

Influence of high temperature stress on starch metabolism in two durum wheat varieties differing in heat tolerance

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Abstract

The formation and characteristics of the grain is largely a function of starch biosynthesis. This study investigated the effect of high temperature during grain development on enzymes, metabolites and components of starch metabolism in two durum wheat (*Triticum durum*) varieties namely WH896 and WH912 differing in their response to high temperature. Activity of starch branching enzyme (SBE) was reduced by 38% in less sensitive variety (WH896) and by 43 per cent in more sensitive variety (WH912). The remarkable difference between the two cultivars was also observed in inorganic pyrophosphate and UDP – glucose level. Differential effects in amount of total soluble sugar and amylopectin were also observed. Magnitude of reduction in ADP-glucose and UDP-glucose metabolites was more in WH 896 (41.0%) and WH 912 (29.3%) under high temperature stress as compared to control. No correlation between the changes in sucrose and ADP – glucose concentration was observed at high temperature.

Keywords: Starch, metabolism, enzymes, metabolites, high temperature

Abbreviations: Days post Anthesis (DPA), ADP-glucose pyrophosphorylase AGPase, Soluble Starch Synthase (SSS), Starch Branching Enzymes (SBE), Glucose-6-phosphate (G-6-P), ADP-Glucose (ADPG), UDP-Glucose (UDPG), Inorganic pyrophosphate (PPi)

Introduction

Starch is the main component which constitutes 75% of the wheat kernel and has a direct effect on the nature and quality of flour and end products of wheat. Reduced starch content accounts for most of the reduction in grain dry matter at high temperature (Yan *et al.*, 2008; Barnabas *et al.*, 2008). Grain filling is mainly a process of starch biosynthesis and accumulation. Extensive studies have been done on the effects of heat stress on the activities of enzymes involved in sucrose to starch metabolism in cereals (Singh *et al.*, 2008 and Zhao *et al.*, 2008). Among the enzymes involved in the pathway of sucrose to starch in wheat endosperms, soluble starch synthase (SSS) is the most sensitive to high temperature (Jenner *et al.*, 1993; Keeling *et al.*, 1993), and it has an unusually low optimum temperature for maximum activity (Denyer *et al.*, 1994).

Below 30°C, however, the loss of the activity of soluble starch synthase alone is not large enough to account in the responses of starch deposition to rising temperatures (Rijven, 1986). Several forms of soluble starch synthase are found in cereal endosperm, and some forms may be more tolerant to high temperature than others (Jenner, 1994).

Keeling *et al.* (1993) concluded that metabolism of sucrose through pentose phosphate pathway and glycolysis and the conversion of sucrose into starch are temperature dependent processes. Much of the work concerned with the biosynthesis of starch in developing cereal grains is done with determination of *in vivo* concentration of both substrates and products simultaneously with measurement of relevant enzyme activities (Truesdale *et al.*, 1999; Zahedi *et al.*, 2003). They reported that such studies might help in understanding the control mechanisms associated with the pathway of starch biosynthesis and thus provide chemical means to manipulate starch content vis-à-vis grain yield.

High temperature stress alters the level of metabolites and corresponding enzymes. Decrease in the level of 3-PGA due to inhibition in activities of AGPase under elevated temperature is one such example (Geigenberger *et al.*, 1998). Keeping this in view the present study was planned with the objective to find out the biochemical basis of high temperature induced changes in sucrose to starch conversion in two durum wheat varieties differing in heat tolerance.

Material and methods

Plant material and growth conditions experiments were laid out in RBD design with three replications and conducted at CCS HAU, Hisar (Haryana), India during year 2006-07. Plants of two durum wheat culture WH 896

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(high temperature tolerant) and WH 912 (high temperature sensitive) were raised in polythene bags under the normal environment during the second week of November, 2006 containing 5 kg of dune sand in screen house. High temperature treatment began after heading and lasted till maturity (45 days after heading), 25 plants of good health were chosen from two cultivar (WH 896 and WH 912) and shifted to polyhouse with maximum temperature 5-9°C higher than ambient control plants (at ambient temperature of 28.7° C).

Enzyme Extraction and Assays: Ears with developing grains at a stage of temperature induced growth impairment

(28 days after post anthesis) were used for preparation of grain extract. Nearly 15-20 developing grains amounting to 0.5 g of the grains were removed from middle portion of earheads and were hand homogenized in a prechilled pestle and mortar at 4°C with 2 ml of chilled buffer on ice. The extraction buffer employed was having composition as 50 mM 3-N-morpholino propane sulphonic acid (MOPS) pH 7.4, 2 mM MgCl₂, 1 mM EDTA and 2 mM Dithiothritol (DTT). The homogenate so obtained was centrifuged at 10,000 x g for 10 min in a refrigerated centrifuge at 4°C. The supernatant was used as grain extract for enzyme analysis.

Table 1. Effect of high temperature on starch metabolism enzymes activity in durum wheat at 28 days post anthesis

Enzymes (nmole min ⁻¹ mg protein ⁻¹)	WH896			WH912			CD at 5%
	Control	High Temperature	%change over the control	Control	High Temperature	% change over the control	
Invertase	6.3±0.27	5.4±0.09	-14.2	3.2±0.18	ND	-	8.9 (T) 5.3 (G)
Sucrose synthase	25.7± 0.5	20.1±0.7	-21.7	18.6±0.5	13.0±0.4	-30.1	16.7 (T) 8.9 (G)
ADPG pyrophosphorylase	78.4±3.6	55.5±0.1	-29.2	70.2±4.3	43.0±1.0	-38.7	50.2 (T) 13.02 (G)
Soluble starch synthase	3.9±0.16	2.1±0.04	-46.1	2.4±0.06	1.7±0.06	-29.1	3.0 (T) 1.19 (G)
Starch branching enzyme	8.5±0.41	5.3±0.14	-37.6	4.9±0.20	2.8±0.06	-42.8	7.5 (T) 3.83 (G)

Each value is the mean of six replication ± SE

ND – Not detectable, T-CD for treatment, G-CD for Genotype

All enzyme assays were optimized for pH and substrate concentration and were within the linear phase with respect to incubation time and protein concentration. Enzymes activities of following were determined: Invertase (Leigh *et al.* 1979), Sucrose synthase (Shannon and Dougherty, 1972), ADP glucose pyrophosphorylase (Kleczkowski *et al.*, 1993), Soluble starch synthase (Leloir *et al.*, 1961) & Starch branching enzyme (Hawker and Downton, 1974). Protein content was determined according to Bradford (1976) using bovine serum albumin as standard. Enzyme activities were expressed on protein basis.

Metabolite extraction and quantification: The metabolites were estimated immediately after the samples were harvested. Any change in the physiological state of the tissue may cause changes in the levels of metabolites. Hence, frozen grains were allowed to drop directly into chilled HClO₄. The metabolites were extracted by the method of Rasi-Caldogno and De-Michelis (1978) with minor modifications. The grains were homogenized in 5 ml of

ice-cold 0.8 N HClO₄. The homogenate was centrifuged at 10,000 x g for 20 min. The supernatant after neutralization with dropwise addition of 5 M K₂CO₃, was centrifuged again at 7,000 x g for 15 min. The resulting supernatant was made to 10 ml with 0.05 M tris-HCl buffer (pH 7.6). During all subsequent work, the extract was maintained at 0°C. The estimation of various carbohydrate components were achieved by standard methods described for Glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate (Latzko and Gibbs, 1969), UDP-glucose and ADP-glucose (Keppler and Decker, 1974), Inorganic pyrophosphate (PPi) (Edwards *et al.*, 1984).

Total and reducing sugars: The estimation of various carbohydrate components was achieved by standard methods described for starch, total soluble sugars (Yemm and Wills, 1954), reducing sugars (Nelson, 1944), sucrose (Johnson *et al.*, 1964), Amylose and Amylopectin (Williams *et al.*, 1970). The results were analyzed by calculating the mean standard error from six independent estimations.

Results and discussion

Enzyme activities: The activities of enzymes in the pathway of starch metabolism were examined to determine the effect of heat stress on altered rates of enzymatic activity. High temperature had little effect on enzyme invertase and sucrose synthase but significantly reduced (>30%) activities of ADP glucose pyrophosphorylase, starch synthase and starch branching enzyme (Table 1). Soluble starch synthase, ADP-glucose pyrophosphorylase, starch branching enzyme, sucrose synthase and acid invertase activity declined more in WH912 than WH896.

High temperature reduces the activity of starch synthase maximally (46.1%). temperature in excess of 30°C reduces

the activity of SSS in the endosperm and the rate of starch synthesis above 30°C is mostly controlled by the activity of SSS. However, the loss of the activity of SSS alone is not large enough to account for the responses of starch deposition to temperature (Rijven, 1986). In this investigation too the greater sensitivity of WH 896 than of WH 912 was not simply associated with temperature response of sucrose synthase.

The overall activity of ADP glucose pyrophosphorylase was found high in both the cultivars. However, the low activity of this enzyme under high temperature probably limits starch accumulation.

Table 2. Effect of high temperature on metabolites of starch metabolism in durum wheat at 28 days post anthesis.

	WH896			WH912			CD at 5%
	Control	High temperature	% change over the control	Control	High temperature	% change over the control	
Glucose	21.0±1.4	18.0±1.0	-14.0	19.5±0.3	16.0±0.4	-17.7	6.8 (T) 2.2 (G)
-6-phosphate Glucose	0.8±0.03	0.7±0.02	-15.6	0.7±0.03	0.6±0.05	-19.2	0.3 (T) 0.1 (G)
-1-phosphate Fructose	4.2±0.3	3.6±0.2	-13.3	4.0±0.3	3.2±0.1	-20.0	1.4 (T) 0.3 (G)
-6-phosphate ADP-Glucose	4.0±0.03	2.9±0.09	-25.9	2.8±0.08	2.1±0.06	-26.3	2.5 (T) 1.2 (G)
UDP-Glucose	12.0±0.43	8.8±0.46	-26.5	8.7±0.53	6.4±0.21	-26.0	7.4 (T) 3.5 (G)
Inorganic pyrophosphate	0.4±0.03	0.3±0.01	-35.4	0.3±0.02	0.1±0.05	-51.4	0.4 (T) 0.2 (G)

Each value is the mean of six replications ± SE.

ND – Not detectable, T-CD for treatment, G-CD for genotype

Table 3. Effect of high temperature on carbohydrate content in durum wheat at 28 days post anthesis

Carbohydrate contents (mg/g dry wt)	WH896			WH912			CD at 5%
	Control	High temperature	% change over the control	Control	High temperature	% change over the control	
Total soluble sugars	52.4±1.5	34.2±1.1	-34.7	38.3±1.0	16.2±1.0	-57.7	47.7 (T) 20.2 (G)
Reducing sugars	15.0±1.1	9.2±0.9	-38.6	10.7±0.9	6.3±0.4	-41.1	11.6 (T) 4.5 (G)
Non reducing sugars	37.4±1.1	25.0±1.1	-33.1	27.6±1.1	9.9±0.9	-64.1	36.4 (T) 15.6 (G)
Sucrose	16.2±1.0	13.4±0.7	-17.2	13.9±0.7	9.8±0.5	-29.4	8.5 (T) 3.7 (G)
Starch	564.3±8.1	406.0±5.1	-28.0	501.5±4.0	332.0±4.7	-33.7	328.3 (T) 86.0 (G)
Amylose	141.1±3.8	150.1±2.1	+6.3	125.1±3.1	140.0±2.3	+11.9	33.1 (T) 16.4 (G)
Amylopectin	423.2±2.7	255.9±4.0	-39.5	376.4±4.6	192.0±3.0	-48.9	341.0 (T) 69.6 (G)

Each value is the mean of six replications ± SE.

Metabolites: The amount of G-6-P was over 25 times higher than that of G-1-P and about 5 times higher than that of F-6-P (Table 2). The ratio of UDPG : ADPG was 1.5 and G-1-P was found double the amount of PPi. The amount of ADPG, UDPG and PPi decreased under high temperature stress. However, the reduction in amount of metabolites was different in two cultivars as the reduction in amount of UDP Glucose was 4.5 times in WH 896 than the WH 912 while inorganic pyrophosphate reduced more in WH 912 than WH 916. Reduced level of ADP glucose at high temperature has also been observed by Jenner (1991) and involving this as a response to an increase in the flux through the pathway of starch synthesis at elevated temperature is one possible interpretation. Very low levels of G-1-P indicate that its mutate may be regulating the rate of starch synthesis by controlling the rate of G-1-P formation (Kumar and Singh, 1984). There was no correlation between the changes in sucrose and ADP-glucose concentration at high temperature (Zahedi *et al.*, 2003).

The amount of ADPG decreased in potato tubers when exposed to elevated temperature ($>35^{\circ}\text{C}$). The decrease in ADPG was correlated with the reduction in starch. There was a strong correlation between sucrose phosphate synthase activation and *in-vivo* level of G-6-P. As the temperature increased from 19°C to 37°C , G-6-P level also increased progressively. No consistent changes were observed in the levels of ADP-glucose, UDP-glucose or PPi. Jenner (1991) reported that the increase in temperature in wheat decreased the level of metabolites. The overall decrease in metabolite levels in wheat during long-term exposure to heat indicates that there may be additional factors besides the inhibition of starch synthase that contribute to a small increase in starch deposition with increasing temperature. Sucrose degradation and starch synthesis are controlled via regulatory signals in response to sucrose and oxygen availability. (i) Sucrose leads to a co-ordinated up-regulation of sucrose synthase and ADP-glucose phosphorylase at the transcriptional and post-transcriptional level (Geigenberger *et al.*, 1998). Tyson and Ap-Rees (1988) demonstrated that amyloplast isolated from wheat endosperm only used glucose 1-phosphate in the synthesis of starch, whereas other C-6 and C-3 compounds did not serve as precursors. Studies of feeding wheat amyloplasts with ^{14}C -labeled hexose phosphates confirmed that, in the presence of ATP, only glucose -1-phosphate is converted into starch (Tetlow *et al.*, 1998). Inorganic phosphate level in the medium had no impact on starch or protein accumulation. A similar response of starch synthesis to phosphate has also been noted by Jenner (1991) and Rijven and Gifford (1983). UDP-glucose and ADP-glucose pyrophosphorylases might be considered rate limiting steps in wheat starch accumulation (Kumar and Singh, 1984).

Carbohydrate composition: The concentration of starch was found significantly higher in WH 896 than WH 912. At high temperature starch content was reduced by 28 and 33.7% in WH 896 and WH 912 (Table 3). Amylopectin component of starch was reduced by 39.5 and 48.9% at high temperature. Significant differences were also found between the amounts of total soluble sugars, reducing sugars, non reducing sugars at the two temperatures in either cultivar. Less sucrose reduction was observed in WH 896 (17.2%) than WH 912 (29.4%) at higher temperature. The effects of elevated temperature on starch deposition are reported by Zahedi (2003). Decrease in the activities of AGPase was observed in Maize kernels and wheat endosperm under heat stress by Zahedi (2003) and correlated with a reduction of starch synthesis. Reduced starch content accounts for most of the reduction in grain dry matter at high temperature (Jenner, 1994). In the present investigation, there was relatively more decrease in soluble starch synthase activity in WH 912 than in WH 896 under high temperature stress (Table 1). The conversion of sucrose to starch is impaired at high temperature and limits starch synthesis (Zhang *et al.*, 2010). Among the enzymes, involved in the pathway of sucrose to starch metabolism in wheat endosperms, soluble starch synthase is the one which is most sensitive to high temperature. The reduction in the rate of grain growth above 30°C is mainly due to the reduced activity of soluble starch synthase (Zahedi *et al.*, 2003). More reduction in starch branching enzyme activity in WH 912 as compared to that in WH 896 was observed. The activity of starch branching enzyme was not significantly affected by the exposure to a temperature of 35°C (Hawker and Jenner, 1993). Jiang *et al.* (2003) reported that the branch chain pattern of amylopectin is changed by temperatures and it is due to reduced activity of branching enzyme at high temperature. There was reduction in carbohydrate content under high temperature stress in both the varieties. The reduction was more in WH 912 than WH 896, except in case of amylose. The amylose percentage increased under high temperature treatment. At 30°C , amounts of both protein and starch were reduced but the reduction in starch was more than that of protein (Bhullar and Jenner, 1985).

In the present study, the reduction in starch was more than that of protein at 28 DPA under high temperature stress as compared to their respective control. It therefore appears that during grain filling at high temperature in addition to reduced synthesis of some starch metabolic enzymes, the catalytic properties of other enzymes are also altered.

Numerous studies involving maize (Keeling *et al.*, 1994;), wheat (Bhullar and Jenner, 1985) and barley (MacLeod and Duffus, 1988) have shown a negative effect of heat stress on starch deposition in the kernel. This may be due to impairment of conversion of sucrose to starch at high temperature and limits starch synthesis (Bhullar

and Jenner, 1985). Correlation was observed between the changes in sucrose and ADPG concentrations at high temperature (Zahedi *et al.*, 2003). Decrease in the activities of AGPase has been observed both in maize kernels and wheat endosperms under heat stresses, thus resulting in a reduction of starch synthesis (Keeling *et al.*, 1993; Wilhelm *et al.*, 1999). High temperature exerts its effects by disturbing sucrose: starch balance in wheat (Bhullar and Jenner, 1985). High temperature decreased soluble sugar content in wheat (Shah and Paulson, 2005). The stability of Granule Bound Starch synthase at moderately high temperature may explain the increase in the percentage of amylose in the grains (Shi *et al.*, 1994; Tester *et al.*, 1995). So, there is progressive increase in the grain amylose percentage over the grain-filling period, especially at high temperature (Zahedi *et al.*, 2004). Despite numerous studies in recent years, determining the aspects of sugar and starch metabolism that are presumably sensitive to conditions of high temperature stress and responsible for the restriction of starch accumulation in heat stressed wheat remained difficult. Synthesis of starch during grain filling of wheat is a very dynamic and complicated process which can be influenced by number of controls. Post anthesis stress, such as restricted assimilates availability or high temperature, can reduce grain filling relative to the potential which prevailed at fertilization. Still it is clear from recent studies that SBE and AGPase are most important for restricting starch synthesis under supra optimal temperatures. Differential response of two wheat cultivars to exposure to high temperature were associated with variation in the availability of substrates for starch synthesis in the grains and also with thermal denaturation of enzymes involved in starch synthesis.

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