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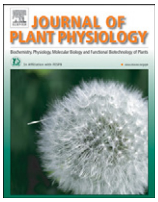

SHORT COMMUNICATION

Short-term high temperature growth conditions during vegetative-to-reproductive phase transition irreversibly compromise cell wall invertase-mediated sucrose catalysis and microspore meiosis in grain sorghum (*Sorghum bicolor*)

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ABSTRACT

Grain sorghum (*Sorghum bicolor*) crop yield is significantly compromised by high temperature stress-induced male sterility, and is attributed to reduced cell wall invertase (CWI)-mediated sucrose hydrolysis in microspores and anthers leading to altered carbohydrate metabolism and starch deficiency in pollen (Jain et al., 2007). Sorghum plants were grown under season-long ambient (30/20 °C day-time maximum/night-time minimum) or high temperature stress (HS, 36/26 °C) environments, or reciprocally transferred for 5–10 days between either temperature regimens through panicle and microspore developmental stages. Quantitative RT-PCR analyses for CWI gene *SbIncw1*, plasma membrane H^+-ATPase (*Mha1*) and sugar transporter proteins (*OsSUT3* and *OsMST7* homologs in sorghum), starch deficiency and pollen sterility data are presented to confirm HS-sensitivity of pre- and post-meiotic stages of sorghum microsporogenesis. Heat stress-induced reduction in Incw transcriptional activity during microspore meiosis was irreversible despite return of optimal growth temperature conditions through further reproductive development.

Introduction

Grain sorghum is the fifth most important cereal crop worldwide and feeds more than 500 million people in the developing countries. It is also an important biofuel crop in the US. Although drought-hardiness makes sorghum an important “failsafe” crop under arid and semi-arid conditions, it is often exposed to episodes of day- and night-time temperatures exceeding 32/22 °C. Persistent and/or episodic high temperature stress (HS) conditions significantly compromise crop performance (Prasad et al., 2006, 2008). The anticipated rise (1.4–5.8 °C) in global mean surface temperatures and postulated spatial increases in mean temperature and its variance or both distributional parameters will result in frequent episodes of high temperature conditions (IPCC, 2007). Hence, a critical evaluation of HS-induced changes is required to better understand crop growth and the physiology and reproductive behavior that ultimately determine grain yields.

Microspores represent a strong photoassimilate sink in cereals, second only to the developing endosperm with respect to assimilate partitioning and its utilization. The first mitotic division of haploid microspores is conspicuously followed by a rapid phase of starch biosynthesis. Starch accumulated during the advanced stages of pollen maturation is regarded as a metabolic marker for pollen maturity (Datta et al., 2001, 2002; Pring and Tang, 2004; Kong et al., 2007). In addition to providing energy for pollen tube germination, the accumulated starch reserves have an implicated role as compatible osmolytes in conferring desiccation tolerance (Hoekstra and Roekel, 1998; Kaplan et al., 2004).

Plant reproductive processes, especially microspore development, are more sensitive to impending stress than vegetative growth, as being shown in cereals (Abiko et al., 2005; Jain et al., 2007; Prasad et al., 2008) and other crops (Frank et al., 2009). Ample evidence corroborates that pollen viability is associated with adequate amounts of starch deposition (Datta et al., 2002; Kong et al., 2007). Stress-induced aberrant starch deposition profiles in microspores resulted in reduced male fertility across

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Abbreviations: Amb, ambient temperature (30/20 °C day-time maximum/night-time minimum); HS, high temperature stress (36/26 °C) stress; DAS, days-after-sowing; CWI (*Incw*), cell wall invertase (gene); Mha1, plasma membrane H^+-ATPase; MST1, monosaccharide transporter; SUT3, sucrose transporter

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various crops including rice (Sheoran and Saini, 1996), wheat (Dorion et al., 1996), barley (Sakata et al., 2000), sorghum (Jain et al., 2007), tomato (Pressman et al., 2006) and bell peppers (Karni and Aloni, 2002). Likewise, targeted antisense repression of anther specific cell wall invertase (CWI) and *SnRK1* genes in tobacco (Goetz et al., 2001) and barley (Zhang et al., 2001), respectively, showed perturbed sugar partitioning and starch deficiency which correlated with subsequent microspore sterility in transgenic plants.

CWI-mediated sucrose inversion is critical for establishment and subsequent maintenance of the microspore sink strength, thus successfully driving source-to-sink assimilate unloading in developing microspores (Goetz et al., 2001). Plasma membrane (PM) H^+-ATPase (Mha1) is the key transporter protein providing energized potential gradient across cell membrane subsequently regulating secondary active ion transport and nutrient trafficking channels. Co-suppression of endogenous and transgenic *PM* H^+*-ATPase* resulted in reduced pollen uptake of sugars and impaired male fertility in tobacco (Zhao et al., 2000). Likewise, coordinated up-regulation of *PM* H^+*-ATPase*, hexose (*OsMST7*) and sucrose transporters (*OsSUT3*), and a high ATP/ADP ratio coincident with accumulation of glucose, fructose and starch deposition were observed during pollen maturation in cytoplasmic male-fertile lines of rice, as opposed to male-sterile lines (Kong et al., 2007).

We have previously shown that season-long HS treatments affected pollen fertility and seed set in grain sorghum. Detailed molecular appraisal of temporal transcription profiles of genes involved in sugar-to-starch metabolic transition showed that CWI-mediated sucrose hydrolysis and subsequent sucrose synthesis was the primary molecular lesion that compromised microspore sink strength and caused male sterility under elevated growth temperature conditions (Jain et al., 2007). Here, we provide further molecular evidence that short-term HS treatments, if coincident with vegetative-to-reproductive phase transition and/or microspore meiosis, are detrimental enough to irreversibly affect CWI-mediated sucrose inversion and starch filling through microspore maturity thus adversely impacting pollen fertility and hence grain yields.

Materials and methods

Plant growth, SPAR chambers and temperature treatments

Semi-dwarf and photoperiod-insensitive grain sorghum (*Sorghum bicolor* L. Moench) cv. DeKalb 28E was grown in sunlit Soil-Plant-Atmospheric-Research (SPAR) growth chambers at a stand density of 20 plants m^{-2} as described (Prasad et al., 2006; Jain et al., 2007). Dry bulb air temperatures were controlled as a time-varying sinusoidal function during the day and logarithmic decay during the night throughout 24-hour cycles. Dew point temperatures were controlled to 10 °C below the respective time-varying dry bulb air temperatures. Day-time CO_2 concentration at ambient set point of 350 µmol mol^{-1} air and relative humidity at 55–58% (at 15:00 h) was maintained across all temperature treatments.

Panicle initiation and emergence in sorghum plants grown under season-long ambient conditions occurred at 25 and 35 days-after-sowing (DAS), respectively. Plants across treatments *A* and *B* were maintained season-long under ambient temperature (Amb, 30/20 °C) or high temperature stress (HS, 36/26 °C) growth conditions ($A{:}Amb^{season\text{-}long}$ and $B{:}HS^{season\text{-}long}$). For plants grown under ambient conditions till phase transition ($Amb^{3\text{–}25DAS}$), the HS treatment labels in the reproductive phase (25–35 DAS) were: $C{:}HS^{25\text{–}30}Amb^{30\text{–}35DAS}$, $D{:}Amb^{25\text{–}30}HS^{30\text{–}35DAS}$ and $E{:}HS^{25\text{–}35DAS}$. Similarly, HS conditions during vegetative growth phase (across treatments *F* through *H*, $HS^{3\text{–}25DAS}$) were interrupted by ambient temperatures during reproductive stages as: $F{:}Amb^{25\text{–}30}HS^{30\text{–}35DAS}$, $G{:}HS^{25\text{–}30}Amb^{30\text{–}35DAS}$ and $H{:}Amb^{25\text{–}35DAS}$. Following panicle emergence (35 DAS) across growth temperature treatments *C* through *H*, the plants were maintained under ambient growth conditions until reproductive maturity. See Fig. 1 for the schematic representation of the time-lines involved in each treatment.

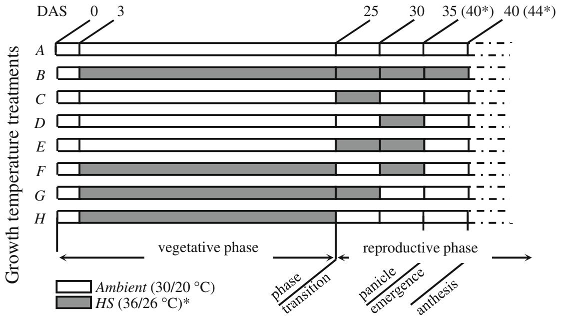

Fig. 1. Schematic showing ambient (30/20 °C day-time maximum/night-time minimum) and elevated (36/26 °C) growth temperature treatments for grain sorghum plants during vegetative and reproductive growth stages. Treatment labels for the reproductive phase after 25 DAS were: $A{:}Amb^{season\text{-}long}$, $B{:}HS^{season\text{-}long}$, $C{:}HS^{25\text{–}30}Amb^{30\text{–}35DAS}$, $D{:}Amb^{25\text{–}30}HS^{30\text{–}35DAS}$, $E{:}HS^{25\text{–}35DAS}$, $F{:}Amb^{25\text{–}30}HS^{30\text{–}35DAS}$, $G{:}HS^{25\text{–}30}Amb^{30\text{–}35DAS}$, and $H{:}Amb^{25\text{–}35DAS}$. See text for further labels for other time-line segments for temperature treatments *A* through *H*. *Asterisk* marks the delayed panicle emergence and anthesis at 40 and 44 days, respectively, under season-long heat stress conditions. *DAS*, days-after-sowing.

Pollen viability and seed set measurements

In vitro pollen germination assays were carried out using a modified germination medium as described (Jain et al., 2007). At maturity, seed set was estimated as the ratio of seed-filled florets to the total number of florets on the tagged panicles, and expressed as a percentage of total potential seed sites.

Harvesting of microspores/young pollen and florets for RNA extraction

The pre-emergent (early) panicles with the tip protruding out of the flag leaf sheath were collected and harvested for microspores as described by Pring and Tang (2004). At this time, the microgametophytes were approximately 7–11 days prior to anthesis, and were represented by young early-mid microspores (pre- to post-meiotic) at the base of the panicle developmentally progressing upwards through post-mitotic young pollen near the panicle tip. Different developmental stages of florets (including the glumes, lemma, palea, lodicule and reproductive whorls) prior to anther dehiscence were plucked off the panicle branches and homogenized under liquid nitrogen for RNA extraction.

Real-time reverse transcription PCR

Total RNA was extracted in Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacture's protocol. Five µg total RNA was digested with RNase free DNase I (Invitrogen), and reverse transcribed using the SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR was performed using the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) and Chromo 4™ CFD supported by Opticon Monitor™ Software version 2.03 (MJ Research, Alameda, CA). The PCR reactions contained 400 nM of gene-specific primers and 2 µL of the 5-fold diluted RT reaction in a final volume of 20 µL. The thermal cycling protocol entailed

Table 1
Primers used for real-time quantitative PCR for genes investigated in the present study.

Gene	GenBank Acc. #	Forward primer (seq. 5′→3′)	Reverse primer (seq. 5′→3′)
SbIncw1	EF177465	GCAAAGTCGGTCACTCTCAGGAA	AAATCCTGCAAATGTCGGGCG
Mha1	U09989	GCAAGGCAGCACATCTTGTCGATT	TTCCTCCGATCAGAAGCACCAACA
OsSUT3	AB071809	ATTCTGGCTGCTCGACTTCTCCAA	AGCCCTGGTCATGAGAAAGGGAAA
OsMST7	AY643749	ATGCTCATCATTGGCCGCATCTTG	AGTTGATGAGGTTGGCGAAGAGGA
ZmTua4	X73980	ACAATCAAGACCAAGCGCACCATC	TGGTGGAGTTGGAGATCATGCACA

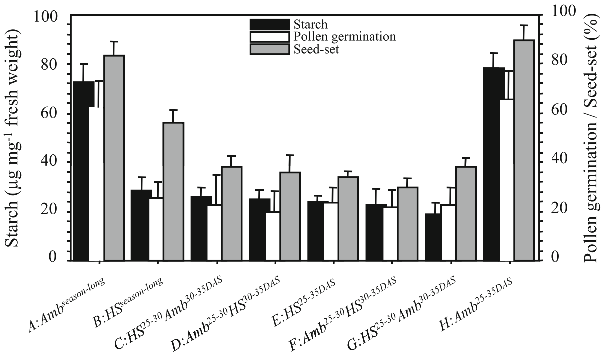

Fig. 2. Effect of time and duration of high temperature growth conditions (HS, 36/26 °C day-time maximum/night-time minimum) on starch content, *in vitro* pollen germination and reproductive success of grain sorghum. For starch quantification, four independent extractions were done with each assay having 5 replications. Pollen germination assays were done on dehisced pollen collected from three randomly tagged panicles, on two different days. Pollen germination was scored as positive, if the length of pollen tube exceeded the pollen grain diameter. The yield data were collected on six randomly tagged plants 60 DAS. The data were collected from two different experiments as described above, and presented as average ± S.D., and *A* through *H* refer to growth temperature treatments as shown in Fig. 1.

50 °C incubation for 2 min, followed by *Tbr* DNA polymerase activation at 95 °C for 15 min. The PCR amplification was carried out for 35 cycles with denaturation at 94 °C for 10 s, and primer annealing and extension at 55 and 72 °C for 30 s each, respectively. Optical data were acquired following the extension step, and the PCR reactions were subject to melting curve analysis beginning at 55 °C through 95 °C, at 0.1 °C s^{-1}. *α-Tubulin* (*Tua4*) was used as an internal reference control for qRT-PCR reactions. Gene-specific primers used for real-time PCR analyses are summarized in Table 1.

Starch quantification

Microspores were homogenized in hot ethanol and centrifuged to separate soluble sugars from starch. Starch content was determined in the pellet fraction following amyloglucosidase digestion (EC 3.2.13, from *Aspergillus niger*, Roche Diagnostics, Indianapolis, IN), followed by quantification of released glucose moieties in a microtitre plate assay as previously described (Datta et al., 2002).

Results

Following seedling emergence at 3 days-after-sowing (DAS) in ambient (30/20 °C day-time maximum/night-time minimum) temperature (Amb), the grain sorghum plants were grown at sinusoidal temperature regimes of 30/20 °C (ambient) and 36/26 °C (HS) in naturally sunlit, controlled environmental growth chambers as described. Vegetative-to-reproductive phase transition (panicle initiation) was visually discernible within 25–27 DAS in more than half of the sampled plants, followed by panicle emergence at 35 and 40 DAS at 30/20 and 36/26 °C treatments, respectively. Anthesis occurred within a 4–5 day period after panicle emergence at both temperature treatments. The short-term HS treatments involved reciprocal shift of growth temperature conditions at 25 and 30 DAS (coincident through panicle and microspore development stages, respectively) (Fig. 1).

The data (obtained from two independent experiments) on total starch content in young pollen grains (post first mitosis, collected from the tip of the pre-emergent panicle), germination potential of dehisced pollen and seed set are shown in Fig. 2. Pollen dehisced under ambient and HS conditions remained turgid, exhibited normal diameter, and app. 76–80% grains stained viable with tri-phenyl tetrazolium chloride across all temperature treatments (data not shown). Season-long or short-term HS treatments adversely reduced sugar-to-starch metabolic transition in developing microspores, resulting in low starch concentration. Consequently, starch-deficient microspores, even though somewhat viable, failed to support germination and growth of pollen tube under high temperature growth conditions. Significant yield penalties (54–64% loss in seed set) caused under short-term HS conditions (treatments *C* through *G*) were attributed to failed fertilization as well as reduced panicle size and necrosis of panicle branches and floral primordia (Jain et al., 2007). Duration and time of incident HS conditions evidently affected starch deposition in developing microspores and pollen fertility, translating accordingly into grain yields. It is noteworthy that no significant differences were discernible with respect to the growth temperature treatments during vegetative growth phase (3–25 DAS), thus confirming that reproductive success is critically dependent upon environmental cues available at the time of onset and further development of reproductive meristems.

Establishment and subsequent maintenance of the source-to-sink assimilate partitioning is contingent upon apoplastic sucrose inversion and sugar uptake across the cell membrane. Real-time RT-PCR data for the CWI gene *SbIncw1* (Jain et al., 2008) in intact floral whorls and young microspores is summarized in Fig. 3a. Significant loss in *Incw* transcriptional activity was evident in floral tissues under HS conditions prevalent during reproductive development (25–35 DAS), especially during the period of early panicle growth and development (25–30 DAS) (treatments $B{:}HS^{season\text{-}long}$, $C{:}Amb^{3–25}HS^{25–30}Amb^{30–35DAS}$, $E{:}Amb^{3–25}HS^{25–35DAS}$ and $G{:}HS^{3–30}Amb^{30–35DAS}$). Notably, developing microspores suffered an irreversible decline in *Incw* transcript abundance due to HS conditions once the meristems progressed towards reproductive transition (treatments *B* through *G*), despite ambient conditions available at the time of microspore meiosis (30–35 DAS) (treatments $C{:}Amb^{3–25}HS^{25–30}Amb^{30–35DAS}$, $G{:}HS^{3–30}Amb^{30–35DAS}$). *Vis-à-vis*, the significant decline in real-time transcript abundance for *Mha1*, *SUT3* and *MST7* encoded plasma membrane H^+-ATPase, sucrose and monosaccharide transporter proteins, respectively, is suggestive of compromised apoplastic sugar uptake by developing microspores under season-long HS

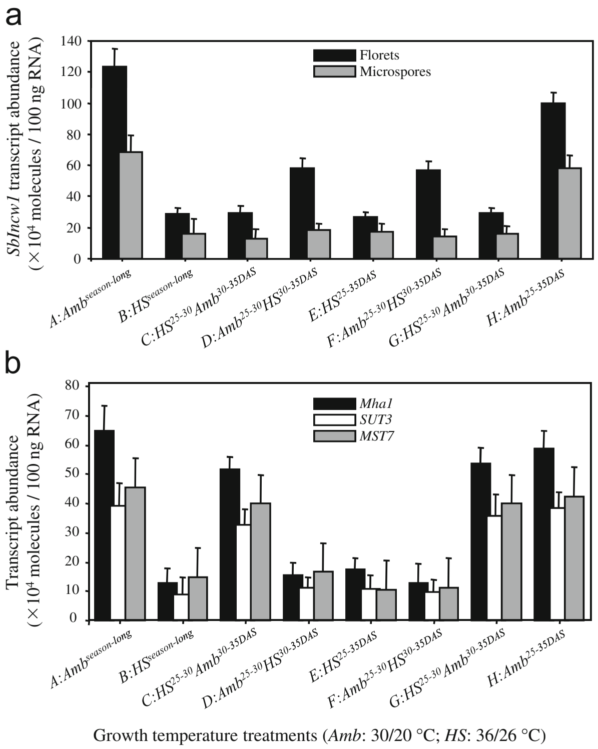

Fig. 3. Quantitative RT-PCR data showing effect of growth temperature regimen on expression of (a) *SbIncw1* (cell wall invertase) in florets and developing microspores (represented by pre- to post-meiotic early-mid microspores at the base developmentally progressing through post-mitotic young pollen at the tip of the panicle) and (b) *Mha1* (plasma membrane H^+-ATPase), *SUT3* (sugar transporter) and *MST7* (monosaccharide transporter) encoded transporter proteins in developing microspores harvested from pre-emergent panicles of sorghum. The data are presented as average ± S.D., for three independently made RT preparations and each having three technical replicates for PCR run (scored independently for two experiments). *A* through *H* refer to growth temperature treatments as shown in Fig. 1.

conditions (treatment *B*) (Fig. 3b). Notably, sugar loading ability from apoplast could be partially recovered as long as the growth conditions during time of microspore development stayed ambient (treatments $C{:}Amb^{3-25}HS^{25-30}Amb^{30-35DAS}$, $G{:}HS^{3-30}Amb^{30-35DAS}$ and $H{:}HS^{3-25}Amb^{25-35DAS}$). The high transcript abundance for treatments *C* and *G* despite poor pollen viability and low grain set indicates that these transporter protein functions are not the critical factors preventing grain set.

Discussion

Consistent with the previously published results, we reiterate a recurring theme underlying (heat, cold, and drought) stress-induced down-regulation in CWI expression and activity causing male sterility across various crop species including tomato (Pressman et al., 2006), rice (Oliver et al., 2005), wheat (Koonjul et al., 2005) and sorghum (present data; Jain et al., 2007).

In cereals like wheat, oat and barley, the high photosynthetic contribution of glumes and hence, its effect on source-sink relationship, declines significantly after anthesis and later during grain filling (Ziegler-Jöns, 1989). A similar temporal decline in *SbIncw1* expression in the glumes was discernible in developing sorghum caryopses (Jain et al., 2008). It is conceivable that symplastic isolation renders developing microspores essentially dependent upon inherent CWI and transporter protein activities for post-phloem assimilate unloading. At this stage, therefore, it is likely that CWI activity in glumes or other green sporophytic whorls will have only minimal, if any, bearing towards microspore sink strength. However, immunolocalization (Jain et al., 2007) and real-time RT-PCR data (Fig. 3a) confirms that *Incw* expression in sorghum florets is under stringent transcriptional regulation and responsive towards environmental growth conditions.

The disconnect in the expression profiles for *Incw1*, *Mha1*, *SUT3* and *MST7*-encoded transporter proteins during short-term HS treatments (Fig. 3a and b) does not conform to the proposed model for coordinated up-regulation of extracellular invertase activity and sugar transport in favor of apoplastic phloem unloading (Ehness and Roitsch, 1997), and may be indicative of functionally divergent regulatory mechanisms controlling sugar partitioning and utilization during male meiosis and overall reproductive development. Uncoupling of sugar cleavage and uploading capacity across microspore membrane may also be reflective of an aggravated respiratory demand in order to maintain structural and physiological integrity of heat-stressed microspores. Accumulation of glucose and fructose observed in the heat-stressed sorghum microspores (Jain et al., 2007) is in agreement with glucose-mediated control of H^+-ATPase activity through phosphorylation-dependent 14-3-3 association in maize (Camoni et al., 2006). This speculated increase in sugar uploading and utilization for energy provision, and concomitant failure of *Incw*-mediated assimilate partitioning and sugar-to-starch metabolic transition can be argued on the basis of discreet spatio-temporal regulation (Fig. 3a) and presence of distinct CWI isoforms in gametophytic and sporophytic floral whorls (Jain et al., 2007). The starch content, pollen fertility and seed set (Fig. 2) and transcript abundance data for *Incw* and sugar transporter proteins (Fig. 3; treatments *C* and *G*, vs. *A* and *H*) clearly reflect that developing microspores may require and upload sugars for maintaining turgor and/or metabolic needs in an *Incw* compromised background, but adequate assimilate partitioning in favor of sugar-to-starch metabolic transition is essentially dependent upon CWI-mediated sucrose catalysis and subsequent sugar uptake that finally determines pollen fertility. Goetz et al. (2001) also showed that exogenous carbohydrate supply could only partially relieve the metabolic arrest in an *in vitro* pollen maturation assay, in transgenic tobacco plants expressing antisense CWI gene under control of tapetum-specific promoter. A "feed forward" mechanism involving perception and amplification of sugar or hormonal signals leading to up-regulation of CWI activity and enhancement of sink strength during microsporogenesis can not be ruled out.

Nonetheless, the role of cell wall bound invertase activity in driving assimilate partitioning from the nutrient rich locular fluid bathing the developing microspores appears to be most critical and under stringent, irreversible transcriptional regulation in response to prevalent environmental cues. Finally, the presented data also indicate that the signals for successful deployment of CWI-mediated sugar-to-starch metabolic transition in developing microspores are perceived by sporophytic tissues early on during phase transition.

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