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ORIGINAL ARTICLE

Effects of season-long high temperature growth conditions on sugar-to-starch metabolism in developing microspores of grain sorghum (*Sorghum bicolor* L. Moench)

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Abstract High temperature stress-induced male sterility is a critical problem in grain sorghum (*Sorghum bicolor* L. Moench) that significantly compromises crop yields. Grain sorghum plants were grown season-long under ambient (30/20°C, day-time maximum/night-time minimum) and high temperature (36/26°C) conditions in sunlit Soil-Plant-Atmospheric-Research (SPAR) growth chambers. We report data on the effects of high temperature on sugar levels and expression profiles of genes related to sugar-to-starch metabolism in microspore populations represented by pre- and post-meiotic "early" stages through post-mitotic "late" stages that show detectable levels of starch deposition. Microspores from high temperature stress conditions showed starch-deficiency and considerably reduced germination, translating into 27% loss in seed-set. Sugar profiles showed significant differences in hexose levels at both "early" and "late" stages at the two temperature regimes; and most notably, undetectable sucrose and ~50% lower starch content in "late" microspores from heat-stressed plants. Northern blot, quantitative PCR, and immunolocalization data revealed a significant reduction in the steady-state transcript abundance of *SbIncw1* gene and CWI proteins in both sporophytic as well as microgametophytic tissues under high temperature conditions. Northern blot analyses also indicated greatly altered temporal expression profiles of various genes involved in sugar cleavage and utilization (*SbIncw1*, *SbIvr2*, *Sh1*, and *Sus1*), transport (*Mha1* and *MST1*) and starch biosynthesis (*Bt2, SU1, GBSS1*, and *UGPase*) in heat-stressed plants. Collectively, these data suggest that impairment of CWI-mediated sucrose hydrolysis and subsequent lack of sucrose biosynthesis may be the most upstream molecular dysfunctions leading to altered carbohydrate metabolism and starch deficiency under elevated growth temperature conditions.

Keywords Cell wall invertase · Grain sorghum · Heat stress · Microsporogenesis · Pollen sterility · Starch biosynthesis

Abbreviations

ADP-Glc	Adenine 5′-diphosphate-glucose
AGPase	ADP-pyrophosphorylase
Bt2	*Brittle 2*
CWI	Cell wall invertase
DAS	Days-after-sowing
Frc	Fructose
GBSS1	*Granule bound starch synthase-1*
Glc	Glucose
INCW	Cell wall invertase protein
Mha1	Plasma membrane H^+ATPase gene
MST1	Monosaccharide transporter gene
SbIncw1	Sorghum cell wall invertase gene

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SbIvr2	Sorghum vacuolar invertase gene
Sh1	*Shrunken 1*
SU1	*Sugary 1*
Suc	Sucrose
Sus1	*Sucrose synthase 1*
SuSy	Sucrose synthase
UDP-Glc	Uridine 5′-diphosphate-glucose
UGPase	UDP-pyrophosphorylase gene

Introduction

Global climate changes, especially the increase in Earth's near-surface temperatures (anticipated to increase by 1.8–5.8°C by end of this century; IPCC 2001) pose an imminent challenge to crop production across the world. Plant reproductive stages are more sensitive to environmental stress than the vegetative phase. Pollen development is one of the most heat-sensitive developmental stages in cereals (Stone 2001; Abiko et al. 2005), vegetable crops (Peet et al. 1998; Aloni et al. 2001; Sato et al. 2002) and grain legumes (Prasad et al. 1999; Porch and Jahn 2001). In small-grain cereals, stress-induced decline in crop yields is often attributed to pollen sterility (Boyer and Westgate 2004). The molecular basis for the high sensitivity of developing pollen grains to heat stress, on one hand, and pollen heat-tolerance, on the other, is poorly understood.

Pollen development is initiated as the microspore mother cell yields a meiotic tetrad of haploid microspores that are held together by callose walls, and are well-nourished through the sporophytic cell layer, tapetum (McCormick 2004). The young microspores become vacuolated after their release from the tetrad and subsequent symplastic isolation from the mother plant. After a large centralized vacuole forms, the first mitotic division produces a large vegetative cell and a generative cell, which further undergoes a second division to form two sperm cells. The pollen is shed after the second mitosis in a trinucleate stage in many plants such as *Arabidopsis*, rice and maize (Raghavan 1988; Tanaka 1997; Twell et al. 1998). The first mitosis is regarded as a key developmental switch in male gametophyte formation and is marked by a decrease in vacuole size, development of subcellular organelles, and extension of the intine layer of the pollen wall (Raghavan 1988; Arizumi et al. 2004). Concurrently, the cytoplasm also undergoes several important metabolic changes, including ribosome biogenesis, lipid and protein biosynthesis (Niewiadomski et al. 2005), and most conspicuously, a rapid phase of starch biosynthesis leaving the cytoplasm heavily "engorged" with starch granules (Datta et al. 2001, 2002). The biochemical, physiological and molecular genetic aspects of sugar-to-starch metabolic transition are well illustrated in the storage sink—the developing seed (James et al. 2003); however, only limited information is available on sugar unloading and subsequent uptake by young microspores during microsporogenesis. Datta et al. (2001, 2002) describe changes in temporal expression profiles for a number of metabolic and regulatory genes involved in sugar-to-starch transition leading to starch deficiency and pollen sterility in cytoplasmic male sterile (CMS) lines of sorghum and maize, respectively. Pollen-sterility in CMS lines of maize was associated with reduced sugar levels throughout microsporogenesis as well as reduced flux of sugars into starch biosynthesis.

Starch accumulated during the advanced stages of pollen maturation is suggested to serve as a reserve source of energy for pollen tube germination and also provides a metabolic checkpoint of pollen maturity (Datta et al. 2001, 2002; Pring and Tang 2004). Microspore development in maize ceases prematurely if starch levels remain lower than a certain threshold level as evident from several cytoplasmic male-sterile mutants where pollen non-viability is associated with starch-deficiency (Wen and Chase 1999; Datta et al. 2002). Lee et al. (1980) showed collapse of pollen structure during starch accumulation phase in a comparative ultrastructural study of the sterile and fertile pollen in S-type cytoplasmic male sterile (S-CMS) lines of maize. Stress-induced aberrant starch deposition profiles during microsporogenesis and reduced male fertility has also been documented in rice (Sheoran and Saini 1996; Saini 1997), wheat (Saini et al. 1984; Dorion et al. 1996; Lalonde et al. 1997), barley (Sakata et al. 2000), tomato (Pressman et al. 2002, 2006; Sato et al. 2002) and bell peppers (Aloni et al. 2001; Karni and Aloni 2002). Perturbed sugar utilization by developing microspores through targeted antisense repression of anther-specific CWI gene in tobacco (Goetz et al. 2001) and *SnRK1* in barley (Zhang et al. 2001) resulted in starch deficient and sterile pollen. Developing pollen grains of the *Nicotiana sylvestris* starch-less mutant NS458 exhibited increased sensitivity to high temperature stress and significant loss in pollen germination capacity, along with low levels of starch accumulation (Firon and Pressman, personal communication). The biochemical significance of starch accumulation towards maintenance of high pollen viability under heat stress conditions is not fully understood. Kaplan et al. (2004) suggested an adaptive role of carbohydrates as compatible solutes and signaling molecules during development and stress tolerance. Increased sucrose levels following starch digestion 1–2 days prior to anthesis is believed to be associated with desiccation tolerance in the developing pollen of *Papaver dubium* L. (Hoekstra and Roekel 1988). Likewise, the differential dehydration sensitivity of maize and *Pennisetum* pollen was accredited to starch-derived sucrose content (Hoekstra et al. 1989).

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most important cereal crop worldwide (http://www.apps.fao.org/default.jsp), and its drought-hardiness makes sorghum an important "failsafe" crop under arid and semi-arid conditions. Sorghum is an important C_4 crop species that is potentially more efficient in carbon assimilation (at current atmospheric CO_2 concentrations) at high temperatures and in water-limited conditions as compared to C_3 crops such as rice and wheat (Kresovich et al. 2005). In grain sorghum, the optimum mean temperature range for seed germination is 21–35°C, and the optimum temperature ranges are between 26–34°C and 25–28°C for vegetative growth/development and reproductive maturity, respectively (Maiti 1996). Adverse environmental conditions, such as cold (Wood et al. 2006) or high temperature stress (Prasad et al. 2006a) have been shown to affect male fertility and compromise grain yield in sorghum. The following research was undertaken in order to determine the threshold levels of chronic (season-long) and acute (short-term, coincident with microsporogenesis) high temperature stress treatments that affect pollen fertility and seed-set in grain sorghum. The specific objectives were to obtain a detailed temporal transcript profiling of sugar-to-starch metabolic transition in developing microspores of sorghum grown under ambient or season-long elevated growth temperature regimes, and to define committed metabolic check-points (if any) that regulate the utilization of sugar into starch biosynthesis and establish microspore sink strength. Genes involved in sucrose cleavage and utilization (*SbIncw1*, *SbIvr2*, *Sh1*, *Sus1*), microspore unloading from enriched locular fluid (*Mha1*, *MST1*), and starch biosynthesis (*Bt2, SU1, GBSS1, UGPase*) were chosen based on the previously published evidence correlating gene expression data, starch deficiency and male-sterility in maize and sorghum microspores (Datta et al. 2001, 2002).

Materials and methods

Plant growth, SPAR chambers and temperature treatments

Semi-dwarf and photoperiod-insensitive sorghum cv. DeKalb 28E was used for all experiments. Sowing, irrigation and fertilizer schedules were as described (Prasad et al. 2006a). Experiments were conducted in eight sunlit Soil-Plant-Atmospheric-Research (SPAR) growth chambers (Pickering et al. 1994), with a stand density of 20 plants per m^2. Air and dew point temperature (10°C below target mean dry bulb air temperature) were typically controlled as a sinusoidal wave function during the day and a logarithmic decay during the night. These environments provided nearly constant relative humidity (55–58%) at 1500 hours in all treatments. Daytime CO_2 concentration was measured continuously for each chamber using infrared gas analyzers (Model Ultramat 21P, Siemens, New York, NY, USA) and controlled at ambient set point (350 ppm) by injecting pure CO_2 from a high pressure cylinder through a mass flow controller (Model 5850i, Brooks Instruments, Hatfield, PA, USA). The data presented in the following research was obtained from two sorghum experiments conducted in summer 2005 and 2006.

Photosynthesis measurements

Individual attached leaf gas exchange measurements were made during mid-day at respective growth temperature conditions at the time of panicle emergence (35–40 DAS) using a LI-COR 6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA). The growth chamber conditions were simulated in the leaf chamber through an integrated Peltier temperature controller and CO_2 injection system. Measurements were conducted on three topmost fully expanded leaves from three different plants on a clear sunny day. The internal light source (6400-02B red/blue) in the LI-COR 6400 was set at 1,800 μmol $m^{-2}s^{-1}$ to have constant and uniform light across all measurements.

Pollen viability and seed set measurements

In vitro pollen germination was carried out as described by Tunistra and Wedel (2000) using a slightly modified germination medium containing 150 mg H_3BO_3, 500 mg $Ca(NO_3)_2{\cdot}4H_2O$, 200 mg $MgSO_4{\cdot}7H_2O$, 100 mg KNO_3, 300 g sucrose and 15 gL^{-1} agar. Pollen was collected prior to anthesis (between 0730 and 0800 hours) and dispersed on slides pre-warmed at 28°C for 30 min, incubated under darkness for 45 min and monitored under microscope. The pollen was scored as germinated if the length of the pollen tube was greater than the diameter of the pollen grain. Pollen viability was also tested using 2% tri-phenyl tetrazolium chloride stain that stains the live pollen reddish purple due to the formation of insoluble red formazan. At maturity, the numbers of filled and unfilled seeds were counted on the tagged panicles. Seed-set was estimated as the ratio of florets with seed to the total number of florets, and expressed as a percentage.

Preparation of microspores/young pollen

Panicles were harvested for microspore isolation as described by Pring and Tang (2004). The pre-emergent panicles were collected following appearance of the panicle tip out of the flag leaf sheath. At this stage, the microgametophytes were approximately 7–11 days prior to anthesis, and included young early-mid microspores (pre- to post meiotic) at the base of the panicle (referred to as *stage D*)

developmentally progressing through post-mitotic young pollen (referred to as *stage A*) near the tip of the panicle. Complete florets including the glumes (at *stages D* through *A* of microsporogenesis) were plucked free from panicle branches and used for RNA extraction.

Northern blot analyses and real-time quantitative PCR

Total RNA was extracted in Trizol Reagent following the manufacture's protocol. RNA was glyoxalated and resolved on a 1.2% (*w/v*) agarose gel, followed by transfer to Nytran membranes (Schleicher & Schull, Keene, NH) and UV cross-linking. Probes were prepared from the full-length/partial cDNA clones and labeled using the random priming method (Prime-It® RmT Random Primer Labeling Kit, Stratagene, LaJolla, CA). Pre-hybridization, hybridization and washing conditions were same as described previously (Datta et al. 2001, 2002). The blots were exposed to X-ray film, in between two intensifying screens, for 1–4 days depending on the transcript abundance. Transcript size was estimated based on the position of the rRNA species visualized in the ethidium bromide-stained gel prior to blotting.

Five µg total RNA was reverse transcribed using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative 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 were prepared according to the manufacturer's instructions and contained 400 nM of both the forward and reverse gene-specific primers and 2 µl of the fivefold diluted RT reaction in a final volume of 20 µl. The thermal cycling protocol entailed 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}. The data are presented as average ±SD of three independently made RT preparations used for PCR run, each having three replicates.

Gene specific primers used for PCR cloning, real-time PCR analyses and cDNA probes are listed in Table 1. The amplification products were cloned in pCR®2.1 vector using the TA Cloning® kit (Invitrogen, Carlsbad, CA), and sequenced to verify the fidelity of the PCR amplification.

Sugar assays

Microspore samples were homogenized and the soluble sugars extracted in hot ethanol to separate soluble sugars from starch. After centrifugation, the supernatant was used for glucose, sucrose and fructose analyses, in a microtiter plate assay as described (Datta et al. 2002). Starch content was determined in the pellet fraction following amyloglucosidase digestion (EC 3.2.13, from *Aspergillus niger*, Roche Diagnostics, Indianapolis, IN), and quantification of released glucose moieties.

Table 1 Primers used for cDNA cloning and 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[a]	EF177465	TGTACTACAAGGGGTGGTACCA	TCAAGCTCCATTCATGAGTGGCTTCTTCAT
SbIncw1[b]	EF177465	GCAAAGTCGGTCACTCTCAGGAA	AAATCCTGCAAATGTCGGGCG
SbIvr2[d]	NA	CTCACCAACTGGACCAAGTACGA	CCATGTAGTCGTGGTTGTATGACG
Sh1[c]	X02400	ACTACAAGGGCACGACGATGATGT	TTCCCGCGATTTCTTGGAATGTGC
Sus1[c]	L29418	TTCCTCAACAGGCACCTGTCATCA	TCCAGGTACGGCCAGACTTCAAAT
Mha1[c]	U09989	ATCCAACACAGGGCTTACCGATCA	TTCAGAGCTGGAGCGTCGTTTACA
MST1[c]	AY683004	AAATCAGTCCGTGCCTGTGTACCT	ATGAGGGAGGCGTCGTTCTTGAAT
Bt2[c]	AF334959	TCAAGAGCCTACGGGAGCAACATT	GCATTTCCTTTGCCCTCACGTCAT
GBSS1[c]	AF079261	AGGGACAAGTACATCGCCGTGAA	TCATGCAGTTCCTCACCATCTCCT
SU1[c]	AF030882	ACATGCTTGCACCTAAGGGAGAGT	ATGACCGTGCCATTTCAACCGTTC
SbTua1[d]	NA	CCTGAGGTTCGATGGTGCTCT	CCATCATCACCTTCCTCACCC

[a] Primers used for RT-PCR cloning of *SbIncw1* genes from 6- to 12 DAP sorghum seed

[b] Primers used for real-time quantitative PCR

[c] Primers used for making cDNA probes from 12 to 16 DAP maize endosperm

[d] The soluble invertase (*SbIvr2*) and *α-tubulin 1* (*SbTua1*) cDNA clones were amplified from immature sorghum microspore RNA

NA Not available

(*UGPase* cDNA was kindly provided by J R Sovokinos, University of Minnesota)

Immunolocalization of cell wall invertase

Florets from pre-emergent panicles were harvested and immediately fixed in cold formalin acetic alcohol (FAA; 3.7% formaldehyde, 5% acetic acid and 50% ethanol) for 24 h, followed by dehydration in a series of ethanol and tertiary butyl alcohol (TBA), infiltration and embedding in Paraplast Plus paraffin (Sherwood Medical Co., St Louis, MO). Paraffin embedded florets were sectioned with a rotary microtome (Microm 325, Carl Zeiss, Germany). Polyclonal antibodies of maize CWI (Shanker et al. 1995) were used for immunodetection of INCW, and the immunogold signal was visualized using the ZYMED kit (Zymed Laboratories Inc, San Francisco, CA).

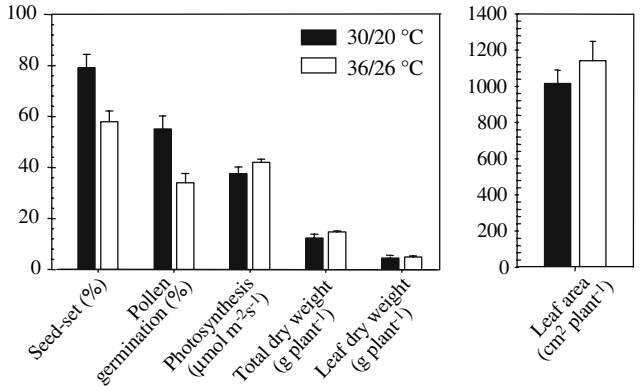

Fig. 1 Effects of ambient (30/20°C) and season-long high (36/26°C) temperature growth conditions on various growth traits as shown. The yield data were collected at 60 days-after-sowing for six randomly tagged plants from two independent experiments, and represent average ± SD

Results

Effects of high temperature conditions on the growth of grain sorghum

Grain sorghum plants were grown at sinusoidal day-time maximum/night-time minimum temperature regimes of 30/20°C (ambient), 36/26°C (high), 40/30°C and 44/34°C (extremely high) in naturally sun-lit, controlled environmental growth chambers equipped with real-time control of relative humidity (55–58% at 1500 hours) and day-time ambient CO_2 set point (350 ppm). Panicle emergence occurred at 35–40 days-after-sowing (DAS) at 30/20 and 36/26°C treatments. Panicle emergence was delayed to 60 DAS and occurred in only 10–12% of plants grown at 40/30°C. Despite a vigorous and highly robust vegetative growth, the floral transition was completely arrested at 44/34°C. Anthesis occurred within a 4–5 day period after panicle emergence at both 30/20 and 36/26°C temperature treatments. The effects of season-long exposure to high growth temperature (36/26°C) conditions imposed from seedling emergence to maturity on leaf photosynthesis, pollen germination, seed-set and grain yield, dry matter production are documented in Figs. 1, 2. Data on leaf photosynthesis, percent seed set, and dry matter production were collected at 60 DAS and showed no significant adverse effects of high temperature on leaf photosynthesis rates, leaf area, leaf dry weight and total plant dry weight accumulation. The highest two temperatures treatments (40/30 and 44/34°C) failed to produce grain, and will not be further discussed here (see Prasad et al. 2006a). Exposure to season-long moderate high temperature stress (36/26°C) resulted in 27% loss in grain yield, and was correlated with significantly reduced germination potential scored in vitro (Fig. 1). Even though the microspores that were collected at both temperature treatments stained viable with tri-phenyl tetrazolium chloride (data not shown), remained turgid and exhibited normal diameter, the microspores at ambient growth temperature were starch-packed whereas the ones at 36/26 °C were deficient in starch (Fig. 2a, b, respectively). Short-term exposure to high temperature at the time of panicle initiation (i.e. 10 or 5 days prior to emergence) resulted in localized sectors of floral necrosis causing significant decrease in panicle length (20–9.2%), diameter (37–27%) and dry weight (52–45%), and 40–35% loss in seed-set (Fig. 2e, g). However, 100-unit-seed weight (or seed size) was not significantly affected by either chronic or short-term high temperature stress, and averaged 2.74 ± 0.43 g at 30/20°C, 2.71 ± 0.21 g at 36/26°C (chronic), and 2.54 ± 0.25 g and 2.53 ± 0.23 g at 36/26°C (imposed at either 10 or 5 days prior to emergence), respectively. Notably, decreased yield at chronic high temperature treatment could be attributed to failed fertilization (as evident by reduced pollen germination and high number of unfilled floral sites, Figs. 1, 2d, f), whereas the panicles exposed to short-term high temperature stress prior to emergence also suffered loss of floral primordia in addition to failed seed set (Fig. 2e, g). Even though the reduced grain yields and harvest index can be ascribed to high temperature stress-induced detrimental effects on pollen quality and reduced pollen germination, decreased pollen production (not determined in the present study) and role of stigma receptivity can not be ruled out. Prasad et al. (2006b) have shown that high temperature reduces pollen production as well as pollen reception by stigma thus lowering spikelet fertility and grain filling in rice. Reduced pollen production and lower viability at high growth temperature can be related to aberrant tapetum development (Suzuki et al. 2001), carbohydrate metabolism (Karni and Aloni 2002; Pressman et al. 2002) and anther indehiscence (Porch and Jahn 2001).

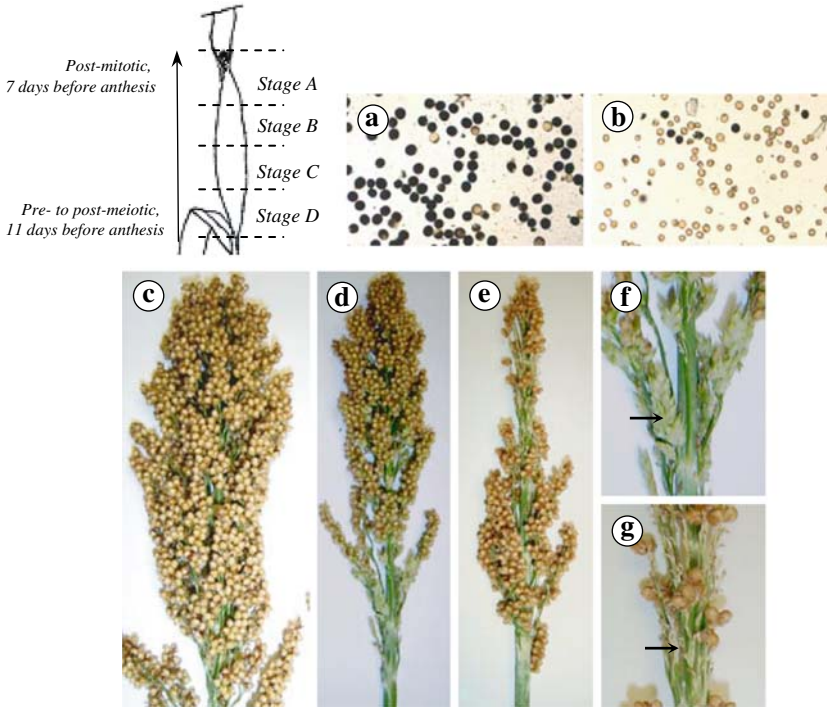

Fig. 2 **a**, **b** Pollen starch and **c**, **g** seed set at ambient (30/20°C) and high (36/26°C) temperature growth conditions. I_2/KI staining for starch in immature pollen, at 7 days prior to anthesis, from plants grown season-long at **a** ambient (30/20°C) and **b** high temperature (36/26°C); seed-set at **c** ambient, **d** season-long high temperature, and **e** short-term (10 days prior to panicle emergence) high temperature growth conditions after ambient environment. *Arrows* indicate sectors of failed seed-set **f** and necrotic florets **g** under season-long and short-term stress treatments, respectively

Effect of growth temperature on carbohydrate profiles during microspore maturation

In sorghum, microsporogenesis and starch deposition profiles are highly synchronized and proceed in a basipetal manner. For all experimental purposes, pre-emergent panicles (approximately 7–11 days prior to anthesis) were sectioned as detailed in Fig. 2. The microgametophytes at the base of the panicle included young early-mid microspores (pre- to post-meiotic) (*stage D*) whereas microgametophytes towards the tip of the panicle (*stage A*) had progressed through first mitotic division and stained positive for starch content. To better understand the effect of high growth temperature on starch biosynthesis in developing microspores, the relative levels of soluble sugars Glc, Frc and Suc, and starch were determined in pre- and post-meiotic (*stage D*, approximately 11 days before anthesis) and post-mitotic (*stage A*, approximately 7 days before anthesis) microspores (Fig. 3). Even though the levels of total soluble sugars at the two developmental stages were not significantly different between either temperature treatments (visually indicated by normal turgor and diameter), the individual profiles of reducing and non-reducing sugars showed notable differences. Remarkably, Suc was below the detection limit in *stage D* at both temperature treatments, and Frc represented nearly the total pool of non-reducing sugars (Fig. 3a). In *stage A* microspores, sucrose was present only under ambient growth temperature conditions (Fig. 3b). Hence total soluble sugars were represented by Glc, Frc and Suc at 30/20°C, and only by Glc and Frc at

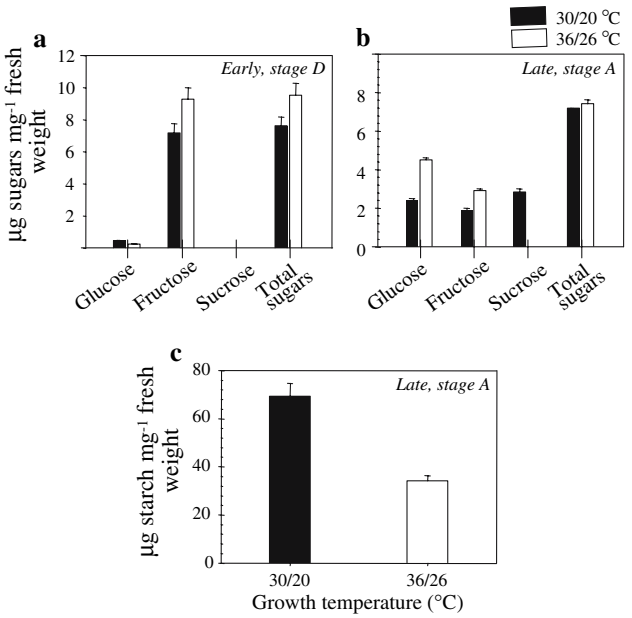

Fig. 3 Carbohydrate profiles of pre- and post-meiotic "early" and post-mitotic "late" microspores (*stages D* and *A*, respectively, in the schematic drawing in Fig. 2) from sorghum plants grown season-long at ambient (30/20°C) and high (36/26°C) temperature growth conditions. The data presented are average ± SD of three independent extractions with each assay having six replicates

36/26°C, with Glc levels being moderately higher than Frc in all the respective microspore populations. At *stage D*, starch was below the detection limit at both the growth temperatures, but was reduced by about 50% in microspores at

high growth temperature at *stage A* (Fig. 3c), as was also evident by reduced I_2/KI staining (Fig. 2b).

Spatiotemporal expression of cell wall invertase gene *SbIncw1* in developing microspores

Invertases catalyze the irreversible hydrolysis of Suc into Frc and Glc and are classified as soluble acid invertases present in vacuoles; whereas the insoluble acid invertases are cell wall-bound and localized to the apoplast. Cell wall invertase-mediated sugar inversion has been shown to be crucial for source-to-sink sucrose unloading, and hence establishment and maintenance of sink strength in developing seeds (Cheng et al. 1996) as well as microspores (Goetz et al. 2001). Figure 4 shows (a) Northern blot and (b) real-time quantitative PCR analyses of CWI gene *SbIncw1* in RNA extracted from young microspores as well as total RNA extracts of intact floral whorls at similar developmental stages. A *SbIncw1* cDNA clone (1.6 kb; GeneBank accession no. EF 177465) from sorghum endosperm was used as the probe (Jain et al. 2007). Under ambient growth temperature conditions, a significant rise in *SbIncw1* expression was observed during the starch deposition phase (*stage A*) in RNA extracted from intact florets, but not in the isolated microspores, reflective of developmental stage-specific *SbIncw1* contribution of sporophytic origin. However, this temporal rise in *SbIncw1* expression during starch-deposition phase was not evident under high temperature stress. Additionally, steady state transcription of the *SbIncw1* gene in microspores through post-mitotic stages was significantly reduced in plants exposed to high temperature stress.

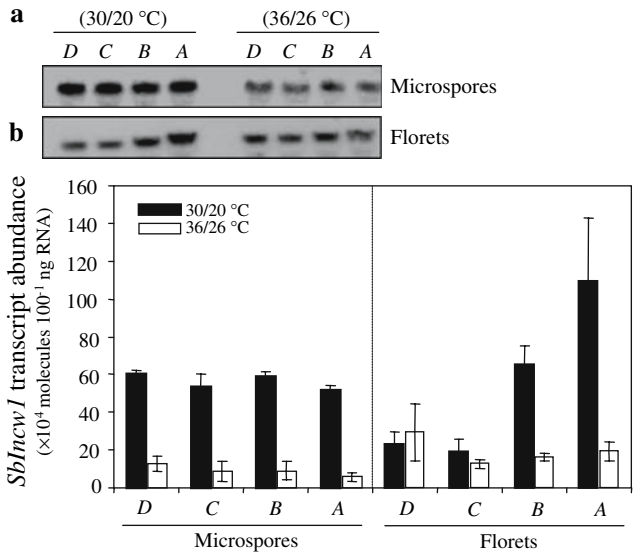

Fig. 4 **a** RNA gel blot and **b** real-time quantitative-PCR analyses showing expression of sorghum cell wall invertase gene, *SbIncw1* (GenBank accession no. EF 177465), in microspores and florets from plants grown at ambient (30/20°C) and season-long high (36/26°C) temperature. The floral and microspore developmental stages are as indicated (Fig. 2, schematic drawing). All lanes were loaded with 15 µg total RNA for the RNA gel blot analyses (**a**). For the q-PCR, the data represents average ± SD (100 ng^{-1} total RNA) for three independently made RT preparations and three replicates for each PCR run (**b**)

Immunolocalization of cell wall invertase in sorghum florets

Polyclonal antisera against maize INCW1 and INCW2 were used to examine spatial localization of CWI proteins in transverse sections of sorghum florets (Fig. 5). Although these antibodies do not discriminate the two isoforms in maize (Shanker et al. 1995), spatially distinctive patterns of immunolocalization were observed with these antibodies in sorghum (Fig. 5a, f). Florets at two developmental stages, "early" (*stage D*, pre- and post-meiotic microspores) and "late" (*stage A*, post-mitotic microspores at the beginning of starch deposition phase) were sampled from the base and the tip of the pre-emergent panicle, respectively. Immunolocalization with the INCW1 antiserum detected antigenic signal in tapetum and vascular parenchyma of anther filaments at the "early" stages (Fig. 5a, b), and also in the microspores at the "late" stages of florets grown at ambient growth temperature conditions (Fig. 5d). However, no cross-reactive antigen was detected under high temperature growth conditions (Fig. 5c, e; respectively). The INCW2-specific signal was detected in the cortical parenchyma in the bifid style and anther filaments (Fig. 5f, g), and in "late" stages the signal was in vascular parenchyma of the anther filaments and microspores in addition to the feathery stigma tissue (Fig. 5i). A significant reduction in cross-reactive protein with INCW2 antiserum was discernible under heat-stress conditions that was restricted only to the female sporophyte (Fig. 5h); and no qualitative differences in antigen presence were observed in anther vasculature and young microspores in "late" florets from high temperature growth conditions (Fig. 5i, j). Overall, high temperature growth conditions resulted in significant loss of the INCW proteins in male sporophytic and gametophytic tissues.

Transcript analyses of genes involved in sugar metabolism, transport, and starch biosynthesis

We also examined the effect of ambient and season-long high growth temperature on comparative transcript profiles of various genes involved in the cleavage, transport and utilization of sucrose during early stages of microsporogenesis through beginning of starch biosynthesis phase (*stage D* through *stage A*, see Fig. 2). Figure 6a shows the RNA expression levels of two other key metabolic enzymes involved in sucrose utilization, namely, soluble invertase (*SbIvr2*) and two maize sucrose synthase orthologs, *Sh1* and *Sus1*. Soluble invertase (*SbIvr2*) expression was

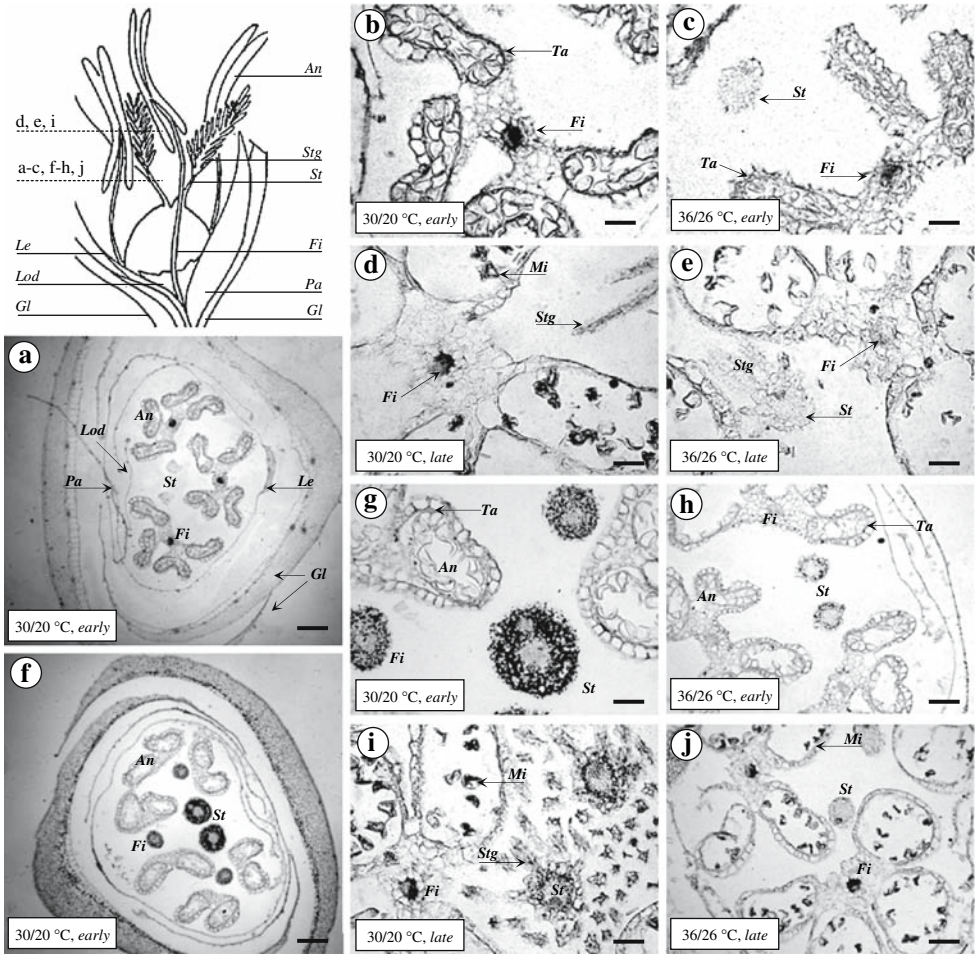

Fig. 5 Immunolocalization of cell wall invertase in sorghum florets grown at ambient (30/20°C) and season-long high (36/26°C) temperature growth conditions. Transverse sections **a-e** and **f-j** were immunodeveloped using polyclonal antisera for maize INCW1 and INCW2, respectively. The developmental stages ("early" vs. "late") and the growth temperatures (ambient vs. high) are marked in the figure. Primary INCW1 and INCW2 antibodies were used at 1:1000 dilution. Presence of cell wall invertase is indicated by precipitation of silver grains. The *line diagram* shows a schematic of floral architecture in sorghum, and the *dotted lines* approximately depict the planes of transverse sections used for immunolocalization. *An* anther lobes, *Fi* filament, *Gl* glume, *Le* lemma, *Lod* lodicule, *Mi* microspores, *Pa* palea, *St* style, *Stg* stigma, *Ta* tapetum. *Magnification bars:* 200 μm (**a**, **f**); 100 μm (**h**, **j**); 50 μm (**b-e**, **g**, **i**)

evident throughout the development of microspores with moderately elevated levels towards beginning of starch deposition phase, and was also upregulated due to high growth temperature. Sucrose synthase (*SuSy*) provides both substrates and precursors for cellulose, callose and starch biosynthesis in various plants. Two *SuSy* genes (maize *Sh1* and *Sus1* orthologs) have been described in sorghum (Chourey et al. 1991). A gradual decrease in steady state transcript abundance of *Sus1* was seen during microspore development; however, the steady state levels at comparable stages were higher in case of high temperature treatment. Likewise, *Sh1* transcription was also considerably reduced during starch deposition stages, but no significant effects of high growth temperature was apparent on the RNA levels; except for in the pre- and post-meiotic *stage D*, where *Sh1* levels were greatly reduced at ambient as compared to the high temperature growth conditions.

Developmental progression of microspores to starch filling phase under ambient growth conditions was associated with coordinated up-regulation of transporter genes plasma membrane H^+ATPase (*Mha1*) and monosaccharide transporter (*MST1*) (Fig. 6b). However, the steady state temporal expression profile of *MST1* was down-regulated during microspore development under high temperature growth conditions. On the other hand, there was a qualitative reversal in the expression profile of *Mha1* under stress conditions. On the whole, such changes are consistent with the energy-dependent role of these transporter proteins in absorption of hexose sugars from the nutrient rich locular broth into the microspore sink (Zhao et al. 2000).

Figure 6c illustrates effects of high growth temperature conditions on the temporal expression profiles of starch biosynthesis genes in sorghum microspores. In cereal seed endosperm, AGPase is a heterotetrameric protein, encoded by two nonallelic genes, *Sh2* and *Bt2* (Bhave et al. 1990) catalyzing the formation of ADP-Glc, the soluble precursor and substrate for starch synthases. We show *Bt2* transcripts as a representative of the AGPase transcript levels. Furthermore, AGPase activity is often coupled to UDP-Glc utilization by UGPase resulting in equimolar production of ADP-Glc from UDP-Glc (Kleczkowski et al. 2004). *Granule-bound starch synthase-1* (*GBSS1*, encoded by the *Waxy* gene in maize) transfers the glucose moieties from ADP-Glc to the non-reducing ends of starch molecules inside the growing starch granules making starch amylose (largely unbranched α-(1 → 4)-linked glucan chains). *Sugary1* (*SU1*) is a starch

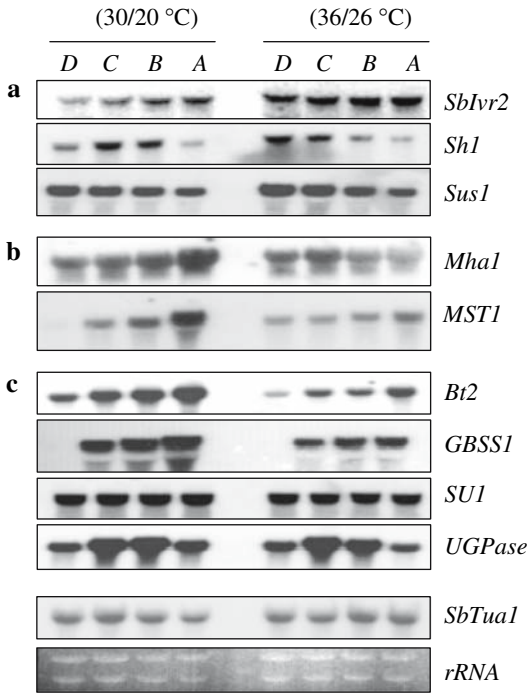

Fig. 6 Effect of season-long high temperature (36/26°C) growth conditions on temporal expression of **a** soluble invertase (*SbIvr2*) and sucrose synthases (*Sh1*, *Sus1*), **b** transporter proteins (*Mha1*, *MST1*), and **c** starch biosynthesis enzymes (*Bt2*, *GBSS1*, *SU1* and *UGPase*) in sorghum microspores at the developmental stages as indicated (Fig. 2, schematic drawing). All lanes were loaded with 15 μg total RNA. RNA loading controls are represented by *α-tubulin 1* (*SbTua1*) and ethidium bromide stained rRNA bands

debranching enzyme hydrolyzing α-(1 → 6)-glucosidic linkages, and is also involved in starch biosynthesis and determines the amylopectin component of starch granule. A gradual temporal increase in *Bt2* transcript abundance was seen during the maturation phase coincident with starch biosynthesis. Although totally undetectable during meiotic stages, *GBSS1* transcript was seen in great abundance during the starch biosynthesis phase under ambient growth conditions. *Sugary1* did not show any temporal variability in expression profile during microspore development. High temperature stress conditions did not affect the temporal specificity of expression, but significantly reduced the steady state transcript levels of all the three genes, *Bt2*, *GBSS1* and *SU1*, at any comparable developmental stage under stress conditions. The abundant increase in the steady state levels of *UGPase* evident prior to the beginning of starch biosynthesis phase under ambient temperature conditions, was transcriptionally repressed under stress conditions.

Discussion

Heat stress-induced reduction in crop yields has previously been documented across a range of crop species including grain sorghum (Prasad et al. 2006), and is attributed to failure of starch deposition consequently impairing pollen development and germination processes. The molecular mechanisms governing heat stress-induced male sterility are sparsely understood. In this regard, our data indicate strong parallels with heat, cold and drought stress-induced down-regulation in CWI expression and activity causing pollen sterility in tomato (Pressman et al. 2006), rice (Oliver et al. 2005) and wheat (Koonjul et al. 2005), respectively. It is axiomatic that post-phloem unloading and assimilate partitioning into symplastically isolated sink tissues via apoplasmic space will rely primarily upon cell wall bound invertase activity, with additional checkpoints at the level of transport across the cell membrane.

Carbohydrate profiles in the developing microspores

The results from sugar analyses (Fig. 3) showed that the total soluble sugar profile at the ambient temperature was represented primarily by Frc during "early" stages of microsporogenesis, and by Glc, Frc and Suc later during development. However, for "late, *stage A*" microspores, the presence of Suc in ambient but not in high temperature plants is of significant interest because this trait is also correlated with starch accumulation; i.e., much higher proportion of pollen from ambient plants exhibit starch accumulation than the high temperature plants. In maize, by contrast, our previous studies showed no sucrose in both early and late microspores, including the genotype that accumulated starch and, ultimately, fertile pollen (Datta et al. 2002). Sucrose accumulation may thus be unique to sorghum and it is most probably synthesized in the microspores from hexoses transported from the sporophytic tapetal cell layer. A "futile cycle" of Suc synthesis and cleavage reactions, similar to developing maize endosperm (Cheng and Chourey 1999), may be operational in the late phases of microspores of plants grown in ambient but not in high temperature plants. The higher hexose sugar levels accumulating in the "late, *stage A*" microspores at high temperature may be an indication that Suc synthesis is blocked which would in turn limit this "futile cycle" of carbon transfer to starch biosynthesis.

Elevated growth temperature-induced inhibition of CWI expression in sorghum florets

Sugar metabolism and its control, and assimilate partitioning are critical to all parts of the plant, including developing seeds and microspores—major sites of sugar utilization. An important role of CWI in providing carbohydrates to the male gametophyte is demonstrated with the presence of anther—expressed CWI isoforms in *Vicia faba* (Weber et al. 1996), *Lilium longiforum* (Clément et al. 1996) and *L. hyb-*

rida (Castro and Clément 2007), potato (Maddison et al. 1999), tobacco (Goetz et al. 2001), tomato (Godt and Roitsch 1997; Pressman et al. 2006), maize (Xu et al. 1996; Kim et al. 2000), wheat (Koonjul et al. 2005), rice (Ji et al. 2005; Oliver et al. 2005) and sorghum (present study). Transcript analyses (Fig. 4a, b) and immunolocalization (Fig. 5) data collectively reemphasize the critical role of INCW activity in driving sucrose unloading from the nutritive tapetum layer into apoplast surrounding the developing microspores and its utilization for starch biosynthesis. However, the possibility of multiple *SbIncw* isoforms in sorghum anthers receptive to differential spatiotemporal and stress-induced signals can not be ruled out. Heat stress-induced transcriptional down regulation of *SbIncw1* expression in young microspores as well as the sporophytic floral whorls (tapetum, anther filaments, stigma and styler tissues) appears to be the most upstream metabolic aberration (Figs. 4, 5) leading to altered sucrose uptake and associated changes in transcriptional activity of genes involved in sugar transport and metabolic transition to starch biosynthesis phase (Fig. 6). Goetz et al. (2001) reported that tapetum-specific antisense repression of CWI gene *Nin88* leads to failure of microsporogenesis beyond the unicellular stage in tobacco. Interestingly, exogenous carbohydrate supply could only partially alleviate the metabolic arrest in an in vitro pollen maturation assay, reflective of an absolute requirement for substrate availability for heterotrophic growth as well as sugar-mediated developmental signal cues for maturation of symplastically isolated microspores. Notably, a similar requirement for INCW2-mediated metabolic release of hexoses was shown to be critical for normal endosperm development in maize (Cheng and Chourey 1999). Koonjul et al. (2005) reported selective transcriptional down regulation of one vacuolar and one cell wall-bound invertase gene and the corresponding enzyme activities in the anthers leading to failed pollen development in water deficient wheat plants. Likewise, in rice, cold-stress induced transcriptional repression of a tapetum-specific CWI gene *OSINV4* and pollen sterility was evident only in the cold-sensitive and not in the tolerant cultivar (Oliver et al. 2005). A few differences in the temporal overlap of transcriptional down-regulation of CWI genes and microsporogenesis events are, however, apparent between wheat, rice, and sorghum. In wheat and rice, stress-induced effects on the transcription of CWI genes and enzyme activity were coincident with the transition of meiospores to early unicellular stage (Koonjul et al. 2005; Oliver et al. 2005). Although a similar role in sorghum is not ruled out, the reduction in sporophytic component of CWI abundance was evident only prior to the starch deposition phase and the microspore-associated changes were observed throughout microsporogenesis (Fig. 4).

The data on heat stress-induced changes in transcriptional levels of *SbIncw1* were further corroborated by our immunolocalization data of distinct CWI isoforms in various floral tissues shown in Fig. 5. It is apparent that starch deficiency of pollen is associated with stress-induced deviation from spatial and developmental specificity of CWI isoforms in tapetum, anther filaments and young microspores. With the onset of tapetal degeneration and isolation of microspores from the mother plant, the apoplastic sugar cleavage and utilization pathway is likely controlled both by the surrounding tapetum layer as well as the developing pollen in a sequential manner (Goetz et al. 2001; Oliver et al. 2005). It is conceivable that heat-stress induced loss of CWI activity in tapetum cells will have deleterious effects on its secretory functions responsible for nutritional enrichment of locular fluids in the apoplast, that are subsequently taken up by microspores as they grow and mature into pollen grains. Also, decreased CWI activity in symplastically discontinuous microspores will adversely affect sugar import from the surrounding locular fluid, further causing changes in carbohydrate metabolism. The residual INCW isoform in the young heat-stressed microspores immunolocalized by INCW2 antiserum (Fig. 5i, j) may only fulfill the partial sucrose requirement for basic respiratory needs and maintaining cell viability. Notably, while cold-stressed anthers in rice showed lack of pollen viability at the time of anthesis (Oliver et al. 2005), dehisced pollen in heat-stressed sorghum score positive upon staining with the vital dye tri-phenyl tetrazolium chloride (data not shown) but display starch-deficient phenotype as a probable cause leading to infertility. Also, adequate CWI activity in the anther filaments may be necessary for assimilate unloading from phloem termini en route to the tapetum cells. Additionally, sucrose inversion in the anther filaments through late stages of flower development may be essential for supporting cellular turgor and expansion leading to exertion of anther lobes prior to anthesis. Such a role of CWI genes has been proposed in drought-stressed rice for coordination of anther dehiscence, panicle exertion and heading (Ji et al. 2005). We also observed heat stress-induced down-regulation in CWI activity in female sporophytic tissue as a probable cause for reduced pollen receptivity and compromised seed set.

Effect of elevated growth temperature on expression of genes involved in sugar transport, metabolism, and utilization

The data summarized in Fig. 6a show that under ambient growth conditions *SbIvr2* transcript levels were associated with a temporal increase in steady state levels during pollen maturation (A stage was greater than the D); whereas, the *Sus1* transcript abundance was slightly higher in younger than the advanced stages of microspores (i.e., D > A). These data agree well with the proposed roles of soluble

invertase and *SuSy* in supporting transient storage of sucrose in the vacuoles. Similar to its role in developing endosperm (Chourey et al. 1998; Jain et al. 2007), it is possible that *Sus1* has only a minor contribution (if any) towards starch biosynthesis in developing microspores and may provide UDP-Glc for callose biosynthesis. By virtue of its reversible reaction kinetics, *SuSy* may also provide for intracellular transient storage of sucrose in the vacuoles.

Previously, Datta et al. (2001) have shown temporal specificity of *Sus1* expression limited to the "early" stages of sorghum pollen derived from male-fertile lines and higher abundance at "late" stages in starch-deficient male-sterile pollen. Also, *Sus1*-encoded mRNA and protein were at higher level in the "early" stages in both male-sterile as well as fertile lines of maize (Datta et al. 2002). Higher *Ivr2* expression levels were reported for male-fertile and sterile lines of sorghum and male-fertile lines of maize (Datta et al. 2001, 2002). In contrast, however, *Ivr2* transcription was nearly undetectable at "late" stages of male-sterile lines of maize (Datta et al. 2002). Differences in *Ivr2* transcript abundance in starch-deficient male-sterile pollen in maize (Datta et al. 2002) and heat-stressed pollen in sorghum (present study) may be reflective of the primary cause of physiological aberration, genetic in the former as compared to abiotic stress in the latter case. Additionally, the heat stress-induced rise in transcriptional activity of *SbIvr2* in sorghum microspores does not agree with the data published in wheat and rice subject to drought stress. Depression in soluble acid invertase activity has been reported preceding any visual developmental anomaly in wheat (Dorion et al. 1996; Lalonde et al. 1997; Koonjul et al. 2005) and rice (Sheoran and Saini 1996) anthers during meiotic-stage water deficit. Water stress-induced inhibition of soluble invertase activity was irreversible and highly specific without any accompanying changes in the activities of other enzymes of starch biosynthesis. This apparent discrepancy may be due to differential isoform sensitivities and their spatial localization (anthers in wheat and rice; and microspores in sorghum), and also differences in the severity of the imposed stress conditions. The observation that vacuolar invertase gene *OsVIN2 i*s repressed during cold stress (Oliver et al. 2005) and up-regulated by drought stress in rice anthers (Ji et al. 2005) further lends credence to such speculations.

The important role of CWI genes in sugar partitioning and regulation of source–sink relationship is functionally coupled and coordinately regulated with expression of transporter proteins (Ehness and Roitsch 1997). Heat-stress induced changes in temporal expression profile of the *SbIncw1* gene closely follows suit with reduced transcriptional activity of transporter genes *MST1* and *Mha1* (Fig. 6b) which indicated less loading ability from apoplast. Zhao et al. (2000) reported co-suppression of endogenous and transgenic *PM H^+-ATPase* in tobacco resulting in reduced pollen uptake of sugars and impaired male fertility in tobacco. Regulation of plasma membrane bound H^+-ATPase activity may also be important as a cellular defense mechanism under stress conditions.

In conclusion, heat-stress induced failure of starch deposition in sorghum microspores is evidently the culmination of altered sugar hydrolysis in CWI compromised background in the secretory tapetum cells and also in the developing microspores, preceding changes in the subsequent sugar uptake and downstream carbohydrate metabolism. Reduced steady-state transcript levels of *Bt2*, *GBSS1* and *SU1* in starch-deficient sorghum pollen under high-temperature growth conditions are reflective of inadequate sucrose synthesis, and subsequent utilization for starch biosynthesis. Under heat stress, the lower loading ability (*MST1* and *Mha1*) would eventually lead to the observed total lack of sucrose in heat stressed pollen and reduced starch accumulation. Even though the importance of CWI-mediated sugar catalysis during microsporogenesis in assuring reproductive success is emerging, the role of pollen receptivity by stigma can not be ruled out (Prasad et al. 2006b). Stress induced-changes in INCW expression seen in female sporophytic tissue in sorghum warrants further work in this direction, especially in view of the documented presence of specific CWI genes in maize (Kim et al. 2000) and tomato (Godt and Roitsch 1997) ovaries.

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