Combined elevated temperature and soil waterlogging stresses inhibit cell elongation by altering osmolyte composition of the developing cotton (*Gossypium hirsutum* L.) fiber

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**A B S T R A C T**

Soil waterlogging events and high temperature conditions occur frequently in the Yangtze River Valley, yet the effects of these co-occurring stresses on fiber elongation have received little attention. In the current study, the combined effect of elevated temperature (ET) and soil waterlogging (SW) more negatively affected final fiber length (reduced by 5.4%–11.3%) than either stress alone by altering the composition of osmotically active solutes (succrose, malate, and K⁺), where SW had the most pronounced effect. High temperature accelerated early fiber development, but limited the duration of elongation, thereby limiting final fiber length. Treatment of ET alone altered fiber succrose content mainly through decreased source strength and the expression of the succrose transporter gene *GhSUT-1*, making succrose availability the primary determinant of final fiber length under ET. Waterlogging stress alone decreased source strength, down-regulated *GhSUT-1* expression and enhanced SuSy catalytic activity for succrose reduction. Waterlogging treatment alone also limited fiber malate production by down-regulating *GhPEPC-1* and elevated temperature and waterlogging limited primary cell wall synthesis by affecting *GhCESAs* genes and showed a negative impact on all three major osmotic solutes through the regulation of *GhSUT-1*, *GhPEPC-1* and *GhKT-1* expression and altered SuSy activity, which functioned together to produce a shorter final fiber length.

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1. Introduction

Heavy rainfall events are occurring with greater frequency and are accompanied by high temperature from May–October in the middle and lower reaches of the Yangtze River, China; furthermore, global temperatures are expected to increase by 2–4 °C in the coming decades [1,2]. Thus, it is expected that co-occurring waterlogging and high temperature stress will have pronounced, negative effects on crop production in this region.

A number of researchers have reported the negative impacts on cotton yield and fiber quality in response to either high temperature [3–5] or waterlogging [6–8] stresses individually, whereas the combined effects of these two stresses are not well studied. For example, it was reported that temperature affected fiber quality more than other meteorological factors [9]. Liakatas showed that fiber length, strength, uniformity and micronaire value were affected by high temperature, especially high daytime temperature [10]. Gipson found the initial stage of fiber elongation was more sensitive to high night temperature [11], and the optimum night temperature range for fiber elongation was between 15 and 21 °C [12]. Waterlogging also showed negative effects on fiber length and strength [13] by changing sucrose metabolism enzyme activities and relative gene expression thereof [14]. The co-effects of high temperature and waterlogging decreased fiber length and increased fiber micronaire value [15], and high temperature exposure was found to decrease fiber length even after removal of the waterlogging stress [16].

As the most important agricultural textile commodity, upland cotton (*Gossypium hirsutum* L.) fiber has been a good model for exploring cell elongation because of its distinct developmental stages including initiation, elongation, secondary cell wall
biosynthesis and maturation [17,18]. Fiber usually elongates rapidly from 0 to 16 DPA (days post anthesis), during which the primary cell wall is deposited [19]. At about 14–16 DPA, the fiber elongation rate begins to slow down with the start of secondary cell wall synthesis, and stops usually at approximately 20–24 DPA [20,21]. It is widely accepted that cell turgor pressure [20,22] and many other factors play important roles in the fiber elongation process. For example, cell turgor is impacted by the transport and metabolism of osmotically active solutes [22,23], the energy required for the transmembrane transport of osmolytes [24], expression of channel proteins, and expression of genes encoding proteins required for cell wall loosening [23,25,26]. Additionally, plasmodesmata (PD) were reported as gateways controlling the generation of fiber cell turgor, which are usually closed between 10 and 15 DPA to block macromolecules from transferring outward. With the high expression of sucrose transporter (SUT) and K+ transporter (KT), osmotically active solutes are highly accumulated in fiber, which leads to increased fiber cell turgor and rapid fiber cell elongation [27]. Both a higher rate and a longer duration of fiber elongation would allow a fiber cell to elongate to a greater final fiber length, which is an important fiber quality characteristic in cotton [20,28]. Soluble sugars, malate, and potassium (K+), together account for about 80% of sap osmolality inside the fiber, and are regarded as the major osmotically active solutes [22,29,30]. Previous studies have shown that soluble sugars and K+ were imported into the cotton fiber from the phloem of the seed coat, whereas malate was synthesized locally through the initial activity of phosphoenolpyruvate carboxylase (PEPC) in fiber cytoplasm [20,24]. Sucrose has been considered the main form of soluble sugar transferred into the fiber and its transport is mediated by sucrose transporter (SUT), while K+ transporters (KT) import K+ into elongating fibers. Besides, pectin precursors have also been reported to play a role in affecting root hair elongation in Arabidopsis [31] and controlling the cell extensibility and elongation in ripening fruits [32].

Most researchers focused on the relation between fiber elongation and sucrose metabolism [33,34], because sucrose supplies energy for fiber elongation in addition to its role as a major osmotically active solute [14]. In elongating fiber cells, sucrose was mainly degraded by sucrose synthase (SuSy, E.C. 2.4.1.13) and vacuolar invertase (VIN, E.C. 3.2.1.26) [23,35]. Ruan showed that fiber elongation was affected by suppression of SuSy gene expression in transgenic cotton (Gossypium hirsutum) ovules [35], and the decreased fiber length was related to the lower SuSy activity under low light [34]. Additionally, VIN was reported to play a role in early fiber elongation by osmotic regulation of vacuole enlargement [23]. Sucrose is the form of carbon universally used for long distance carbon transport in vascular plants employing the apoplastic pathway of phloem loading, and its transporters have vital roles in plant growth and development. Many types of sucrose transporters have been discovered since SoSUT-1 was first identified by Riesmeier et al. using a screening system with yeast mutant [36,37]. Ruan et al. identified the essential role of sucrose transporter GhSUT-1 in the elongating cotton fiber [20]. Moreover, sucrose can also be degraded to uridine diphosphate glucose (UDPG), which is used to synthesize cellulose and callose during the secondary cell wall biosynthesis [38]. The synthesis of cellulose is regulated by cellulose synthase, while callose is synthesized by callose synthase (mainly regulated by GhCalS5) [39,40]. Li [41] reported that GhCESA1 and GhCESA8 played an important role during the secondary cell wall biosynthesis, while GhCESA3, GhCESA5, and GhCESA6 mainly worked for primary cell wall synthesis, and some recent data indicated the possible association of SuSy and CESA complexes for high cellulose production [42]. Due to the importance of K+ as an osmoregulator, field studies showed that soil K deficiency reduced K concentrations in the cotton fiber cell [43], which led to decreased final fiber length [44–46]. Similar to sucrose, K+ in the fiber cell is mainly transferred from the underlying seed coat cells by K+ transporters [20]. GhKTI-I, encodes a cotton K+ transporter, and may function along with GhSUT-1 for the maintenance of fiber cell turgor pressure required to drive fiber elongation [20,25,47]. Unlike sucrose and K+, malate was synthesized within fiber cells through phosphoenolpyruvate carboxylase (PEPC) [48]. Li et al. indicated that high activity of PEPC can affect cotton fiber elongation, likely through the expression of GnPPEC-1 and –2 genes [48]. When considering the impact of waterlogging and high temperature stress on fiber development and carbon dynamics, it is also important to document the impact of these co-occurring stresses on source strength. In cotton, greater than 60% of the carbohydrate requirement for boll development comes from the leaf that subtends the boll on a fruiting branch [49]. Thus, abiotic stresses (i.e. high temperature) that negatively impact the photosynthetic rate of the subroduct leaf also limit the carbohydrate content of the immediately adjacent reproductive structure [50] and would be expected to limit carbohydrate supply to the developing fiber.

Previous studies mainly addressed the effects of high-temperature or waterlogging individually, or focused on fiber elongation without considering how the three major osmotically active solutes respond to these abiotic stresses during fiber development. Studies that provide a comprehensive assessment of waterlogging and high temperature stress effects on fiber development in relation to source strength and osmolyte impacts on the developing fiber are to our knowledge, nonexistent. In the current study, it was hypothesized that: (1) elevated temperature (ET) and soil waterlogging (SW) would exhibit a more pronounced negative effect on fiber elongation and final fiber length than either stress in isolation; (2) that fiber elongation responses to these combined stresses will be related to source limitations, alterations in osmotically active solute content and related enzymes and genes participating in fiber elongation. Consequently, our objectives in the current study were to (1) quantify the co-effect of elevated temperature (ET) and soil waterlogging (SW) on fiber elongation and final fiber length; (2) to characterize the impact of ET, SW and combined ET and SW on the photosynthetic rate of the subroduct leaf and fiber osmotically active solutes; and (3) identify the key enzymes and gene isoforms participating in fiber elongation under combined ET and SW conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Experiments were conducted in plots (each one is 4 m in length, 4 m in width and 1.5 m in height) with a transparent waterproof top for three cotton growing seasons from 2013 to 2015 at Pailou Experimental Base (118°50′E, 32°02′N), Nanjing Agricultural University, Nanjing, China. The yellow brown soil used in plots was collected from the upper 30 cm of topsoil layer at the experimental base. The soil contained 69.5, 74.3, and 77.8 mg kg−1 alkali-hydrolysable nitrogen (N); 17.1, 20.3, 18.5 mg kg−1 phosphorus (P) and 101.4, 107.8, 112.5 mg kg−1 potassium (K), from 2013 to 2015, respectively. Cotton seeds (cv. Siza 3) were planted on 7 April in all growing seasons, and individual uniform seedlings with three true leaves were transplanted into ponds. Each pond contained 5 rows, and the inter-row and intra-row plant spacing was 75 × 25 cm, respectively.

2.2. Experimental designs and treatments

The experiment was conducted as a completely randomized and full factorial design with two temperature regimes and three levels of soil water management. Both elevated temperature regime
and waterlogging treatments were not imposed until white flowers were initially observed in the first position (FP1st) closest to the main stem along the sympodial branch at the 6th mainstem (FB6th) node above the cotyledons. The elevated temperature treatment was maintained until the boll at this position opened, while the waterlogging treatments were only imposed for 3 or 6 days, depending on the treatment.

The temperature control system [51] (OTC, Southeast Co. Ltd, Ningbo, China) was used to maintain the microclimate temperature and to make sure the temperature at 6th main stem node above the cotyledons was 2–3 °C higher for the elevated temperature zone than that of the ambient temperature zone. The real-time temperature data was recorded in 20 min intervals. The mean daytime/nighttime temperature (MDTday/MDTnight) was obtained from the three experimental years for the elevated temperature regimes 34.1/29.0 °C, while it was 31.6/26.5 °C for ambient temperature treatment during flowering and boll development. Both the elevated temperature zone and ambient temperature zone contained three water treatments, with 0, 3 and 6 days of soil waterlogging. The 0 day waterlogging treatment was the well-watered control with soil relative water content maintained at 70–80% of field capacity. The 3 and 6 day waterlogging treatments contained a 1–2 cm layer of water on the top of soil surface which lasted 3 and 6 days, and then water was removed through the opening holes at the bottom of ponds.

The treatments in this experiment were: (a) well-watered control under ambient temperature (A = 31.6/26.5 °C) (AC); (b) 3 and 6 days soil waterlogging treatments under 31.6/26.5 °C (AW3, AW6); (c) well-watered control under elevated temperature (E = 34.1/29.0 °C) (EC) and (d) 3 and 6 days soil waterlogging treatments under 34.1/29.0 °C (EW3, EW6).

2.3. Meteorological data and net photosynthesis (Pn)

The mean daily air temperature (MDT), mean daily maximum and minimum air temperature (MDT_{max}, MDT_{min}), mean daytime and night-time air temperature (MDT_{day}, MDT_{night}) from anthesis to boll opening were collected from the temperature control system during the cotton growing seasons. These data were quoted from another publication by our group [52] and were provided in Supplementary Information Table S1. Across the three experimental years, the boll development period was shortened about 1–5 days after combined elevated temperature and waterlogging treatments. The air temperature at the 6th main-stem fruiting branch in 2013 was much higher at those in 2014 and 2015 from anthesis to boll opening stage, and MDT, MDT_{day}, MDT_{night}, MDT_{max} and MDT_{min} under ambient temperature were 1.9–2.9 °C lower than those under elevated temperature from 2013 to 2015. In the year of 2013, MDT under elevated temperature was up to 33.6 °C, which was significantly higher than the 32 °C threshold defined by Balls [53] for normal cotton fiber development.

To determine the effects of elevated temperature and waterlogging treatments on subtending leaves of tagged bolls, net photosynthesis (Pn) was measured with a portable photosynthesis system (LI-6400, LI-COR, Lincoln, NE, USA) at 5, 15, 25 DPA from 0900 – 1100 at, 380 ± 5 ppm reference CO2, 65 ± 5% relative humidity, 1500 μmol m⁻²s⁻¹ photosynthetically active radiation (PAR), and chamber block temperature = air temperature. Leaves were not removed from chamber until a steady photosynthesis value was obtained.

2.4. Sampling and processing

White flowers at the sympodial fruiting position 1 at the 6th main-stem node above the cotyledons were tagged for all cotton plants, with the flowering dates and boll opening dates listed on the tags. About 6 – 8 tagged bolls were collected at 0900 – 1000 h every 5 days from 5 day post anthesis (DPA) to 25 DPA, and were used for determining fiber length and subsequent biochemical and molecular analyses. One locule from each boll was used to measure fiber length, whereas the other locules were used for chemical analyses. Fibers were excised using a scalpel in an ice bath, frozen in liquid nitrogen, and finally stored at –80 °C for the determination of enzyme activities and gene expression. Once the analyses were finished, fibers were then dried at 40 °C to a constant weight for sucrose, malate and K⁺ determination. Additionally, another 3–4 bolls were collected from 31 DPA to boll opening for fiber length determination.

2.5. Fiber length determination

The locules of each boll younger than 30 DPA was boiled in water with 0.1% HCl to separate cotton seeds and the fibers inside, after which length (mm) of the fiber was measured as described below. 3–5 seeds were randomly selected for the measurement from each locule. Each seed was placed on the convex surface of a watch glass, after which fibers were streamed out with a water jet. Fiber length was then measured using a digital caliper from the seed epidermis (at least 3 positions along the axis of the seed) to the end of the fiber. Cotton fiber after 30 DPA was dried in an oven at 60 °C for 0.5 h and then at 40 °C at least 48 h. Then, fiber was transferred to a standard testing room with constant humidity (65 ± 2%) and temperature (20 ± 2 °C) for 48 h. The length of fiber was measured with a Y-146 fiber photodometer (Taicang Electron Apparatus Co., Ltd., China). Length of fiber at boll opening was measured with the USTER HVI MF100 (Uster Technologies Co., Ltd., Switzerland) cotton fiber quality measurement system.

2.6. Enzymatic analyses

Enzyme extraction and the assay of SuSy and vacuolar invertase activity were conducted according to a modified method from King et al. [54]. Briefly, take about 0.5 g fresh weight (FW) fiber samples and ground into fine powder with liquid nitrogen. Then, the cold extraction buffer (5: 1, v/w, 4 °C) containing 1 mM ethyleneglycol bis-(2-aminoethylether)-tetraacetic acid (EGTA), 0.5% (w/v) bovine serum albumin (BSA), 10 mM MgCl2, 1 mM ethylenediamine tetraacetatic acid (EDTA), 50 mM N- (2-hydroxyethyl) piperazine-N′- (2-ethanesulfonic acid) –NaOH (Hepes–NaOH) (pH = 7.5) 0.1% (v/v) Triton X-100, 2% (w/v) polyvinylpyrrolidone (PVP), 2 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added. The homogenate was obtained and then centrifuged at 4 °C at 15,000g for 20 min, and the supernatant obtained was used for the subsequent analysis.

Fiber soluble protein concentration was determined with the dye-binding method (Bradford, 1976) [55], in which BSA was used as a protein standard. Fiber protein was firstly solubilized using 0.05% (v/v) Triton X-100 before adding the dye reagent (Bio-Rad), and the final concentration of Triton X-100 in the protein mixture was 0.005% (v/v).

The SuSy activity was analyzed according to Shu et al. [56]. The total volume of the reaction was 650 μl, which contained 20 mM of piperazine-N, N’-bis (2-ethanesulfonic acid) – KOH (Pipes–KO H, pH = 6.5), 450 μl of 100 mM sucrose, 2 mM UDP and 200 μl of the above fiber enzyme extract. The reactions were started by incubating at 30 °C for 30 min, and were terminated with adding 250 μl of 0.5 M N-tris-(hydroxymethyl) methylglycine–KOH (Tricine–KOH, pH = 8.3), and finally heated for 10 min in boiling water. The final amount of fructose in the SuSy reactions was determined according to King et al. [54].

Activity of vacuolar invertase was determined with a reaction initiated from incubating a total volume of 2.5 ml mixtures
containing 1 M sucrose, 200 mM acetic acid–NaOH (pH = 5.0) and 100 μL of the above fiber extract at 30 °C for 30 min [56]. Then, reaction was stopped after adding 1 ml of 3,5-dinitro salicylic acid (DNS) and being heated in boiled water for 5 min. Final glucose content was determined with a spectrophotometer (xMarkTM, BIO-RAD, Japan) by analyzing absorbance at 540 nm.

Reaction of determination of PEP [EC 4.1.1.31] activity was started by the addition of the enzyme extract and was spectrophotometrically performed at 340 nm at 24 °C according to Smart [24]. Malate dehydrogenase (EC 1.1.1.37) was enzymatically coupled in the reaction, and the NADH oxidation rate was monitored with the presence of 10 mm magnesium chloride, 25 mm BTP–Mes (pH 8.0), 0.2 mm NADH, 10 mm NaHCO3, 3 mm PEP, 5 mm DTT and 10 units of malate dehydrogenase.

2.7. Gene expression by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cotton fiber with a modified method according to Wu and Liu [57]. A Primer-Script First Strand cDNA synthesis kit (TaKaRa, Japan) was used to synthesize the first-strand cDNAs according to the manufacturer’s protocol in a total volume of 20 μl reaction mixture containing 1 μg of total RNA, 4 μl 5X PrimeScript RT Master Mix and RNase-free ddH2O. Gene-specific primers were designed with primer 5.0 software (Premier Biosoft International, USA) and synthesized by Invitrogen (Shanghai, China). All primer details are provided in Table 1. The quantitative real-time PCR program was conducted on a Roche 480 Realtime detection system (Roche Diagnostics, Switzerland) following the manufacturer’s instructions, and qRT-PCR was conducted in a total volume mixture of 15 μl, which contained 2 μl cDNA, 200 nM of the forward and reverse primers, and 7.5 μl 2X SYBR Premix Ex Taq (TaKaRa, Japan). The amplification program was conducted as below: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extension at 72 °C for 20 s. Each experiment was conducted with three replications. The relative gene expression values were calculated with the 2−ΔΔCt method following Livak and Schmittgen [58]. The housekeeping gene Ubiquitin was used as the internal control.

2.8. Fiber sucrose, malate and K+ content determination

Sucrose extraction and quantitation was performed using a modified method according to Pettigrew [59]. Dried, 0.3 g fiber samples were triple extracted with 5 ml of 80% ethanol. Briefly, samples were incubated with ethanol for 30 min at 80 °C, followed by centrifuging at 10 000 × g for 10 min, and then the three aliquots of supernatant were combined together and brought to a final volume of 25 ml for the final analysis. The sucrose assay was conducted according to Shu [56].

Malate was extracted and measured using the method of Famiani [60]. In summary, 50 μg of dried fiber was ground into powder with liquid nitrogen, and then incubated in 1.5 ml of 80% ethanol including 20 mM MgCl2 and 100 mM Heps–KOH (pH 7.1) at 80 °C for 1 h. After cooling at room temperature, the primary extract was centrifuged. The supernatant was recovered, stirred and centrifuged at 12,000 g for 5 min after adding 150 μl of charcoal suspension (100 mg ml−1). The supernatant was stored at −20 °C for the subsequent analysis. Then, 200 μl of the above extract was mixed together into 1 ml assay buffer, which contained 40 mM glutamate (pH 9.9), 50 mM 2-amino–2-methypropanol, and 1 mM NAD+. The reaction was started by adding 10 U (in 100 μl) of glutamate-oxidaloacetate transaminase (GOT, from pig heart) and 0.7 U (in 100 μl) of L-malate dehydrogenase (L-MDH, from pig heart) into the mixture. After a 1 h reaction time at 20–25 °C, absorption of the solution was determined at 340 nm.

The fiber K+ content was determined using an atomic absorption spectrophotometer (SpectAA-50/55, Varian, Australia) after the fiber was digested by H2SO4-H2O2 [61].

3. Statistical analysis

Data were analyzed with SPSS statistics package ver. 17.0. The effect of water treatment, temperature, and the interaction between the two treatments was assessed using a two-way analysis of variance. Post hoc comparisons were conducted using Fisher’s least significant difference (LSD) test. For all analyses alpha = 0.05. Figures were drawn using the Origin 9.0 software program.

Dynamic changes of fiber length can be described with a logistic function [62,63] (1). In the Eq. (1), Len, Lenm represent fiber length and the theoretical maximal fiber length, respectively, while a and b are two coefficients. Vmax (maximum fiber elongation rate) and FRED (fiber rapid elongation duration) are calculated with the formulae (2) and (3), respectively, in which DPA1 represents the initiation DPA of fiber rapid elongation, and DPA2 means the termination DPA of fiber rapid elongation.

4. Results

4.1. Net photosynthetic rate (Pn) of leaves subtending to cotton boll

The Pn (net photosynthetic rate) of subtending leaves was not affected at 5 DPA but was significantly decreased after 10 DPA for well watered plants under 34.1/29.0 °C (EC) compared with well watered plants under 31.6/26.5 °C (AC) (Fig. 1). Both the 3 and 6 days of soil waterlogging treatment under 31.6/26.5 °C (AW3, AW6) or under 34.1/29.0 °C (EW3, EW6) significantly lowered Pn compared with AC. And the average Pn across DPA from 2013 to 2015 was decreased by 13.4%-19.2%, 27.9%-33.2%, 7.3%-9.2%, 39.1%-49.2% and 60.0%-65.5% for AW3, AW6, EC, EW3 and EW6, respectively, relative to AC.

4.2. Dynamic changes of fiber length and eigenvalues (Vmax, FRED, Lenmax) during fiber elongation

Fiber length increased dramatically between 0 and 24 DPA, and the maximum velocity was observed at about 10–15 DPA, while increased little after 24 DPA and had nearly attained the final length at that time (Fig. 2). Both soil waterlogging and elevated temperature treatments shortened the theoretical maximal fiber length and final fiber length, and fibers were shorter under the combined two treatments than under them individually. Across the three years, final fiber length was reduced by 2.9%–4.7% for EC, and 2.2%–4.4%, 4.4%–6.7% for AW3 and AW6, respectively, while a 5.4%–7.8% and 8.9%–11.3% reduction was observed for EW3 and EW6, respectively, relative to AC. Furthermore, fibers were shorter for waterlogging treatments after 5 DPA until boll opening when compared with AC, especially under AW6. However, unlike waterlogging, between 15 and 20 DPA (10–15 DPA in 2013) fiber length was greater under
elevated temperature in the absence of waterlogging stress than ambient temperature in the absence of waterlogging stress; therefore, fibers were shorter for the elevated temperature treatment. The process of fiber elongation can be regressed with a logistic function, and the data in this study also fit a sigmoidal model very well (Table 2). Eigenvalues of the maximum fiber elongation rate ($V_{\text{max}}$) and the fiber rapid elongation duration (FRED) were calculated and are provided in Table 2. It was observed that elevated temperature enhanced the $V_{\text{max}}$ and decreased the FRED compared with AC. In contrast, waterlogging lowered the $V_{\text{max}}$ and increased

### Table 1
Specific primers and conditions in quantitative real-time PCR program.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Sequence (5′ - 3′)</th>
<th>Length of amplified DNA (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GhSUT-1</td>
<td>AF191025</td>
<td>Forward: CTGCCAATTTGGATGGTG&lt;br&gt;Reverse: CTTGGCCTTACCCGCACTAGA</td>
<td>333</td>
<td>54</td>
</tr>
<tr>
<td>GhPEPC-1</td>
<td>AF008939</td>
<td>Forward: GGAACCCCGGCTGTAICTCCT&lt;br&gt;Reverse: CTGCAAGCCACATGTAATACCTCA</td>
<td>125</td>
<td>62</td>
</tr>
<tr>
<td>GhPEPC-2</td>
<td>AF008940</td>
<td>Forward: CCTCTGTAAGAATGTCACCTG&lt;br&gt;Reverse: CCCCCTATGTGAAATGACCTG</td>
<td>83</td>
<td>62</td>
</tr>
<tr>
<td>GhKT-1</td>
<td>AJ224961</td>
<td>Forward: AACCATGTAAGTCCACTG&lt;br&gt;Reverse: CCCCCTATGTGAAATGACCTG</td>
<td>417</td>
<td>60</td>
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<tr>
<td>GhCESA1</td>
<td>US8283</td>
<td>Forward: CCTCAACAAAGGTACAGG&lt;br&gt;Reverse: CAACACTGACCAAAGGACAAAC</td>
<td>128</td>
<td>62</td>
</tr>
<tr>
<td>GhCESA3</td>
<td>AF150630</td>
<td>Forward: AGCTTTTGGTTATGCGG&lt;br&gt;Reverse: AGTCAAGCGAGACGCGGG</td>
<td>142</td>
<td>62</td>
</tr>
<tr>
<td>GhCESA5</td>
<td>JQ345693</td>
<td>Forward: ATGACTGATGGGAGATGAC&lt;br&gt;Reverse: CCAATACCGTGGTAGGGAGGG</td>
<td>124</td>
<td>62</td>
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<tr>
<td>GhCESA6</td>
<td>JQ345694</td>
<td>Forward: TTCTTCTCTCTCTATTACAG&lt;br&gt;Reverse: TGAGACACACACCACACCT</td>
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<td>62</td>
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<tr>
<td>GhCESA8</td>
<td>JQ345696</td>
<td>Forward: CCCCCTATGTGAAATGACCTG&lt;br&gt;Reverse: TGAGACACACACCACACCT</td>
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<td>62</td>
</tr>
<tr>
<td>GhCalS5</td>
<td>XP016698828.1</td>
<td>Forward: CATGATCGACGAGGCGG&lt;br&gt;Reverse: CGATCTCTTTGGCAATGCGG</td>
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<td>62</td>
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<tr>
<td>Ubiquitin</td>
<td>AY189972</td>
<td>Forward: AGACCTTACACCGCAAGCG&lt;br&gt;Reverse: CTCTTTCCTCAGCCTCTGAACCT</td>
<td>196</td>
<td>62</td>
</tr>
</tbody>
</table>

![Fig. 1](image1.png)

**Fig. 1.** The $P_n$ changes in the leaves subtending the cotton bolls under the combination of elevated temperature and soil waterlogging from 2013 to 2015. AC, AW$_3$, AW$_6$, EC, EW$_3$, and EW$_6$ represent ambient temperature plus well-watered control, ambient temperature plus waterlogging for 3 days, ambient temperature plus waterlogging for 6 days, elevated temperature plus well-watered control, elevated temperature plus waterlogging for 3 days and elevated temperature plus waterlogging for 6 days, respectively.

![Fig. 2](image2.png)

**Fig. 2.** Dynamic changes of fiber length under the combination of elevated temperature and soil waterlogging from 2013 to 2015. AC, AW$_3$, AW$_6$, EC, EW$_3$, and EW$_6$ represent ambient temperature plus well-watered control, ambient temperature plus waterlogging for 3 days, ambient temperature plus waterlogging for 6 days, elevated temperature plus well-watered control, elevated temperature plus waterlogging for 3 days and elevated temperature plus waterlogging for 6 days, respectively.
### Table 2
Main eigenvalues ($V_{\text{max}}$, FRED, $\text{Len}_{\text{max}}$) of the fiber elongation logistic growth function under the combination of elevated temperature and soil waterlogging from 2013 to 2015.

<table>
<thead>
<tr>
<th>Year</th>
<th>Temperature Regimes</th>
<th>Days of Soil Waterlogging</th>
<th>n</th>
<th>$R^2$</th>
<th>$V_{\text{max}}$ (mm d$^{-1}$)</th>
<th>FRED (d)</th>
<th>$\text{Len}_{\text{max}}$ (mm)</th>
<th>$\text{Len}_{\text{obs}}$ (mm)</th>
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</thead>
<tbody>
<tr>
<td>2013</td>
<td>Ambient</td>
<td>0</td>
<td>7</td>
<td>0.9979</td>
<td>2.6</td>
<td>7.9</td>
<td>31.7</td>
<td>31.9 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>7</td>
<td>0.9962</td>
<td>2.3</td>
<td>8.4</td>
<td>29.8</td>
<td>30.5 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>7</td>
<td>0.9960</td>
<td>2.1</td>
<td>9.1</td>
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$V_{\text{max}}$, the maximum velocity of fiber elongation; FRED, the fiber rapid elongation duration; $\text{Len}_{\text{max}}$, the theoretical maximal fiber length; $\text{Len}_{\text{obs}}$, the observed final fiber length. Values followed by different letters within the same column are significantly different at $P=0.05$ probability level.

the FRED, and the negative effect of waterlogging was enhanced by a longer duration of waterlogging or when combined with elevated temperature.

### 4.3. Contents of sucrose, malate and K$^+$ in fiber

After 5 DPA, sucrose content in fiber cell decreased gradually with days post anthesis (DPA) (Fig. 3A). The AW$_3$ and AW$_6$ treatments significantly lowered the sucrose content in fiber, while elevated temperature enhanced sucrose content before 10 DPA and reduced it after 10 DPA. Sucrose content declines were greater for EW$_3$ and EW$_6$ than for AW$_3$ and AW$_6$ when compared to EC and AC, respectively.

Malate content in the fiber cell showed a unimodal curve which peaked at 10 DPA (Fig. 3B). It was obvious that EC treatment decreased malate content after 10 DPA, while no significant effect was found before 10 DPA, compared to AC. All the AW$_3$, AW$_6$, EW$_3$ and EW$_6$ treatments lowered malate content in the fiber, and the negative effect was highest at 10 DPA. The average malate content in fiber at 10 DPA across the three years was 91.2, 89.1, 85.9, 88.8 and 82.0 Unit mg$^{-1}$ protein for AC, AW$_3$, AW$_6$, EW$_3$ and EW$_6$, respectively.

Similar to malate, the K$^+$ content in fiber also displayed a unimodal curve which peaked at 10 DPA (Fig. 3C). However, we didn’t see any significant difference under either elevated temperature alone or waterlogging (only significant at 10 DPA for AW$_6$ in 2013) alone relative to AC, while a significant decrease occurred at 10 DPA for EW$_3$ and EW$_6$ (not significant for EW$_3$ in 2014) treatments.

### 4.4. Activities of sucrose degradation enzymes and PEPC in fiber

Sucrose synthase activity increased with days post anthesis until peaked at 15 DPA, and was decreased under EC compared with AC (Fig. 4A). In contrast, waterlogging treatment significantly enhanced fiber SuSy activity, and it was much higher for AW$_3$ than AW$_3$ compared to AC. Furthermore, SuSy activities for EW$_3$ and EW$_6$ were much higher than for AW$_3$ and AW$_6$, respectively.

The vacuolar invertase activity declined with fiber development (Fig. 4B). The elevated temperature and waterlogging decreased the VIN activity relative to AC, especially after the combination of these two treatments. The average activity of VIN across DPA among the three years was decreased by 3.1%-4.0%, 6.8%–7.6%, 2.9%–4.1%, 6.0%–7.6%, and 11.1%-15.0% for AW$_3$, AW$_6$, EC, EW$_3$ and EW$_6$, respectively.

Fiber PEPC activity peaked at 15 DPA (Fig. 4C). Elevated temperature enhanced PEPC activity before 15 DPA, and decreased it after 15 DPA, while activity of PEPC was decreased under waterlogging treatment after 5 DPA, especially when accompanied with elevated temperature.

### 4.5. Gene expressions during fiber elongation

Relative expression of GhsUTF-1 was significantly up-regulated at 10 DPA and down-regulated after 10 DPA for EC compared to AC but was down-regulated under waterlogging treatments and the combination of elevated temperature and waterlogging (Fig. 5A). Expression of GhPEPC-1 and GhPEPC-2 were down-regulated under all the treatments in this experiment, and GhPEPC-1 was more sensitive than GhPEPC-2 (Fig. 5B & C). The negative effect on GhPEPC-1 was most evident on 15 DPA for EC, which showed a 0.31- and 0.25-fold decrease for GhPEPC-1 and GhPEPC-2, respectively, compared with AC. However, waterlogging treatment especially when accompanied by elevated temperature lowered GhPEPC-1B2 expressions more at 10 DPA, and the negative effect weakened after 10 DPA. GhPEPC-1 expression at 10 DPA was down-regulated by 0.22-, 0.35-, 0.42- and 0.55-fold for AW$_3$, AW$_6$, EW$_3$ and EW$_6$, respectively. Both elevated temperature and waterlogging treatment alone showed no significant differences in the expression of GhKT-1, while the combined treatments decreased GhKT-1 expression by 0.34- and 0.42-fold for EW$_3$ and EW$_6$ on 10 DPA, respectively (Fig. 5D). In 15 DPA fiber, GhCESA8 was more sensitive to stresses than GhCESA1 (Fig. 6), which was significantly decreased for AW$_6$, EW$_3$ and EW$_6$, compared with AC. However, we didn’t see any significant differences on GhCESA1 expression under waterlogging or elevated temperature alone, while it was significantly decreased under the combined two stresses (0.28-fold for EW$_3$ and 0.43-fold for EW$_6$). Expression of GhCaLS5 was significantly up-regulated under waterlogging alone and combined two stresses in 15 DPA fiber. Waterlogging alone down-regulated GhCESA3 and GhCESA6 expression levels and had no significant difference on GhCESA5 in 10 DPA fiber, relative to AC. Elevated temperature alone slightly enhanced GhCESA3 expression and
significantly enhanced *GhCESA6* expression level. However, *GhCESA3, GhCESA5* and *GhCESA6* were significantly down-regulated under combined two stresses.

5. Discussion

Both waterlogging and high temperature are a worldwide agricultural problem, and more frequent flooding events are occurring accompanied with high temperature from June to October in the middle and lower reaches of the Yangtze River Valley, which would severely affect cotton production and fiber development. The underlying mechanisms associated with altered fiber length or cellulose synthesis under elevated temperature or soil waterlogging separately have been described previously [14,51], whereas the combined effect of these two stressors on fiber elongation remained unaddressed.

Previous studies have pointed out that 15°C to 21°C is the optimum night temperature range for fiber elongation [3], and 23–32°C is the optimal thermal kinetic window for upland cotton enzyme activity [62]. In the current experiment, temperature in 2013 was highest across the three years, and the MDT, MDT$_{day}$, MDT$_{night}$, MDT$_{max}$ and MDT$_{min}$ were much higher than the temperature range noted above after the temperature was elevated (Table S1). The boll development period was significantly shortened after elevated temperature (ET), soil waterlogging (SW) and combined ET and SW treatments.

Both ET treatment alone and SW treatment alone significantly decreased the final fiber length, and the negative effect was significantly enhanced under the combined treatments (Table 2). The dynamic change of fiber length is described by a Logistic growth curve [63], and both $V_{max}$ and FRED can affect the fiber elongation process, whereas which one plays a dominated role is uncertain. It was reported that higher fiber cell turgor may lead to a higher $V_{max}$ [64], which was similar to research addressing the relation between cell turgor and cell elongation in Barley leaves [65]. FRED was controlled by the closure duration of plasmodesmata (PD) and the timing of secondary cell wall synthesis initiation [66]. In the present study, even though both ET and SW treatment alone decreased the final fiber length, they showed a different effect on $V_{max}$ and FRED (Table 2). The ET treatment alone led to higher $V_{max}$ and shorter FRED, while the SW treatment alone resulted in lower $V_{max}$ and longer FRED. Interestingly, the ET treatment alone enhanced fiber length during the early phase of elongation and decreased it subsequently, and there seemed to be a turning point between 15 – 20 DPA (10–15 DPA in 2013) (Fig. 2). Even though fibers had higher $V_{max}$ of elongation under ET alone, this did not...
compensate for the negative effect of shorter FRED. The decreased boll maturation period may be responsible for the shorter FRED. Besides, the track of fiber length over time provided detailed data to support a hypothesis from Lokhande and Reddy [3], in which they reached the same conclusion that high temperature accelerates boll development (higher rate of heat unit accumulation) but limits the time frame over which development occurs, thereby resulting in shorter final fiber lengths. However, fibers under SW alone showed a shorter boll maturation and longer FRED than AC fibers, which indicated that SW treatment may postpone the initiation of fiber secondary cell wall synthesis [14]. Importantly, \( V_{\text{max}} \) under SW treatment may play a more important role in fiber elongation than FRED, where lower \( V_{\text{max}} \) led to decreased final fiber length.

The content of osmotic solutes influences cell turgor and to a large extent, drives cell elongation during fiber development [47]. As an important osmotic solute, sucrose has been the focus of studies concerning fiber development [51,67]. The fiber sucrose content was increased initially, and was then lowered after 10 DPA for EC compared with AC (Fig. 3A). There are two explanations for this result: (1) altered activity of sucrose degradation enzymes; and (2) the changes in import of sucrose from cotton source leaves to fiber cells. In the current study, both the activities of sucrose synthase and vacuolar invertase were decreased for EC compared with AC, which resulted in the accumulation of fiber sucrose. Furthermore, \( Pn \) of subtending leaves was not affected at 5 DPA but was decreased at 15 and 25 DPA under EC treatment. This indicated that sucrose transferred from leaves to cotton bolls may be limited due to the low photosynthetic rates of the subtending leaf under high temperature [68]. Moreover, as sucrose was mainly transferred into the fiber through sucrose transporter (SUT), the declined \( GhsuT-1 \) expression further indicated that sucrose transport was blocked under ET alone (Fig. 5A). Thus, we speculated that the photosynthetic rates of the subtending leaf may have affected the fiber \( GhsuT-1 \) expression. Unlike elevated temperature, the waterlogging treatment decreased fiber sucrose content in different ways. For example, sucrose imported inside the fiber cell was decreased through lower \( Pn \) of leaves subtending the cotton bolls. Additionally, the enhanced \( SuSy \) activity in cotton fiber degraded more sucrose, which resulted in lower fiber sucrose content. Increased \( SuSy \) activity can produce more energy used for cotton fiber development and helps mitigate the effects of waterlogging stress, which was agrees with studies reporting that \( SuSy \) was the main sucrose degradation enzyme affected by waterlogging [14]. Interestingly, \( SuSy \) activity was enhanced under waterlogging stress, which indicated more UDPG was pro-

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**Fig. 4.** Dynamic changes of fiber sucrose synthase activity (A), vacuole invertase activity (B), and PEPC activity (C) under the combination of elevated temperature and soil waterlogging from 2013 to 2015. AC, \( AW_3 \), \( AW_6 \), EC, \( EW_3 \) and \( EW_6 \) represent ambient temperature plus well-watered control, ambient temperature plus waterlogging for 3 days, ambient temperature plus waterlogging for 6 days, elevated temperature plus well-watered control, elevated temperature plus waterlogging for 3 days and elevated temperature plus waterlogging for 6 days, respectively.
Fig. 5. Relative expression levels of GhSUT-1 (A), GhPEPC-1 (B), GhPEPC-2 (C) and GhKT-1 (D) in elongating fiber under the combination of elevated temperature and soil waterlogging. AC, AW3, AW6, EC, EW3 and EW6 represent ambient temperature plus well-watered control, ambient temperature plus waterlogging for 3 days, ambient temperature plus waterlogging for 6 days, elevated temperature plus well-watered control, elevated temperature plus waterlogging for 3 days and elevated temperature plus waterlogging for 6 days, respectively.

Produced and more cellulose should be synthesized. However, we didn’t see an significant enhance on GhCESA1 and GhCESA8 expression (Fig. 6). Previous studies reported that fiber cellulose content was decreased and callose content was increased under waterlogging stress [14]. In this study, we found GhCalS5 was significantly up-regulated under waterlogging stresses (Fig. 6). We speculated that carbons from sucrose was more transferred to callose rather than cellulose under waterlogging stress, which was also supported by another paper in our lab focusing on cellulose synthesis under combined waterlogging and elevated temperature [52]. Moreover,
the SW treatment also significantly decreased fiber malate content by changing PEPC activity and GhPEPC-1 & -2 expression, and GhPEPC-1 was more sensitive to stresses than GhPEPC-2 (Fig. 5B&C). Furthermore, the combined stresses also showed negative effects on fiber cell K⁺ content (Fig. 3C). Similar to sucrose, K⁺ in the fiber is mainly imported from underlying seed coat cells by K⁺ transporters [20]. Down-regulated GhKT-1 under combined stresses finally led to the decreased fiber K⁺ content. In addition to the affected osmotic solutes content in fiber, combined ET and SW also significant limited primary cell wall synthesis through down-regulating expression levels of GhCESA3, GhCESA5 and GhCESA6 (Fig. 7).

Although ET and SW decreased fiber length in different ways by regulating fiber osmotically active solutes, the waterlogging treatment had a more pronounced effect than elevated temperature alone, and the combination of waterlogging and high temperature together were more deleterious than either stress in isolation. Based on our study, we verified the essential role of osmotic solutes in osmoregulation of fiber elongation. The changed content of all the three osmotic solutes finally led to the reduction of final fiber length under combined elevated temperature and waterlogging stresses.

6. Conclusions

We concluded that both elevated temperature and soil waterlogging treatment can decrease cotton final fiber length although the mechanisms associated with the disruption of the fiber cell elongation process were quite different. Elevated temperature (34.1/29.0 °C) accelerated fiber elongation rate but shortened the duration of fiber rapid elongation, while an opposite trend occurred under waterlogging treatment, especially under the combined two stressors. Elevated temperature enhanced fiber length first and decreased fiber length subsequently after 15 DPA, which was mainly due to the alteration of fiber sucrose content through the regulation of GhSUT-1 expression. Waterlogging treatment not only limited the import of sucrose into the fiber cell by down-regulating GhSUT-1 expression, but also enhanced SuSy reverse catalytic activity for sucrose reduction. Furthermore, combined elevated temperature and waterlogging negatively impacted sub-tending leaf photosynthesis and the three major osmotically active solutes through the regulation of GhSUT-1, GhPEPC-1 & -2 and GhKT-1 expression and SuSy activity, which worked together to produce shorter fiber length.
Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.01.001.

References


