray and validated using qRT-PCR. Differential expression of genes associated with metabolism, photosynthesis and mitochondrial electron transport between cultivars Sicot 53 and Sicala 45 grown under high temperatures in the growth cabinet indicate the presence of cultivar-specific gene expression in response to heat stress. Furthermore, the number of genes differentially expressed under high temperatures for Sicala 45 (Figure 7-5) was greater compared with Sicot 53 (Figure 7-5), thereby indicating a more severe heat stress response and may partially explain decreased photosynthesis and electron transport rate (Chapter 5) and enzyme viability and cell structural integrity (Chapter 6) under high temperature stress in the growth cabinet. These genes may be then validated using qRT-PCR and then assessed under stress conditions in the field to determine for suitability for targeting in directed breeding programs for enhanced stress tolerance.

Using microarray analysis, a high number of genes associated with heat-shock protein expression were found to be up-regulated for Sicala 45 under high temperatures in the growth cabinet (Figure 7-5). This potentially indicates a higher level of stress compared to Sicot 53 and a greater need for up-regulation of stress-mediating biochemical and physiological responses, particularly protection proteins. This cultivar specificity for heat shock protein generation under heat stress was also described for cotton by de Ronde *et al* (1993), who used a protein-extraction method. Strong down regulation of several genes involved in carbohydrate and lipid metabolism for Sicala 45 (Table 7-10) suggests that these protective mechanisms are not sufficient to protect and maintain energy production (cf. Kant *et al.* 2008; Taiz

and Zeiger 2006). Cultivar differences in gene expression have been found for salt stress in rice (Sahi *et al.* 2003), drought stress in sorghum (Sharma *et al.* 2006) and heat stress in fescue (Zhang *et al.* 2005) however, there have been no studies evaluating cultivar differences in global gene expression under high temperature stress in the growth cabinet.

Verification of Rubisco activase (G*h*RCAα2) expression using qRT-PCR indicates that this gene is down-regulated to a greater degree for Sicala 45 than Sicot 53 under high temperatures (42 ^{o}C) in the growth cabinet (Figure 7-1) and may limit photosynthesis (Demirevska-Kepova *et al.* 2005; Kim and Portis 2005; Kurek *et al.* 2007; Salvucci and Crafts-Brandner 2004a). Rubisco activase transcript levels were higher for Sicot 53 compared with Sicala 45 plants under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet (Figure 7-1). Relative expression of Rubisco activase in Sicala 45 leaf tissue decreased under high temperature conditions after 0.5 h incubation and this decrease was in addition to a natural diurnal down-regulation in expression of Rubisco activase in response to photoperiod (DeRidder and Salvucci 2007). This suggests severe protein denaturation and subsequent decrease in overall plant function, thus contributing to a relatively low level of thermotolerance. Cultivar specificity for Rubisco activity has been reported in field cotton (Pettigrew and Turley 1998) and Zhou *et al.* (2006) found that Rubisco content and activity of cucumber cultivar JY4 decreased with chilling stress, whereas cultivar JCH3 was unaffected. Conversely, the relative expression of Rubisco activase was not different in the leaf tissue of Sicot 53 plants after 1 h incubation at optimal (32 ^{o}C) and high temperature (42 ^{o}C) conditions (Figure 7-1). This indicates that Sicot 53 was able to maintain enzyme function under short term (1 h) high temperature stress, interpreted as a high level of thermotolerance. Rubisco activase expression

decreased to a minimum at 3 h into the treatment period however, this decrease was comparable to Sicala 45 and indicates a natural diurnal decline associated with the photoperiod and is fully reversible after return to optimal temperatures and is thus is not an indicator of heat tolerance (DeRidder and Salvucci 2007).

Assuming that a decrease in gene expression associated with Rubisco activase activity is limiting to photosynthesis, decreased expression for Sicala 45 compared with Sicot 53 supports the results in Chapter 5 whereby the photosynthetic rate was lower for Sicala 45 compared with Sicot 53 for leaf material exposed to high temperatures under the tents in the field and growth cabinets. However electron transport rate also decreased under high temperatures in the growth cabinet and under the tents in the field and to a greater degree for Sicala 45 compared with Sicot 53. Hence, validation of genes associated with electron transport rate may also identify underlying processes contributing to photosynthetic capacity under high temperature stress.

Several genes involved in the mitochondrial electron transport chain were also down regulated under high (42 ^{o}C) temperatures in the growth cabinet (Table 7-8) and these genes may be candidates for verification using qRT-PCR for identification of cultivar specific heat tolerance. Genes involved in metabolite transport, electron transfer from complex III to complex IV via cytochrome C and uncoupling proteins, responsible for the movement of H+ ions across the membrane (Table 7-8) were down regulated under high temperatures in the growth cabinet thus potentially limiting limit mitochondrial electron transport and plant respiratory potential (Bartoli *et al.* 2005). Decreased expression of genes associated with mitochondrial respiration supports under high temperatures in the growth cabinet supports the findings in Chapter 6 where the 2,3,5-triphenyltetrazolium (Abs_t) assay showed decreased mitochondrial enzyme activity and respiratory potential under high temperatures in the field and in

the growth cabinet. Cytochrome C is a moderately soluble protein that is loosely attached to the plasma membrane (Taiz and Zeiger 2006) and may shift out of position with increasing membrane fluidity under heat stress. Hence, decreased expression of genes associated with cytochrome C may be indicative of membrane damage was determined to be greater for Sicala 45 compared with Sicot 53 under high temperatures in the growth cabinet and under the tents in the field, using the membrane integrity assay (Chapter 6).

The expression of mitochondria-localised heat shock proteins was up-regulated under high temperature stress in the growth cabinet (Table 7-7). Salvucci (2008) suggested that this may provide protection against degradation of this electron transport pathway and photosynthesis and may warrant further validation and investigation as a potential source of cultivar specific protection against the deleterious effects of heat stress in cotton cultivars.

Genes associated with metabolism, development, electron flow and ATP production that are differentially expressed under high temperatures in the growth cabinet (Figure 7-2) may be involved in the expression of cultivar specific heat tolerance in cotton. However, these candidate genes require verification by qRT-PCR as it is a more specific and sensitive measure of gene expression than microarray determination (cf. Dowd *et al.* 2004). Quantitative RT-PCR is sufficiently sensitive to resolve subtle cultivar differences in gene expression which may be overshadowed by large temperature treatment differences when using microarray gene profiling. Although time series analysis of candidate genes may provide an overall picture of single gene regulation in response to heat stress, evaluation of a broader genotypic range including *G. hirsutum* and *G. barbadense* cultivars for gene expression under high

temperature stress may implicate a greater number of genes involved in heat tolerance.

7.5 **Conclusion**

Up-regulation of genes associated with protection against heat stress and down-regulation of genes associated with plant function under high temperatures in the growth cabinet indicated that Sicala 45 may be relatively less heat tolerant than Sicot 53. Validation of a Rubisco activase (G*h*RCAα2) gene indicated that Sicala 45 plants may have lower potential capacity for photosynthesis under high temperature stress in the growth cabinet compared with Sicot 53.

Gene determination methods such as microarray and qRT-PCR quantification are repeatable and rapid, with a short lag time between multi-generational analyses and thus make these approaches appealing for genotypic screening for thermotolerance. However, stress responses in variable environments involve a cascade of biochemical and physiological responses (Chinnusamy *et al.* 2005; Larkindale *et al.* 2005) and multi-gene interactions under stress can be oversimplified or ignored (Humphreys and Humphreys 2005). In addition plant performance in the field is largely dependent on seasonal environmental variables and seasonally-dependant adaptation to long term stress which are not generally represented in growth cabinet experiments (Chinnusamy *et al.* 2005). These issues therefore highlight the importance of global approaches to detection of stress tolerance. Hence, to account for genotype by environment interactions, thermally responsive gene expression should also be extended from growth cabinet studies to field studies.

Significant opportunities exist to support molecular assisted breeding programs with activities that undertake physiological and yield-based characterisation of specific

genotypic thermotolerance. It would be beneficial to identify a range of cultivar specific thermally responsive genes using molecular techniques and then exploit plant physiology to explain the biological significance of these data. This issue is discussed in more detail in Chapter 8.

Chapter 8 General Discussion

High temperature stress adversely affects multiple physiological and biochemical pathways that contribute to growth and development and ultimately limit yield. There is strong interest in the development of stress tolerant cotton cultivars that can maintain high yield and fibre quality under adverse conditions in the field. Although breeding programs have generally focused on yield as a cultivar selection tool, there exists potential for the development of stress screening tools specifically for the identification of heat tolerant cotton cultivars.

This study was the first step in using a multi-scale approach to understand the performance of cultivars in response to high temperature stress in the growth cabinet and in the field. Although physiological and biochemical tools for determining high temperature tolerance have been described for plants grown under glasshouse conditions, there has been little extension of this knowledge to field conditions. Complex interactions between environmental variables, agronomic management and individual plant responses indicate that mechanisms contributing to high temperature tolerance in the field may not be identified in growth cabinet measurements and assays (Marcum 1998). Furthermore, there has been little research as to whether differences in thermotolerance that are identified through these methods actually contribute to yield.

This chapter discusses how understanding of plant responses to high temperature stress can be used to develop plant-based screening tools for determining heat tolerance from a crop, whole plant, leaf, cell and single gene perspective, by utilising a range of agronomic, physiological, biochemical and molecular tools under growth cabinet and field conditions.

The primary goal of this thesis was to evaluate screening tools for the identification of cultivar specific heat tolerance. This dissertation used multi-level analysis to build a body

of evidence using a range of approaches to indicate potential cultivar specific tolerance of heat stress, rather than provide a 'silver bullet'. As such, it is important to consider this thesis in its entirety, rather than consider specific measurements or, seasons in isolation when interpreting results.

Current literature suggests that there is great emphasis on the development of genocentric research aimed at identifying specific genes or gene groups that contribute to heat stress tolerance (Alia *et al.* 1998; DeRidder and Salvucci 2007; Gao *et al.* 2008; Larkindale *et al.* 2005; Lee *et al.* 1995; Lohmann *et al.* 2004; Schramm *et al.* 2006). Similarly, stress tolerance determination through quantification of a single enzyme, cell integrity or physiological trait in isolation has been widely used to ascertain cultivar specific tolerance. However, these approaches may trivialise the complexity of whole plant and environment interactions (Sinclair and Purcell 2005). Caution should be exercised when using a 'bottom up' approach to stress tolerance research as although large treatment differences may be determined at a genetic or leaf level, it is likely that the magnitude of these differences diminishes at each increasing level of plant function due to initiation of alternate biological compensatory pathways or confounding influence of environment to a point where translated differences in yield may be negligible. Hence, it is important to consider higher level plant function and whole plant systems biology before interpretation of results arising from detailed molecular, biochemical and physiological measurements as an indicator of potential benefits relevant to actual agricultural production systems (Boote and Sinclair 2006).

Thus the framework of this thesis firstly utilised a 'top down' approach to identify differences in genotypes with known and differing levels of heat tolerance (Figure 8-1). By implementing this approach, the underlying physiological, biochemical and genetic

factors contributing to actual differences in cultivar yield under varying thermal environments were explored. This approach identified cultivar differences at lower levels of plant function which may be indicative of, or which may partially account for actual differences in yield, subsequently rendering them significant to actual production system. This approach may also highlight cultivar differences which have the potential to affect other factors contributing to yield, such as cultivar specific water flux permitting decreased irrigation frequency in warm or hot seasons. The 'top down' approach enabled identification of multiple opportunities at different levels of plant function for the development of tools to identify cultivar specific heat tolerance. Secondly, this 'top down approach' provided a framework for 'bottom up' approach including validation under a range of environments and subsequent confirmation of tolerance for a range of cultivars.

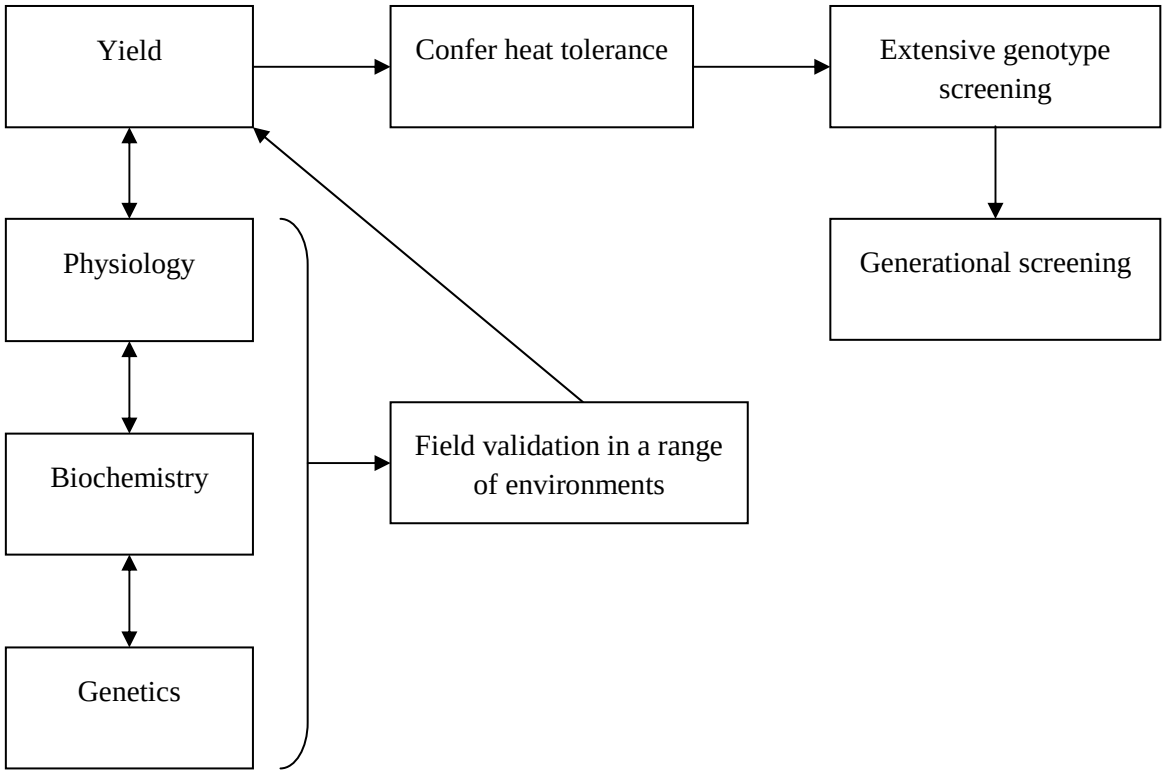

Figure 8-1 The 'top-down' and 'bottom-up' approach for determination of cultivar specific heat tolerance and validation under a range of environments for a range of genotypes

Utilising this approach, Sicot 53 was the best performing cultivar for photosynthesis and electron transport (Chapter 5) and relative electrical conductivity (Chapter 6) under the tents which generated on average a 1.7 ^{o}C increase in temperature and also under high temperatures in the growth cabinet. Furthermore, gene profiling indicated that a higher proportion of total genes, including genes involved in heat shock protein expression, metabolism and cell development were affected by high temperature stress in Sicala 45 compared with Sicot 53 (Chapter 7). This may indicate a higher degree of stress in Sicala 45 plants and hence more severe mobilisation of compensatory pathways. Consistent cultivar differences at a whole plant, leaf, cell and single gene level indicate Sicot 53 has a higher capacity for thermotolerance.

Pooled analysis for photosynthesis across the 3 seasons indicated that Sicot 53 was the better performing cultivar under high temperatures in the field (Figure 5-1) and expression quantification using qRT-PCR indicates that this cultivar specificity may be partially attributed to variable regulation of genes involved in Rubisco activase activity (Figure 7-1). Stomatal conductance and transpiration rate were higher for Sicala 45 compared with Sicot 53 but this is most likely attributed to higher capacity for heat avoidance and may not confer heat tolerance (Lu *et al.* 1998) or determine photosynthetic capacity. However, similar photosynthetic and electron transport capacity under field and growth cabinet conditions indicate that electron transport rate may also be a primary limitation to photosynthesis under high temperatures. Furthermore, the relationship between electron transport rate and yield under field conditions (Figure 5-12) indicates that electron transport and subsequently energy generation may contribute to thermotolerance under field conditions.

Electron transport rate is a reliable and repeatable measurement for stress tolerance determination. This may be because fluorescence-based measurements such as electron transport rate are not influenced by environmental conditions inside the measurement chamber, as is the case with determination of photosynthesis. However, the cost of this test is considerable. There is a significant initial investment associated with the purchase of a Li-6400 portable photosynthesis system and fluorescence attachment. Alternatively, use of pulse amplitude modulated (PAM) fluorometer may decrease the initial investment cost. Other methods of fluorescence determination including maximum efficiency of photosystem II (Fv/Fm) and quenching analysis may be potentially useful for use in screening programs (Bibi *et al.* 2008; Ducruet *et al.* 2007) and thus warrant further investigation. Furthermore, there is potential for investigation of the use of fluorometry for cultivar screening for drought (Burke 2007; Clavel *et al.* 2006; O'Neil *et al.* 2006), light (Bjorkman and Schafer 1989; Lambreva *et al.* 2005), cold (Warner and Burke 1993), salinity (Jiang *et al.* 2006), biotic or a combination of stresses for a complete stress screening program.

Biochemical assays for membrane integrity and enzyme viability were used to determine the underlying limitations to electron transport under high temperature stress (Chapter 6). Membrane integrity decreased under high temperatures in the growth cabinet as well as under the tents and may possibly limit electron flow through photosynthetic and respiratory pathways (Taiz and Zeiger 2006). Furthermore, relative electrical conductivity was lower for Sicot 53 under both field and growth cabinet conditions compared with Sicala 45. Up or down-regulation of genes associated with membrane integrity was identified using microarray gene profiling and these genes may be validated for cultivar specificity using qRT-PCR for development of specific gene targeting for membrane integrity under high temperature stress.

Relative electrical conductivity correlated with yield during season 1 (Figure 6-5), as described for wheat (Blum and Ebercon 1981), beans (Schaff *et al.* 1987), sorghum (Sullivan 1971) and cotton (Rahman *et al.* 2004). Decreases in membrane integrity have been reported for drought (Bajji *et al.* 2002; Blum and Ebercon 1981; Rahman *et al.* 2008) and cold (Cottee *et al.* 2007; McDowell *et al.* 2007; Wulff *et al.* 1994) stress thereby indicating potential development of this assay for broad-spectrum stress tolerance screening.

As a laboratory assay, determination of REC is rapid and reliable with few initial associated input costs (Marcum 1998), thus indicating potential for development of this method for genotype screening for thermotolerance (Bibi *et al.* 2008). Furthermore, similar results for REC under field and growth cabinet conditions indicate that there is potential for the development of growth cabinet and laboratory assays for the identification of stress tolerant cultivars for potential incorporation into breeding programs for industry-wide production.

Dehydrogenase activity also decreased under high temperature stress in the growth cabinet (Figure 6-7) which is consistent with the findings of previous research (Chen *et al.* 1982; de Ronde and van der Mescht 1997; Porter *et al.* 1995; Schaff *et al.* 1987). This suggests that heat stress may increase membrane permeability, thus decreasing electron transport associated with membrane associated proteins and enzymes (Taiz and Zeiger 2006).

Genes associated with cytochrome C and uncoupling proteins as well as genes involved in dehydrogenase and carboxylase activity were down-regulated under high temperatures in the growth cabinet (Table 7-8) thereby indicating a possible mechanism for reduced photosynthetic and respiratory capacity in the plant. Down-regulation of respiratory electron chains may contribute to lower potential for ATP production and energy

generation. Validation of genes associated with mitochondrial electron transport using qRT-PCR may indicate potential genes for targeting for stress screening programs under high temperature stress. However, cultivar differences for enzyme viability were not detected under high temperature stress in the field, thereby suggesting that this assay is not sufficiently sensitive to be used as a screening tool for thermotolerance in the field.

Sicot 53 outperformed Sicala 45 in terms of yield, photosynthesis, electron transport, membrane integrity and enzyme viability under high temperature stress in the growth cabinet as well as under tents in this study. These findings are consistent with the work of Rahman (2005) who found similar patterns in stomatal conductance under high temperature stress under field conditions and in the glasshouse. This suggests that assays and measurements for determination of thermotolerance in the glasshouse may be indicative of functionality under field conditions. However, measurements of photosynthesis, electron transport, stomatal conductance, membrane integrity and enzyme viability under field conditions were greatly variable across the 3 seasons, 2 locations and multiple days of measurement which may be attributed to environmental variability (Marcum 1998).

Although not indicative of plant capacity through an entire season, point-in-time survey measurements of gas exchange, membrane integrity are indicators of cultivar performance under high temperature stress at a specific point in time which, when replicated at various developmental stages may provide a snapshot of cultivar performance in response to plant and environmental variables throughout a season. Furthermore, although the magnitude of treatment differences for these measurements was relatively small, it is likely that the regulation of specific biological pathways is either an indicator of, or contributes to changes in overall plant function. It is not suffice to say that any difference at any level is

indicative of overall plant thermotolerance, but rather that by measuring these components of plant functionality, cultivars with potential to maintain production under the environments for which they are tested can be identified. Furthermore, this research does not attempt to identify cultivar heat tolerance under all thermal environments that would be realistically encountered under field production systems, but rather attempts to find tools for cultivar discrimination under multiple high temperature environments including five Solarweave® tent events over 3 seasons as well as and a high temperature growth cabinet.

There was a high degree of variation associated with determination of physiological and biochemical function under high temperatures in the field across and within the 3 seasons. Plant variation under field conditions is widely recognised as a primary limitation to the repeatability of field experiments, this variation may be minimised by evaluation of cultivar performance at a greater number of field sites over a greater number of seasons and with a larger number of measurements during each season for a higher number of replicates. This approach is currently used by plant breeders but may be applied to specific stress tolerance breeding programs, thus providing a more accurate representation of cultivar performance under high temperature stress in local environments and under long term production systems.

The greatest resolution between temperature treatments and cultivars was determined under the tent 1 in seasons 1 and 2 and under high (42 ^{o}C) temperatures in the growth cabinet. Plants grown under these tents had in excess of 60 hr exposure to temperatures exceeding the high temperature threshold (35 ^{o}C) for cotton and over 20 hr under extreme (45 ^{o}C) high temperature stress (Table 3-6). This suggests that screening tools for thermotolerance are more effective when implemented in the presence of stress, which is

consistent with studies by Lopez *et al*. (2003b) and Rahman (2005). This highlights the importance of incorporating a large number of measurements under variable environmental conditions to ensure measurements are taken under the target stress and to identify subtle differences in plant function under high temperatures effectively. Hence growth cabinet studies need to be supplemented by field screening techniques to ensure that thermotolerant cultivars are correctly identified.

The tents increased air temperatures, which may have initiated heat shock within the first few hours of plant exposure to heat stress and subsequent heat acclimation after several consecutive days of exposure under the tents. However, it is likely that heat stress was not imposed in isolation. The 18% nominal shade value of Solarweave® fabric may have altered the light environment, but Solarweave® simultaneously increases the proportion of diffuse radiation thereby resulting in an increase in radiation use efficiency by the crop (Healey *et al.* 1998). Furthermore, Solarweave® has sufficient durability to withstand unforseen environmental extremes, particularly high wind speeds and heavy precipitation events and was hence deemed the most suitable for field experiments. Disruption of air circulation under the tents or interception and diffusion of solar radiation may induce water logging or humidity stress following an irrigation or precipitation event. However, care was taken to ensure the tents were only constructed after at least 4 days post irrigation to allow sufficient drainage and aeration of roots thus minimising water logging stress for the measurement period. The tents were raised off the ground to facilitate air flow down the rows but the relative humidity under the tents still exceeded the level for the control. Although exposure to relative humidity stress was minimised, it was not excluded and is a limitation of imposing high temperature stress using Solarweave® tents. It is likely that by taking measurements 4 days after an irrigation event, the onset of drought stress was minimised during the measurement period. Evaluation of alternate methods to increase *in-*

situ temperatures in the field may help alleviate problems with strict control of temperature and relative humidity during the treatment phase.

All field experiments were conducted under full irrigation and growth cabinet experiments were conducted at field capacity in an attempt to attain relatively uniform leaf temperatures (cf. Gardner *et al.* 1981) within the ambient or tent temperature treatments. However, it is likely that leaf temperature of plants in response to the air temperature treatment was not uniform. This is highlighted in the growth cabinet study whereby cultivar differences were evident under control (32 ^{o}C) temperatures but not high (42 ^{o}C) temperatures in the growth cabinet (Figure 5-15). Cultivar dissimilarities in leaf temperature may be further exacerbated by instances of mild water deficit stress in the field or decreased incident radiation under the tents. The relationship between air and leaf temperature did not differ for plants grown under control and tent temperature regimes for measurements using the Li6400 portable photosynthesis system. For measurements of photosynthesis, electron transport rate, stomatal conductance and transpiration rate, variables such as light, water vapour pressure, humidity and carbon dioxide delivery were set within the sensor head and were thus sufficiently similar between the treatments. However, the imposition of tents may alter incident radiation, wind speed and vapour pressure to modify leaf temperature to a degree that surpasses equivalent increases in air temperature under ambient conditions thus affecting yield. Future research incorporating leaf and canopy temperature measurements may help explain temperature treatment and cultivar differences in plant morphology, physiology, biochemistry and gene expression under high atmospheric temperatures that were identified in this research.

This thesis evaluated thermotolerance of cotton cultivars at a crop, plant, leaf, cell and gene level. Due to the multi-level and broad-scale theme of this research, only two

cultivars were selected for in-depth physiological determination. Sicot 53 was selected as a relatively tolerant cultivar whilst Sicala 45 was considered to have a lower level of heat tolerance on the basis of yield data in warm and hot growing regions. These cultivars had similar morphological characteristics e.g. leaf shape and are both used in current breeding programs. However, it is unlikely that these cultivars are representative of the upper and lower limit of thermotolerance in cotton cultivars and are also not widely grown as commercial cultivars in a market that is currently dominated by transgenic cultivars. These cultivars were sufficiently similar to facilitate molecular comparisons of gene expression under high temperature stress but were dissimilar to a level that facilitated cultivar discrimination for yield, physiological and biochemical function under high temperature stress. It is likely that inclusion of a greater number of genotypes will increase the resolution of these tolerance mechanisms as a result of greater genetic differences and capacity for thermotolerance.

For the recommendations of this thesis to be applied in practical situations, it is necessary to verify the efficacy of these screening tools for a greater range of germplasm from diverse backgrounds and subsequent generations of crosses in a range of actual production systems. These tools may then be used to complement traditional breeding programs, rather than replace them to screen large genotypic populations at various growth stages for tolerance to heat stress as well as other abiotic stresses encountered under field production systems that may contribute to yield at the end of the season.

8.1 **Suggested future work**

This study has evaluated a broad range of screening tools for determination of cultivar specific thermotolerance at a crop, whole plant, leaf, cell and gene level. However, there are several opportunities for further research as a result of this study, as summarised below:

i. Screening of a wider range of commercially available cultivars and breeding lines, including genotypes originating from other countries and other cotton species such as *Gossypium barbadense* to identify a broad range of potential candidates for thermotolerance targeted breeding.

ii. Screening cotton cultivars for thermotolerance in a wider array of environments and production areas for the development of locally adapted genetic material.

iii. Investigations into the effects of rapid increases in temperature compared with slow increases in temperature to evaluate acquired thermotolerance.

iv. Further evaluation of cultivar differences in recovery after exposure to stress.

v. Further development of fluorescence as a possible screening tool for thermotolerance using pulse amplitude modulated fluorometry.

vi. Further development of simple methods to impose heat stress in the laboratory, such as incubators (Burke 2007) and the use of infra-red heaters in the field (Nijs *et al.* 1996) to minimise increases in relative humidity under the tents.

vii. Measurements of canopy and leaf temperature should be taken to explain changes in plant morphology, physiology, biochemistry and gene expression under high temperatures in the field.

viii. Application of screening tools for thermotolerance, to other stresses for the identification of drought, radiation, cold and biotic stress tolerance to aid the development of a simple tool for screening overall stress tolerance.

ix. Validation of genes associated with heat stress and high temperature tolerance that have been determined in this study by global gene profiling to confirm their role in regulation of the heat stress response.

x. Validation of gene expression for thermotolerance under field conditions to confirm the role of genes associated with high temperature tolerance in the growth cabinet, to the complex field environment.

xi. Identification of genes associated with fruit retention and fibre quality under high temperature stress for identification of thermotolerance at the flowering, boll set, boll and fibre development stages.

xii. Development of molecular markers for marker assisted breeding.

8.2 **Concluding remarks**

This study found cultivar differences in morphological, physiological, biochemical and molecular function in response to high temperature stress in the field and in the growth cabinet using a multi-level approach, encompassing crop, whole plant, leaf, cell and gene level measurements and assays.

Electron transport rate determined by fluorescence measurements and membrane integrity determined by the relative electrical conductivity assay were correlated with yield in seasons 1 and 3. These methods were simple, rapid and reliable. Hence, electron transport rate and relative electrical conductivity were the most effective methods for cultivar determination of thermotolerance under high temperature conditions and may have

potential for incorporation into future breeding programs for superior yield under heat stress.

Sicot 53 had consistently higher photosynthesis, electron transport and membrane integrity compared with Sicala 45 plants grown under the tents and high (42 ^{o}C) temperatures in the growth cabinet. A greater number of genes associated with metabolism, photosynthesis, mitochondrial electron transport and protective proteins were up- or down-regulated in response to high temperatures in the growth cabinet for Sicala 45 compared with Sicot 53 indicating a more severe heat stress response. Consistent cultivar differences across measurement levels and environments indicate that Sicot 53 has relatively higher thermotolerance compared with Sicala 45. This multi-level approach provides a comprehensive knowledge base as to the contributing factors to heat tolerance from a single gene to a whole crop level and highlights multiple opportunities for the development of screening methods to enable the identification of heat tolerant cultivars.

In conclusion, this study has identified consistent cultivar differences in heat tolerance at a crop, whole plant, leaf, cell and molecular level of organisation, thus providing a foundation for the development of a multi-level approach for identification of heat tolerance in cotton cultivars.

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Appendix Gene expression tables for microarray analysis

Table 1 Relative expression of stress genes up-regulated for cultivars Sicot 53 and Sicala 45 under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Biotic stress	DN804462	1.327	4.00E-02	At1g33970.1	expressed protein	8.24E-41
Biotic stress	DW236162.1	1.351	2.25E-02	At4g05440.1	D123 -related protein protein D123	1.00E-130
Biotic stress	DT463926	1.459	2.30E-03	At5g51700.1	RAR1 disease resistance protein	8.33E-74
Biotic stress	DT053436	1.504	2.77E-03	At4g37000.1	accelerated cell death 2 (ACD2)	4.87E-48
Biotic stress	DW507678.1	1.562	3.10E-02	At1g64140.1	expressed protein	4.14E-151
Biotic stress	AI726851	2.418	8.34E-04	At5g47120.1	Bax inhibitor-1 (BI-1)	1.14E-89
Cold stress	DT048302	2.664	3.33E-02	-	-	-
Drought/salt stress	CO127394	1.389	2.61E-02	At2g03480.1	dehydration-induced protein-related	2.77E-132
Drought/salt stress	DV849352	1.515	3.61E-02	At5g49230.1	drought-induced protein	8.97E-43
Drought/salt stress	DT455898	1.643	1.33E-02	At4g16390.1	chloroplastic RNA-binding protein P67, putative	2.15E-140
Drought/salt stress	DT468710	1.999	2.23E-02	At2g03480.1	dehydration-induced protein-related	1.03E-89
Heat stress	DT467391	1.353	2.74E-02	At4g10250.1	endomembrane-localized small heat shock protein	1.03E-45
Heat stress	CO070759	1.37	2.42E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	2.89E-60
Heat stress	DR462570	1.394	4.23E-02	At5g18750.1	DnaJ domain-containing protein	5.53E-08
Heat stress	CO075693	1.399	4.61E-02	At3g44110.1	DnaJ protein AtJ3	2.62E-104
Heat stress	DN758088	1.404	1.08E-02	At3g44110.1	DnaJ protein AtJ3	3.72E-146
Heat stress	DT465440	1.488	4.65E-02	At4g11660.1	heat shock factor protein 7 (HSF7)	4.33E-34
Heat stress	DW516953.1	1.489	2.03E-03	At2g35795.1	DnaJ domain-containing protein	1.05E-42
Heat stress	DW516183.1	1.509	3.37E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	1.40E-34
Heat stress	DW516183.1	1.509	3.37E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	1.40E-34

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Heat stress	DR456019	1.567	4.01E-02	-	-	-
Heat stress	DR456432	1.623	4.89E-02	At4g24280.1	heat shock protein cpHsc70-1 heat shock 70 protein	1.20E-76
Heat stress	DT560821	1.672	4.85E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	0
Heat stress	DT560821	1.672	4.85E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	0
Heat stress	DT048776	1.681	2.88E-02	At3g44110.1	DnaJ protein AtJ3	2.52E-111
Heat stress	DN761905	1.695	1.40E-02	At5g09590.1	heat shock protein mtHsc70-2 (Hsc70-5)	2.45E-24
Heat stress	DT460619	1.705	4.87E-02	At2g32120.2	heat shock protein hsp70t-2	2.26E-53
Heat stress	CO070151	1.77	1.46E-02	At5g62020.1	heat shock transcription factor 6 (HSF6)	1.16E-75
Heat stress	CO125371	1.805	2.31E-02	At4g24280.1	heat shock protein cpHsc70-1	2.63E-107
Heat stress	DW496653.1	1.963	2.33E-02	At3g14200.1	DnaJ protein family	6.09E-06
Heat stress	DT460963	2.102	6.83E-03	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Heat stress	CA993412	2.312	2.02E-02	-	-	-
Heat stress	DT051231	2.389	8.64E-04	At4g24190.1	shepherd (SHD)	3.48E-44
Heat stress	CO108164	2.545	1.88E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	5.03E-127
Heat stress	CO108164	2.545	1.88E-02	At5g56030.1	heat shock protein 81-2 (HSP81-2)	5.03E-127
Heat stress	AW186892	2.635	2.71E-03	At4g24190.1	shepherd (SHD)	1.14E-107
Heat stress	DR457102	2.643	1.41E-04	-	-	-
Heat stress	CA992849	2.956	1.28E-03	At3g44110.1	DnaJ protein AtJ3	1.54E-170
Heat stress	CO092638	2.956	1.21E-02	At3g23990.1	chaperonin (CPN60/HSP60)]	5.37E-72
Heat stress	DT457869	3.406	8.96E-04	At2g29500.1	small heat shock protein	2.54E-45
Heat stress	DT545357	3.428	3.47E-04	At4g25200.1	mitochondrion-localized small heat shock protein	4.99E-62

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Heat stress	DW237876.1	3.473	3.66E-02	At5g59720.1	heat shock protein family	1.48E-43
Heat stress	DT046481	3.559	4.55E-03	At5g56030.1	heat shock protein 81-2 (HSP81-2)	0
Heat stress	DT046481	3.559	4.55E-03	At5g56030.1	heat shock protein 81-2 (HSP81-2)	0
Heat stress	DW487642.1	3.813	1.26E-02	At3g23990.1	chaperonin (CPN60/HSP60)	5.39E-90
Heat stress	DT047459	3.994	1.06E-04	At5g62020.1	heat shock transcription factor 6 (HSF6)	2.88E-09
Heat stress	DW496991.1	4.792	6.07E-03	At1g54050.1	heat shock hsp20 protein family	2.77E-33
Heat stress	CO132723	4.901	1.25E-05	At4g11660.1	heat shock factor protein 7 (HSF7)	1.05E-57
Heat stress	DT050385	4.981	6.49E-06	At3g23990.1	chaperonin (CPN60/HSP60)	1.35E-10
Heat stress	DW505128.1	6.712	3.08E-04	At4g27670.1	small heat shock protein, chloroplast precursor(HSP21)	1.96E-25
Heat stress	CA992712	7.539	7.06E-04	At5g52640.1	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	8.99E-66
Heat stress	DT456116	8.919	5.54E-04	At2g26150.1	heat shock transcription factor family	2.66E-72
Heat stress	DT049115	9.451	2.65E-04	At2g29500.1	small heat shock protein	3.03E-56
Heat stress	DT467180	9.761	2.25E-05	At5g12020.1	class II heat shock protein	1.12E-35
Heat stress	DW230150.1	10.084	4.11E-04	At5g52640.1	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	1.12E-54
Heat stress	DR455939	10.441	7.24E-04	At5g12020.1	class II heat shock protein	3.71E-31
Heat stress	DW513189.1	15.343	1.80E-04	At4g25200.1	mitochondrion-localized small heat shock protein	2.47E-59
Heat stress	DN760566	15.345	3.66E-04	At5g52640.1	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	7.11E-76
Heat stress	DN757824	17.311	2.53E-04	At5g37670.1	heat shock protein family	2.20E-38
Heat stress	DT527234	18.079	2.89E-03	At5g59720.1	heat shock protein family	4.55E-36
Heat stress	CA993514	18.212	5.77E-04	At2g29500.1	small heat shock protein	9.90E-55
Heat stress	DT465978	20.649	3.94E-05	At2g29500.1	small heat shock protein	1.39E-56

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Heat stress	DT457869	21.789	2.38E-04	At2g29500.1	small heat shock protein -related	2.54E-45
Heat stress	DT049773	22.439	1.38E-05	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Heat stress	DW234293.1	23.818	5.77E-04	At2g29500.1	small heat shock protein	4.32E-46
Heat stress	DW503063.1	28.87	1.17E-04	At5g52640.1	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	2.42E-112
Heat stress	DW503063.1	28.87	1.17E-04	At5g52640.1	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	2.42E-112
Heat stress	DR455451	30.145	2.24E-03	At4g27670.1	small heat shock protein, chloroplast precursor(HSP21)	8.71E-64
Heat stress	DW517704.1	35.33	1.84E-04	At4g10250.1	endomembrane-localized small heat shock protein	2.11E-15
Heat stress	CA992719	38.989	1.19E-04	At4g10250.1	endomembrane-localized small heat shock protein	3.92E-31
Heat stress	DW503697.1	83.99	7.78E-07	At1g07400.1	heat shock protein, putative	1.40E-49
Light stress	DW505008.1	1.817	2.22E-02	At5g11580.1	expressed protein rjs protein	8.90E-80
Touch/wounding	DR456264	1.368	3.39E-02	At3g07230.1	wound-induced basic protein	1.79E-13
Touch/wounding	BF272159	1.532	4.02E-02	At3g07230.1	wound-induced basic protein	6.52E-15
Unspecified stress	M19379.1	1.248	4.92E-02	-	-	-
Unspecified stress	AI055725	1.68	4.39E-02	At1g28290.1	prolin-rich protein	1.44E-33
Unspecified stress	DT571171	1.7	1.13E-02	At1g11360.2	expressed protein	1.75E-65
Unspecified stress	AJ513421	1.738	1.39E-03	At1g01170.1	expressed protein	8.98E-16
Unspecified stress	DN801667	4.432	2.85E-02	At1g44760.1	expressed protein	1.18E-19

Table 2 Relative expression of stress genes up-regulated for cultivars Sicot 53 and Sicala 45 under optimal (32 ^{o}C) and high (42 ^{o}C) temperatures in the growth cabinet

Pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-Value
Biotic stress	DW509680.1	0.405	2.81E-02	At1g58170.1	disease resistance response protein-related/ dirigent protein-related	9.89E-57
Biotic stress	DT465458	0.493	1.92E-02	At3g54420.1	glycosyl hydrolase family 19 (class IV chitinase)	1.46E-70
Biotic stress	DW488405.1	0.575	1.09E-02	At2g21340.1	enhanced disease susceptibility 5 (salicylic acid induction deficient 1)	7.20E-140
Biotic stress	DV849720	0.622	6.29E-03	At5g61240.1	leucine rich repeat protein family	6.29E-110
Biotic stress	AY040533.1	0.673	2.10E-02	At5g61240.1	leucine rich repeat protein family	9.56E-106
Biotic stress	DT050959	0.769	4.39E-02	At5g17680.1	disease resistance protein (TIR-NBS-LRR class)	1.07E-24
Biotic stress	DT050959	0.769	4.39E-02	At5g17680.1	disease resistance protein (TIR-NBS-LRR class)	1.07E-24
Cold stress	DT465699	0.653	1.40E-02	At4g13850.1	glycine-rich RNA-binding protein (AtGRP2)	6.61E-19
Cold stress	DW511523.1	0.665	1.11E-02	At4g13850.2	glycine-rich RNA-binding protein (AtGRP2)	5.29E-32
Drought/salt stress	AY641991.1	0.457	3.50E-02	At5g25610.1	dehydration-induced protein RD22	5.78E-35
Drought/salt stress	CO091414	0.459	3.61E-02	At5g25610.1	dehydration-induced protein RD22	1.63E-80
Drought/salt stress	AY641990.1	0.468	4.16E-02	At5g25610.1	dehydration-induced protein RD22	4.71E-48
Drought/salt stress	AI731201	0.474	4.15E-02	At5g25610.1	dehydration-induced protein RD22	1.32E-61
Drought/salt stress	DT054238	0.515	2.53E-02	At3g23300.1	dehydration-induced protein-related	4.93E-95
Drought/salt stress	DW520205.1	0.635	8.33E-03	At4g18030.1	dehydration-induced protein family	2.26E-80
Drought/salt stress	DW224875.1	0.665	4.45E-02	At4g14360.1	dehydration-induced protein	1.08E-146
Drought/salt stress	DT462193	0.757	2.02E-02	At5g64030.1	dehydration-induced protein-related	4.39E-75
Heat stress	CO086335	0.694	1.56E-02	At1g76700.1	DnaJ protein family	4.14E-82
Heat stress	DT466906	0.763	4.29E-02	At1g67970.1	heat shock transcription factor 5 (HSF5)	5.37E-28
Unspecified stress	DT467978	0.567	2.98E-02	At2g21620.1	auxin-regulated protein	1.30E-66

Table 3 Relative expression of metabolism genes, up-regulated under high (42 °C) and compared to optimal (32 °C) temperatures in the growth cabinet for cultivars Sicot 53 and Sicala 45.

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Carbohydrate metabolism	DT465672	4.67	2.75E-02	At1g23870.1	trehalose phosphatase family	3.79E-35
Carbohydrate metabolism	DT464481	1.361	1.18E-02	At1g06690.1	aldo/keto reductase family	3.24E-20
Carbohydrate metabolism	DT462221	2.061	3.84E-03	-	-	-
Carbohydrate metabolism	AY628139.1	3.007	4.44E-02	At1g23870.1	trehalose phosphatase family	0
Carbohydrate metabolism	DT050177	1.775	1.25E-02	-	-	-
Carbohydrate metabolism	DT050909	1.442	4.21E-02	-	-	-
Carbohydrate metabolism	DT049671	1.294	3.63E-02	At4g28300.1	proline-rich protein family	2.57E-22
Carbohydrate metabolism	DW238688.1	5.903	4.13E-02	At2g47180.1	galactinol synthase, putative	2.29E-126
Carbohydrate metabolism	DR463306	1.871	2.09E-02	At5g19730.1	pectinesterase family	4.23E-95
Carbohydrate metabolism	DR462102	1.624	2.75E-02	At4g02500.1	transferase - related	2.75E-13
Carbohydrate metabolism	AI726514	1.857	3.34E-03	At3g02210.1	predicted GPI-anchored protein	0
Carbohydrate metabolism	AW186880	2.417	1.16E-02	At5g41870.1	polygalacturonase, putative	6.02E-40
Carbohydrate metabolism	DT456785	2.079	4.77E-02	-	-	-
Carbohydrate metabolism	DW227981.1	2.936	4.29E-02	At5g65730.1	xyloglucan endotransglycosylase, putative	2.46E-105
Carbohydrate metabolism	CO122431	3.178	3.18E-02	At4g37800.1	xyloglucan endotransglycosylase, putative	5.40E-47
Electron flow / ATP	DN800322	2.237	1.20E-02	At1g06680.1	photosystem II oxygen-evolving complex 23 (OEC23)	5.11E-85
Electron flow / ATP	DT051416	1.599	2.29E-02	At1g07890.2	ascorbate peroxidase, putative (APX)	2.26E-101
Electron flow / ATP	DR458096	2.003	4.73E-04	At2g28000.1	RuBisCO subunit binding-protein alpha subunit/	0
Electron flow / ATP	DV849478	3.338	2.27E-04	At2g28000.1	RuBisCO subunit binding-protein alpha subunit	8.87E-23
Electron flow / ATP	DW508175.1	1.742	6.83E-03	At1g75270.1	dehydroascorbate reductase	1.75E-32

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Electron flow / ATP	DW497212.1	1.798	3.46E-02	At1g03600.1	photosystem II protein family	2.14E-43
Electron flow / ATP	DW509246.1	1.241	4.12E-02	At1g63460.1	glutathione peroxidase	7.62E-70
Electron flow / ATP	DN817738	1.4	4.75E-02	At4g20130.1	expressed protein	4.62E-49
Electron flow / ATP	DN817738	1.4	4.75E-02	At4g20130.1	expressed protein	4.62E-49
Electron flow / ATP	CO072814	2.449	3.50E-03	At2g28000.1	RuBisCO subunit binding-protein alpha subunit	1.67E-154
Electron flow / ATP	CO121719	2.455	1.37E-02	At2g28000.1	RuBisCO subunit binding-protein alpha subunit	3.75E-107
Electron flow / ATP	DW226042.1	1.543	9.27E-03	At5g14590.1	isocitrate dehydrogenase [NADP+]	2.94E-33
Lipid metabolism	DT047253	1.289	4.84E-02	At1g54580.1	acyl carrier protein (ACP), chloroplast	4.38E-23
Lipid metabolism	CA993580	1.479	1.18E-02	At4g33030.1	UDP-sulfoquinovose synthase	1.69E-33
Lipid metabolism	CA993580	1.818	2.30E-03	At4g33030.1	UDP-sulfoquinovose synthase	1.69E-33
Lipid metabolism	DT466441	1.975	9.08E-04	At2g19450.1	diacylglycerol O-acyltransferase	1.15E-56
Lipid metabolism	AY138250.1	1.32	2.29E-02	At2g42010.1	phospholipase D (PLDbeta)	0
Lipid metabolism	DR452394	1.486	1.59E-02	At1g01710.1	acyl CoA thioesterase -related	8.86E-65
Lipid metabolism	DW508235.1	1.578	4.23E-03	At5g65940.1	3-hydroxyisobutyryl-coenzyme A	3.18E-144
Protein metabolism	DR454255	3.276	5.79E-04	At1g80160.1	glyoxalase family	1.02E-63
Protein metabolism	DT052636	1.642	1.02E-02	-	-	-
Protein metabolism	DT050254	1.482	3.15E-02	At1g43710.1	histidine decarboxylase -related	2.83E-102
Protein metabolism	DT049512	1.729	2.55E-02	At4g16210.1	enoyl-CoA hydratase/isomerase family	2.67E-89
Protein metabolism	DT049512	1.729	2.55E-02	At4g16210.1	enoyl-CoA hydratase/isomerase family	2.67E-89
Protein metabolism	DT459731	1.551	2.22E-02	-	-	-
Protein metabolism	DT463796	1.616	5.56E-03	At5g47720.2	acetyl-CoA C-acetyltransferase	9.-96E-81

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Protein metabolism	DW513900.1	1.442	4.84E-03	At1g53580.1	glyoxalase II, putative (hydroxyacylglutathione hydrolase)	3.25E-55
Protein metabolism	DW508235.1	1.578	4.23E-03	At5g65940.1	3-hydroxyisobutyryl-coenzyme A hydrolase	3.18E-144
Protein metabolism	DW508235.1	1.578	4.23E-03	At5g65940.1	3-hydroxyisobutyryl-coenzyme A hydrolase	3.18E-144
Protein metabolism	CO125254	1.735	2.95E-02	At4g34030.1	3-methylcrotonyl-CoA carboxylase non-biotinylated subunit	0
Protein metabolism	DT463182	2.11	1.33E-02	At3g59760.3	Mitochondria cysteine synthase	1.65E-16
Protein metabolism	DW228504.1	1.534	3.65E-02	At5g11880.1	diaminopimelate decarboxylase	0
Protein metabolism	CO123242	1.623	3.89E-02	At3g47340.1	glutamine-dependent asparagine synthetase	8.71E-85
Protein metabolism	CO086852	1.919	1.18E-02	At1g55880.1	pyridoxal-5'-phosphate-dependent enzyme, beta family	1.63E-136
Protein metabolism	DR458982	1.586	1.06E-02	At1g31860.1	phosphoribosyl-ATP pyrophosphohydrolase (At-IE)	1.57E-16
Protein metabolism	DT049512	1.729	2.55E-02	At4g16210.1	enoyl-CoA hydratase/isomerase family	2.67E-89
Protein metabolism	DT050039	9.65	2.24E-04	At1g64660.1	methionine/cystathionine gamma lyase -related	1.14E-69
Protein metabolism	AI728424	1.3	1.87E-02	At2g44520.1	UbiA prenyltransferase family	2.19E-124
Protein metabolism	DT050567	1.295	4.50E-02	At1g20270.1	oxidoreductase, 2OG-Fe(II) oxygenase family	4.09E-106
Protein metabolism	DT463796	1.616	5.56E-03	At5g47720.2	acetyl-CoA C-acetyltransferase	9.96E-81
Protein metabolism	DW508235.1	1.578	4.23E-03	At5g65940.1	3-hydroxyisobutyryl-coenzyme A hydrolase	3.18E-144
Protein metabolism	CO085474	1.579	6.59E-04	At5g53120.3	spermidine synthase	3.66E-12
Protein metabolism	DR457948	1.446	3.66E-02	At4g33510.1	2-dehydro-3-deoxyphosphoheptonate aldolase (DHS2)	7.43E-22
Protein metabolism	CA993771	1.717	8.79E-03	At3g26900.1	shikimate kinase family	5.00E-12
Protein metabolism	CK640599	1.817	8.69E-03	At3g26900.1	shikimate kinase family	5.00E-12
Protein metabolism	DW224301.1	1.307	1.46E-02	At5g12200.1	dihydropyrimidinase	7.03E-118
Secondary metabolism	DT468970	1.672	3.93E-03	At3g02780.1	isopentenyl-diphosphate delta-isomerase II (IPP2)	1.56E-102

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Secondary metabolism	DT463796	1.616	5.56E-03	At5g47720.2	acetyl-CoA C-acetyltransferase	9.96E-81
Secondary metabolism	DW489433.1	1.504	1.37E-02	At5g06060.1	short-chain dehydrogenase/reductase family protein	2.81E-80
Secondary metabolism	DV849305	1.35	4.79E-03	At1g65020.1	hypothetical protein	2.41E-08
Secondary metabolism	DW508496.1	1.493	2.83E-02	At2g33590.1	cinnamoyl-CoA reductase family	8.57E-67

Table 4 Relative expression of metabolism genes, down-regulated under high (42 °C) and compared to optimal (32 °C) temperatures in the growth cabinet for cultivars Sicot 53 and Sicala 45.

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Carbohydrate metabolism	AJ864707.1	0.622	2.68E-02	At4g02500.1	transferase - related	0
Carbohydrate metabolism	CO091591	0.52	2.66E-02	At3g02230.1	reversibly glycosylated polypeptide-1	6.20E-179
Carbohydrate metabolism	CO091591	0.52	2.66E-02	At3g02230.1	reversibly glycosylated polypeptide-1	6.20E-179
Carbohydrate metabolism	DV849226	0.643	2.04E-02	At5g60920.1	phytochelatin synthetase	1.64E-162
Carbohydrate metabolism	AI730691	0.423	1.39E-02	At4g31590.1	glycosyltransferase family 2	8.59E-157
Carbohydrate metabolism	CO124872	0.548	4.25E-03	At5g60920.1	phytochelatin synthetase	1.67E-156
Carbohydrate metabolism	CO074429	0.71	2.42E-02	At2g37770.1	aldo/keto reductase family	3.34E-120
Carbohydrate metabolism	DW493894.1	0.491	1.26E-02	At5g53580.1	aldo/keto reductase family	5.76E-113
Carbohydrate metabolism	CO090978	0.543	4.65E-02	At4g31590.1	glycosyltransferase family 2	2.52E-108
Carbohydrate metabolism	DT560269	0.339	2.86E-03	At5g15650.1	reversibly glycosylated polypeptide-3	4.88E-107
Carbohydrate metabolism	CO085470	0.673	4.55E-03	At5g07720.1	transferase - related	2.19E-101
Carbohydrate metabolism	DW515371.1	0.635	9.94E-03	At3g53520.2	NAD-dependent epimerase/dehydratase family	8.04E-101
Carbohydrate metabolism	AI727768	0.703	3.30E-02	At5g37850.1	pfkB type carbohydrate kinase protein family	7.54E-93
Carbohydrate metabolism	DT467596	0.629	2.72E-02	At5g57330.1	aldose 1-epimerase family	1.27E-90
Carbohydrate metabolism	DW230406.1	0.348	2.73E-02	At3g29030.1	expansin, putative (EXP5)	3.87E-90
Carbohydrate metabolism	DW230406.1	0.309	1.18E-02	At3g29030.1	expansin, putative (EXP5)	3.87E-90
Carbohydrate metabolism	CO123503	0.496	2.62E-03	At3g53520.2	NAD-dependent epimerase/dehydratase family	3.59E-89
Carbohydrate metabolism	AI730914	0.555	3.99E-02	At2g21250.1	mannose 6-phosphate reductase (NADPH-dependent)	7.52E-81
Carbohydrate metabolism	DW487656.1	0.514	2.29E-02	At1g74910.2	ADP-glucose pyrophosphorylase family	2.37E-71
Carbohydrate metabolism	DW502713.1	0.734	2.09E-02	At2g36850.1	callose synthase (1,3-beta-glucan synthase) family	3.44E-66

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Carbohydrate metabolism	CO101937	0.675	2.66E-02	At1g53840.1	pectinesterase family	3.71E-62
Carbohydrate metabolism	DW515687.1	0.572	2.17E-02	At4g03550.1	callose synthase (1,3-beta-glucan synthase) family	1.58E-61
Carbohydrate metabolism	CO121156	0.569	8.20E-03	At2g16730.1	glycosyl hydrolase family 35 (beta-galactosidase)	1.65E-61
Carbohydrate metabolism	CO072739	0.619	1.70E-02	At4g24780.1	polysaccharide lyase family 1 (pectate lyase)	1.04E-60
Carbohydrate metabolism	DT047184	0.492	2.68E-02	At3g59480.1	fructokinase	1.84E-58
Carbohydrate metabolism	AW187367	0.672	2.78E-02	At5g61760.1	inositol polyphosphate 6-/3-/5-kinase 2b (IPK2b)	3.46E-58
Carbohydrate metabolism	DT461699	0.589	4.92E-02	At5g51970.1	L-iditol 2-dehydrogenase (sorbitol dehydrogenase)	1.07E-56
Carbohydrate metabolism	CO123504	0.729	2.43E-02	At3g53520.2	NAD-dependent epimerase/dehydratase family	3.75E-49
Carbohydrate metabolism	DW502455.1	0.66	1.16E-02	At3g47810.1	calcineurin-like phosphoesterase family	1.06E-43
Carbohydrate metabolism	AI729695	0.531	3.34E-02	At5g03760.1	glycosyltransferase family 2	6.08E-41
Carbohydrate metabolism	DT048325	0.65	3.44E-02	At5g07370.3	inositol polyphosphate 6-/3-/5-kinase 2a (IPK2a)	7.61E-30
Carbohydrate metabolism	AI727392	0.692	4.13E-02	At4g39210.1	glucose-1-phosphate adenylyltransferase, large subunit 3	1.43E-26
Carbohydrate metabolism	DW496260.1	0.28	2.62E-04	At2g40835.1	4-alpha-glucanotransferase -related	1.08E-24
Carbohydrate metabolism	DT048308	0.346	3.08E-04	At2g40840.1	glycosyl hydrolase family 77 (4-alpha-glucanotransferase)	2.73E-20
Carbohydrate metabolism	DT467597	0.767	2.98E-02	At5g57330.1	aldose 1-epimerase family	7.14E-20
Carbohydrate metabolism	DT462436	0.505	1.63E-02	At5g15650.1	reversibly glycosylated polypeptide-3	6.09E-09
Electron flow / ATP	DW520074.1	0.431	2.05E-02	At5g21105.1	L-ascorbate oxidase	0
Electron flow / ATP	AI727260	0.508	1.41E-02	At1g65930.1	isocitrate dehydrogenase [NADP+]	0
Electron flow / ATP	DT570696	0.565	7.93E-03	At3g52990.1	pyruvate kinase	0
Electron flow / ATP	CO079284	0.813	4.76E-02	At1g65930.1	isocitrate dehydrogenase [NADP+]	0
Electron flow / ATP	AI729460	0.644	6.68E-03	At5g56350.1	pyruvate kinase	1.34E-180

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Electron flow / ATP	DN758099	0.731	3.86E-02	At5g43330.1	malate dehydrogenase, cytosolic	3.27E-157
Electron flow / ATP	DR460112	0.67	4.62E-02	At3g02360.2	6-phosphogluconate dehydrogenase -related	8.07E-153
Electron flow / ATP	CA992949	0.546	1.08E-02	At2g22500.1	mitochondrial carrier protein family	3.32E-113
Electron flow / ATP	CD486706	0.528	1.06E-02	At4g35260.1	isocitrate dehydrogenase [NAD+] subunit 1	5.35E-108
Electron flow / ATP	DW482562.1	0.555	2.53E-02	At1g63940.2	monodehydroascorbate reductase	9.39E-103
Electron flow / ATP	AI731438	0.475	1.89E-03	At5g58970.1	uncoupling protein (AtUCP2)	1.58E-101
Electron flow / ATP	DN779701	0.55	5.39E-04	At3g12290.1	tetrahydrofolate dehydrogenase/cyclohydrolase	6.82E-81
Electron flow / ATP	CO122289	0.648	2.33E-02	At1g12230.1	expressed protein	5.45E-77
Electron flow / ATP	CO121880	0.701	4.73E-02	At5g05980.1	folylpolyglutamate synthase (fpgs2)	1.54E-62
Electron flow / ATP	DR457529	0.576	2.44E-02	At4g29210.1	gamma-glutamyltransferase	5.58E-58
Electron flow / ATP	DW233286.1	0.747	4.73E-02	At3g54110.1	uncoupling protein (ucp/PUMP)	8.49E-58
Electron flow / ATP	DR452595	0.694	4.08E-02	At5g13420.1	transaldolase	2.85E-54
Electron flow / ATP	DT049717	0.614	6.12E-03	At5g56350.1	pyruvate kinase	3.35E-25
Electron flow / ATP	DT462190	0.595	2.83E-02	At1g26340.1	cytochrome b5, putative	4.70E-25
Electron flow / ATP	DW516468.1	0.692	2.25E-02	At1g36370.1	hydroxymethyltransferase -related	4.32E-23
Electron flow / ATP	DW516468.1	0.692	2.25E-02	At1g36370.1	hydroxymethyltransferase -related	4.32E-23
Electron flow / ATP	DW516487.1	0.614	1.51E-03	At3g12290.1	tetrahydrofolate dehydrogenase/cyclohydrolase	8.63E-18
Electron flow / ATP	DN804599	0.776	3.05E-02	At2g06520.1	expressed protein	3.76E-12
Electron flow / ATP	DW502424.1	0.559	1.26E-02	At2g30570.2	photosystem II reaction center 6.1KD protein	7.97E-10
Lipid Metabolism	CO088408	0.743	4.88E-02	At5g05580.1	omega-3 fatty acid desaturase, chloroplast precursor (FAD8)	3.96E-82
Lipid Metabolism	DW483924.1	0.702	4.25E-03	At4g38570.1	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	5.43E-81

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Lipid Metabolism	DW497734.1	0.624	2.64E-03	At5g46800.1	mitochondrial carrier protein	3.89E-73
Lipid Metabolism	DW512425.1	0.637	7.43E-03	At2g20900.2	diacylglycerol kinase -related	5.07E-59
Lipid Metabolism	CA993116	0.407	9.27E-03	At1g54580.1	acyl carrier protein (ACP), chloroplast	1.12E-25
Not assigned/ Miscellaneous	CD485841	0.587	4.39E-02	At5g17380.1	2-hydroxyphytanoyl-CoA lyase-related protein	5.71E-79
Not assigned/ Miscellaneous	DT048493	0.723	3.55E-02	At5g17380.1	2-hydroxyphytanoyl-CoA lyase-related protein	9.60E-44
Protein metabolism	CO125029	0.64	1.26E-02	At5g11520.1	aspartate aminotransferase, chloroplast (transaminase A/Asp3)	0
Protein metabolism	DR457196	0.489	1.96E-02	At1g02500.2	s-adenosylmethionine synthetase	0
Protein metabolism	CO121832	0.543	1.17E-02	At5g17920.1	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0
Protein metabolism	CO085576	0.514	1.85E-02	At5g17920.1	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0
Protein metabolism	DT051155	0.425	3.34E-02	At4g13940.1	adenosylhomocysteinase	0
Protein metabolism	CD486388	0.498	3.38E-02	At4g13940.1	adenosylhomocysteinase	0
Protein metabolism	DT047727	0.694	4.61E-03	At3g54470.1	UMP synthase	0
Protein metabolism	AI726035	0.533	1.21E-02	At1g48850.1	chorismate synthase	0
Protein metabolism	DT463094	0.508	2.25E-02	At5g53460.1	glutamate synthase [NADH], chloroplast	2.05E-169
Protein metabolism	CO125856	0.646	2.64E-02	At5g07440.1	glutamate dehydrogenase 2	3.26E-169
Protein metabolism	CO109339	0.655	2.06E-02	At1g79230.1	mercaptopyruvate sulfurtransferase (Mst1/Rdh1)	1.50E-158
Protein metabolism	DT567362	0.495	4.87E-02	At2g02010.1	glutamate decarboxylase	4.22E-147
Protein metabolism	DT046700	0.68	4.36E-02	At5g17920.1	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	1.02E-142

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Protein metabolism	DR458557	0.534	7.24E-03	At5g18280.1	apyrase	2.03E-141
Protein metabolism	DT561935	0.611	2.55E-02	At4g34200.1	D-3-phosphoglycerate dehydrogenase (3-PGDH)	8.26E-126
Protein metabolism	DT046719	0.727	1.22E-02	At5g18900.1	oxidoreductase, 2OG-Fe(II) oxygenase family	1.07E-125
Protein metabolism	CO091894	0.618	2.06E-02	At3g15290.1	3-hydroxybutyryl-CoA dehydrogenase	1.81E-119
Protein metabolism	CO092415	0.514	3.33E-02	At3g53620.1	inorganic pyrophosphatase	2.53E-108
Protein metabolism	DN760012	0.629	1.51E-02	At1g15690.1	inorganic pyrophosphatase -related	4.84E-104
Protein metabolism	DW477912.1	0.547	4.14E-02	At3g17760.1	glutamate decarboxylase	2.60E-102
Protein metabolism	CO091240	0.558	4.76E-02	At2g17630.1	phosphoserine aminotransferase -related	2.92E-102
Protein metabolism	AF009568.1	0.478	4.64E-03	At1g15690.1	inorganic pyrophosphatase -related	5.26E-97
Protein metabolism	CO497384	0.513	1.61E-02	At3g23580.1	ribonucleoside-diphosphate reductase small chain	3.91E-95
Protein metabolism	AI055377	0.49	3.33E-02	At5g11520.1	aspartate aminotransferase, chloroplast (transaminase A/Asp3)	2.45E-91
Protein metabolism	DT456038	0.446	5.90E-03	At5g11160.1	adenine phosphoribosyltransferase	2.39E-85
Protein metabolism	DW229616.1	0.627	2.54E-02	At2g16500.1	arginine decarboxylase	9.80E-84
Protein metabolism	AI727870	0.7	3.58E-02	At5g19550.1	aspartate aminotransferase, cytoplasmic isozyme 1	9.80E-79
Protein metabolism	DW227402.1	0.209	1.34E-02	At3g22890.1	ATP sulfurylase -related	4.73E-78
Protein metabolism	DW509152.1	0.426	8.88E-04	At3g53620.1	inorganic pyrophosphatase -related protein	4.90E-77
Protein metabolism	CO086679	0.616	8.79E-03	At1g15690.1	inorganic pyrophosphatase	2.79E-68
Protein metabolism	DT527501	0.539	4.46E-02	At3g46940.1	dUTP pyrophosphatase-related protein	9.01E-65
Protein metabolism	DW502728.1	0.551	2.20E-03	At1g19920.1	sulfate adenylyltransferase	3.04E-62
Protein metabolism	DT543331	0.772	2.11E-02	At1g22410.1	2-dehydro-3-deoxyphosphoheptonate aldolase	5.28E-56
Protein metabolism	DW510782.1	0.565	2.31E-02	At4g34200.1	D-3-phosphoglycerate dehydrogenase (3-PGDH)	6.22E-54

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Protein metabolism	AI728979	0.704	3.22E-02	At5g10330.1	histidinol-phosphate aminotransferase	6.69E-51
Protein metabolism	AI728979	0.704	3.22E-02	At5g10330.1	histidinol-phosphate aminotransferase	6.69E-51
Protein metabolism	DT560839	0.439	1.68E-02	At1g19920.1	sulfate adenylyltransferase	1.19E-45
Protein metabolism	DT463008	0.197	1.52E-02	At3g22890.1	ATP sulfurylase -related	3.97E-37
Protein metabolism	AI731024	0.452	2.51E-03	At5g11160.1	adenine phosphoribosyltransferase	3.63E-36
Protein metabolism	DT051246	0.525	2.60E-02	At2g36880.1	s-adenosylmethionine synthetase -related	4.49E-36
Protein metabolism	DW498800.1	0.232	8.23E-04	At1g15410.1	expressed protein (Aspartate-glutamate racemase family)	5.52E-36
Protein metabolism	DW498800.1	0.232	8.23E-04	At1g15410.1	expressed protein (Aspartate-glutamate racemase family)	5.52E-36
Protein metabolism	CO126813	0.687	4.48E-02	At2g43750.1	cysteine synthase, chloroplast	1.83E-34
Protein metabolism	CO091929	0.469	4.96E-02	At4g13940.1	adenosylhomocysteinase	4.04E-30
Protein metabolism	DW516468.1	0.692	2.25E-02	At1g36370.1	hydroxymethyltransferase -related	4.32E-23
Protein metabolism	DT049585	0.59	2.82E-02	At1g09795.1	AtATP-PRT2 mRNA for ATP phosphoribosyl transferase	6.29E-11
Protein metabolism	CA993525	0.367	3.61E-03	-	-	-
Protein metabolism	CA993525	0.367	3.61E-03	-	-	-
Protein metabolism	DV849287	0.179	1.13E-02	-	-	-
Protein metabolism	DV849287	0.179	1.13E-02	-	-	-
Secondary metabolism	CO090127	0.718	4.65E-02	At1g68530.1	very-long-chain fatty acid condensing enzyme (CUT1)	0
Secondary metabolism	CO125011	0.766	2.06E-02	At1g44446.1	chlorophyll a oxygenase (chlorophyll b synthase)	1.01E-145
Secondary metabolism	CO091962	0.465	5.81E-05	At4g20840.1	FAD-linked oxidoreductase	6.77E-144
Secondary metabolism	BQ404875	0.376	4.79E-02	At5g22020.1	strictosidine synthase	6.91E-141
Secondary metabolism	AI728347	0.617	5.92E-03	At4g30210.1	NADPH-ferrihemoprotein reductase	8.60E-127

Major pathway	Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Secondary metabolism	DR455920	0.641	6.09E-03	At5g57800.1	CER1 protein	6.26E-122
Secondary metabolism	CO496470	0.697	2.49E-02	At1g67730.1	short chain dehydrogenase/reductase family protein	3.47E-113
Secondary metabolism	CD486444	0.528	3.14E-02	At2g38700.1	mevalonate diphosphate decarboxylase	9.66E-75
Secondary metabolism	CO126046	0.657	1.08E-02	At5g55350.1	long-chain-alcohol O-fatty-acyltransferase (wax synthase)	9.56E-74
Secondary metabolism	DR463129	0.566	2.31E-02	At5g54010.1	glycosyltransferase family	1.60E-68
Secondary metabolism	AI729300	0.244	3.35E-03	At1g02190.1	CER1 protein	6.14E-67
Secondary metabolism	DT547279	0.709	2.26E-02	At1g77670.1	aminotransferase family	1.40E-66
Secondary metabolism	CO083496	0.676	4.04E-02	At2g23910.1	cinnamoyl-CoA reductase-related	2.22E-64
Secondary metabolism	CO084738	0.504	3.62E-02	At4g12330.1	cytochrome P450 family	5.32E-63
Secondary metabolism	AI730798	0.312	2.32E-02	At5g22020.1	strictosidine synthase family	1.33E-62
Secondary metabolism	CO123007	0.262	4.58E-02	At5g14700.1	cinnamoyl-CoA reductase-related	3.46E-61
Secondary metabolism	AI728058	0.371	4.23E-03	At5g17050.1	glycosyltransferase family	3.02E-50
Secondary metabolism	DR452522	0.697	2.29E-02	At2g23910.1	cinnamoyl-CoA reductase-related	5.89E-47
Secondary metabolism	DT049270	0.264	4.04E-02	At2g37700.1	CER1 protein	3.20E-28
Secondary metabolism	AI054687	0.281	4.55E-02	At1g08470.1	strictosidine synthase-related	3.92E-18
Secondary metabolism	DT052242	0.483	2.54E-02	-	-	-

Table 5 Relative expression (>2 fold) of genes associated with mitochondrial electron transport for cultivars Sicot 53 and Sicala 45 plants grown under high (42 ^{o}C) temperatures, compared to optimal (32 ^{o}C) temperatures in the growth cabinet.

Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Up-regulated genes					
DT456151	1.823	2.53E-02	At2g43400.1	electron transfer flavo protein ubiquinone oxidoreductase -related	5.68E-66
CO091076	1.558	2.83E-02	At5g17400.1	mitochondrial ADP, ATP carrier protein	1.85E-34
Down-regulated genes					
CO127818	0.4801	3.02E-02	At5g14040.1	mitochondrial phosphate transporter	1.69E-105
AI731438	0.475	1.89E-03	At5g58970.1	uncoupling protein (AtUCP2)	1.58E-101
CA992949	0.546	1.08E-02	At2g22500.1	mitochondrial carrier protein family	3.32E-113
DW500449.1	0.639	6.91E-02	At4g10040.1	cytochrome c several plant cytochrome c	9.94E-56
DW512717.1	0.641	6.70E-02	At2g29990.1	NADH dehydrogenase family	2.88E-91
DR463298	0.569	3.19E-02	At2g22500.1	mitochondrial carrier protein family	5.76E-104
DT053610	0.669	3.99E-02	At4g01100.1	mitochondrial carrier protein family	7.09E-23
DW233179.1	0.675	2.32E-02	At3g62650.1	expressed protein putative mitochondrial carrier protein	9.79E-25
AI726701	0.649	4.57E-02	At5g14040.1	mitochondrial phosphate transporter	6.03E-98
BQ412199	0.631	9.94E-03	At5g19760.1	mitochondrial 2-oxoglutarate/malate translocator	2.42E-125
DW485545.1	0.689	3.12E-02	At2g47490.1	mitochondrial carrier protein family	1.46E-57

Table 6 Relative expression (2-fold) of genes associated with the photosynthetic pathway for Sicot 53 and Sicala 45 plants grown under high (42 °C) temperatures compared to optimal (32 °C) temperatures in the growth cabinet.

Public ID	Relative expression	Adjusted P value	Arabidopsis hit	Arabidopsis hit description	E-value
Up-regulated genes					
DN800322	2.237	1.20E-02	At1g06680.1	Oxygen-evolving enhancer protein 2; calcium ion binding	5.11E-85
CO072814	2.449	3.50E-03	At2g28000.1	RuBisCO subunit binding-protein α subunit, chloroplast 60 kDa chaperonin	1.67E-154
CO121719	2.455	1.37E-02	At2g28000.1	RuBisCO subunit binding-protein α subunit, chloroplast 60 kDa chaperonin	3.75E-107
DR458096	2.003	4.73E-04	At2g28000.1	ATP binding protein binding	0
DV849478	3.338	2.27E-05	At2g28000.1	RuBisCO subunit binding-protein alpha subunit/60 kDa chaperonin alpha	8.87E-23
Down-regulated genes					
DV848944	0.344	1.68E-01	At1g42970.1	glyceraldehyde-3-phosphate dehydrogenase B subunit	0

Table 7 Genes for Sicot 53 that are up-regulated or down-regulated in response to heat stress in the growth cabinet

Pathway	Public ID	Relative expression	P value	Arabidopsis Hit Name	Description	E-Value
Up-regulated genes						
Lipid metabolism	DR458005	2.31	4.87E-02	-	-	-
Miscellaneous	AI729487	3.16	4.57E-02	At5g53430.1	trithorax 5 (TX5)	3.22E-163
Not assigned	CA993412	2.47	4.57E-02	-	-	-
Not assigned	DT465816	3.16	4.57E-02	-	-	-
Not assigned	DW507443.1	2.98	4.69E-02	At2g19310.1	small heat shock protein -related	2.36E-24
Not assigned	AI727913	3.31	4.87E-02	At1g70090.1	glycosyltransferase family	5.08E-132
Not assigned	BM358962	3.12	4.45E-02	-	-	-
Not assigned	DR456718	3.27	4.87E-02	-	-	-
Not assigned	DR460676	2.25	4.84E-02	-	-	-
Not assigned	DT049773	20.35	4.45E-02	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Not assigned	DT050039	6.52	4.57E-02	At1g64660.1	methionine/cystathionine gamma lyase	1.14E-69
Not assigned	DT050385	5.15	4.69E-02	At3g23990.1	chaperonin (CPN60/HSP60), mitochondrial precursor	1.35E-10
Not assigned	DT458200	3.35	4.57E-02	-	-	-
Not assigned	DT467180	9.02	4.57E-02	At5g12020.1	class II heat shock protein	1.12E-35
Not assigned	DW503697.1	89.07	4.45E-02	At1g07400.1	heat shock protein	1.40E-49
Not assigned	DW520166.1	2.76	4.69E-02	At2g31080.1	reverse transcriptase family protein	1.57E-10
Protein metabolism	DN781218	13.36	4.57E-02	At5g07330.1	68412.m00774 expressed protein	2.33E-32
Protein metabolism	DT456271	2.82	4.45E-02	-	-	-
Protein metabolism	DW509391.1	5.83	4.69E-02	At3g25270.1	68410.m02887 hypothetical protein	2.90E-08
Protein metabolism	DW511109.1	4.25	4.84E-02	-	-	-
Stress	DT046994	4.81	4.69E-02	At3g16050.1	ethylene-inducible protein	5.09E-24

Pathway	Public ID	Relative expression	P value	Arabidopsis Hit Name	Description	E-Value
Stress	DT048069	2.52	4.69E-02	At2g41540.2	glycerol-3-phosphate dehydrogenase	6.12E-19
Stress	DW506829.1	3.41	4.45E-02	At5g65260.1	RNA recognition motif (RRM)	1.68E-90
Down-regulated genes						
Not assigned	CO076413	0.07	4.57E-02	At5g54770.1	thiazole biosynthetic enzyme precursor (ARA6) (sp Q38814)	2.81E-140
Protein metabolism	CA993457	0.06	4.57E-02	At5g54770.1	thiazole biosynthetic enzyme precursor (ARA6) (sp Q38814)	3.59E-142
Stress	DT054070	0.25	4.57E-02	At2g21660.2	glycine-rich RNA-binding protein (AtGRP7) SP|Q03250	3.74E-38
Stress	DT461768	0.26	4.45E-02	At2g21660.2	glycine-rich RNA-binding protein (AtGRP7) SP|Q03250	2.52E-36

Table 8 Genes for Sicala 45 that are up-regulated or down-regulated under high temperature stress in the growth cabinet

Pathways	Public ID	Relative expression	P value	Arabidopsis Hit	Description	E-Value
Up-regulated genes						
Carbohydrate metabolism	DT465978	19.62	4.44E-02	At2g29500.1	small heat shock protein -related	1.39E-56
Cell & Development	DN760147	2.87	1.99E-02	-	-	-
Lipid metabolism	DR453388	5.01	4.44E-02	-	-	-
Not assigned	DR460975	2.47	4.10E-02	-	-	-
Not assigned	DT047239	4.25	1.99E-02	-	-	-
Not assigned	DT050039	14.78	1.99E-02	At1g64660.1	methionine/cystathionine gamma lyase	1.14E-69
Not assigned	DT465816	3.37	4.03E-02	-	-	-
Not assigned	DW506829.1	4.12	2.71E-02	At5g65260.1	RNA recognition motif (RRM) - containing protein	1.68E-90
Not assigned	AI727895	2.18	4.03E-02	At1g16210.1	For proteins ESTs gb|T04357 and gb|AA595092	4.85E-52
Not assigned	DN757824	18.36	2.26E-02	At5g37670.1	heat shock protein family	2.20E-38
Not assigned	DN759807	3.03	4.10E-02	-	-	-
Not assigned	DN760566	14.87	4.33E-02	At5g52640.1	heat shock protein 81-1	7.11E-76
Not assigned	DR455377	2.32	4.03E-02	-	-	-
Not assigned	DR460676	2.68	3.97E-02	-	-	-
Not assigned	DT050385	4.75	2.27E-02	At3g23990.1	chaperonin (CPN60/HSP60)	1.35E-10
Not assigned	DT456529	2.74	4.44E-02	At2g23090.1	expressed protein	6.34E-12
Not assigned	DT463334	8.43	1.54E-02	-	-	-
Not assigned	DT463678	2.65	4.10E-02	-	-	-
Not assigned	DT463965	11.03	3.97E-02	-	-	-
Not assigned	DT545357	3.2	2.86E-02	At4g25200.1	mitochondrion-localized small heat shock protein	4.99E-62
Not assigned	DW493548.1	3.13	4.57E-02	At4g02450.1	glycine-rich protein	1.11E-30

Pathways	Public ID	Relative expression	P value	Arabidopsis Hit	Description	E-Value
Not assigned	DW501150.1	3.38	3.97E-02	At1g14980.1	10 kDa chaperonin (CPN10)	4.21E-41
Not assigned	DW503063.1	34.94	4.93E-02	At5g52640.1	heat shock protein 81-1	2.42E-112
Not assigned	DW506473.1	10.2	3.97E-02	At5g18650.1	expressed protein	3.33E-114
Protein metabolism	DN780838	3.26	3.97E-02	At3g03150.1	expressed protein	5.74E-28
Protein metabolism	DR455485	3.07	3.97E-02	At1g01940.1	expressed protein	2.73E-79
Protein metabolism	DT048450	2.71	4.03E-02	At2g32520.1	carboxymethylenebutenolidase -related	3.65E-105
Protein metabolism	CA992712	7.12	3.32E-02	At5g52640.1	heat shock protein 81-1	8.99E-66
Protein metabolism	CO087722	3.13	1.99E-02	At2g32520.1	carboxymethylenebutenolidase -related	3.46E-111
Protein metabolism	DW503054.1	5.67	2.26E-02	-	-	-
Protein metabolism	DW503697.1	96.75	1.54E-02	At1g07400.1	heat shock protein, putative	1.40E-49
Protein metabolism	DT052182	2.71	4.44E-02	At2g31080.1	reverse transcriptase family protein	7.86E-06
Protein metabolism	DR458062	2.28	3.32E-02	At5g27620.1	cyclin family similar to SP|P51946 Cyclin H	5.01E-37
Protein metabolism	DT464151	2.49	3.95E-02	-	-	-
Protein metabolism	DW520572.1	2.63	4.10E-02	-	-	-
Protein metabolism	CO123341	2.23	4.44E-02	At1g62740.1	stress inducible protein (sti), putative	9.59E-82
Protein metabolism, Cell & development	DT048369	3.36	4.44E-02	-	-	-
Protein metabolism, Cell & development	CO132723	5.02	1.99E-02	At4g11660.1	heat shock factor protein 7 (HSF7)	1.05E-57
Secondary metabolism	AI731478	5.06	1.99E-02	At5g02810.1	pseudo-response regulator, APRR7	6.41E-22
Secondary metabolism	CA992719	56.33	3.93E-02	At4g10250.1	endomembrane-localized small heat shock protein	3.92E-31
Secondary metabolism	DW505128.1	6.98	4.44E-02	At4g27670.1	small heat shock protein, chloroplast precursor(HSP21)	1.96E-25
Secondary metabolism	DW514802.1	3.67	4.44E-02	-	-	-

Pathways	Public ID	Relative expression	P value	Arabidopsis Hit	Description	E-Value
Signaling & transport	DT457280	2.94	3.97E-02	-	-	-
Stress	DR459244	3.48	3.92E-02	-	-	-
Stress	DT047015	3.29	3.97E-02	At5g20720.2	chloroplast Cpn21 protein	1.00E-45
Stress	DT049773	25.71	4.23E-02	At2g32120.2	heat shock protein hsp70t-2	6.89E-45
Stress	DT456083	2.53	4.44E-02	-	-	-
Stress	DT456194	2.35	4.44E-02	-	-	-
Stress	DT458200	3.16	4.44E-02	-	-	-
Stress	DV850132	15.42	4.44E-02	At5g47220.1	ethylene responsive element binding factor 2	7.88E-43
Stress, Protein metabolism	DR454255	4.14	3.95E-02	At1g80160.1	glyoxalase family protein	1.02E-63
Stress, Protein metabolism	DW511109.1	4.35	4.44E-02	-	-	-
Down-regulated genes						
Cell & Development	DW485677.1	0.47	4.10E-02	At2g18960.1	ATPase 1, plasma membrane-type	0
Miscellaneous	DT051905	0.26	4.44E-02	At2g01830.2	histidine kinase -related	6.46E-58
Not assigned	AI727247	0.42	3.32E-02	At4g39220.1	AtRer1A	1.76E-72
Not assigned	CA993457	0.04	1.54E-02	At5g54770.1	thiazole biosynthetic enzyme precursor (ARA6)	3.59E-142
Not assigned	CO127856	0.24	1.54E-02	At5g08260.1	serine carboxypeptidase II	4.51E-165
Not assigned	DR457567	0.34	4.10E-02	At4g16370.1	isp4 like protein	1.69E-151
Not assigned	DW225341.1	0.24	4.44E-02	-	-	-
Not assigned	DR455218	0.39	3.32E-02	At5g03300.1	pfkB type carbohydrate kinase protein family	6.49E-82
Not assigned	DT048453	0.19	4.23E-02	At2g46210.1	delta-8 sphingolipid desaturase	4.44E-52
Not assigned	DT052025	0.2	4.10E-02	-	-	-
Not assigned	DT054070	0.2	4.44E-02	At2g21660.2	glycine-rich RNA-binding protein (AtGRP7)	3.74E-38
Protein metabolism	DW224339.1	0.24	4.03E-02	At5g27150.1	sodium proton exchanger (NHX1)	8.76E-15

Pathways	Public ID	Relative expression	P value	Arabidopsis Hit	Description	E-Value
Protein metabolism	DN799904	0.47	4.10E-02	At4g03230.1	receptor kinase -related	4.75E-101
Protein metabolism	DT461573	0.48	4.10E-02	-	-	-
Protein metabolism	DW225422.1	0.3	4.44E-02	At1g17345.1	auxin-induced (indole-3-acetic acid induced) protein	1.60E-25
Protein metabolism, Cell & development	DN779370	0.12	1.99E-02	At1g15060.1	expressed protein	1.88E-72
Secondary metabolism	DW513352.1	0.49	4.44E-02	At1g50430.1	sterol delta-7 reductase (7-dehydrocholesterol reductase)	2.93E-45
Secondary metabolism	CO091962	0.43	4.44E-02	At4g20840.1	FAD-linked oxidoreductase family	6.77E-144
Secondary metabolism	DW503233.1	0.47	4.03E-02	At3g02750.1	protein phosphatase 2C (PP2C)	2.62E-114
Signaling & transport	DT048308	0.29	4.33E-02	At2g40840.1	glycosyl hydrolase family 77	2.73E-20
Stress	CO076413	0.04	1.99E-02	At5g54770.1	thiazole biosynthetic enzyme precursor (ARA6)	2.81E-140
Stress	DT461768	0.23	4.05E-02	At2g21660.2	glycine-rich RNA-binding protein (AtGRP7)	2.52E-36
Stress	DW510614.1	0.37	3.92E-02	At2g17610.1	reverse transcriptase family protein	1.91E-12