***In vivo* studies on artificial induction of thermotolerance to detached panicles of wheat (*Triticum aestivum* L) cultivars under heat stress**

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Abstract The mechanism imparting thermotolerance by gibberellic acid (GA₃) and abscisic acid (ABA) is still unresolved using either spraying technique or *in vitro* conditions. Alternative way of studying these effects under near *in vivo* conditions is through the use of liquid culturing technique. Effects of GA₃ and ABA (100 µM) on sucrose metabolism (invertase and sucrose synthase) and aminotransferases (GOT and GPT) in relation to starch and protein accumulation were studied in detached panicles of three wheat (*Triticum aestivum* L.) cultivars PBW 343, C 306 (heat tolerant) and WH 542 (heat susceptible) cultured in a liquid medium. Ears were subjected to heat shock treatment (45 °C for 2 h) and then maintained at 25 °C for 5 days. Heat shock treatment resulted in a significant decline in starch content but caused a marked build-up of total free sugars and protein content in grains of all cultivars. However, activities of acid and neutral invertases increased only in tolerant cultivars but reduced in susceptible cultivar. Following treatment with GA₃ contents of starch and free sugars increased in grains maintained at 25 °C but free sugar content decreased in stressed grains compared to control. ABA application showed inhibitory effect on starch accumulation under normal temperature but was promotory under stress conditions. Concomitantly, soluble protein content also increased in conjunction with an increase in the activities of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT). Apparently, the wheat grain responds to heat shock mediated disruption of carbon metabolism by a compensatory effect on nitrogen

metabolism. GA₃ stimulated grain sink activity both under stress and non stress condition while ABA was promotory only under stress condition.

Keywords Abscisic acid · Aminotransferases · Gibberellic acid · Sucrolysis · Heat shock · Wheat

Introduction

High temperature during grain filling stage is an important yield limiting factor in wheat (Zhao et al. 2007). It has been reported that single grain mass falls by 3–5% for every 1 °C rise in temperature above 18 °C (McDonald et al. 1983). Plaut et al. (2004) reported that high temperature reduced the rate of transport of dry matter from vegetative organs to kernel, but sensitivity to high-temperature stress differed with varieties. The adverse effects of heat stress can be mitigated by developing crop plants with improved thermotolerance.

A major perturbation of plant protection under heat stress is ascribed to carbon metabolism (Blum and Sinmena 1994; Asthir et al. 1998c) which is manifested in premature cessation of starch deposition in endosperm (Bhullar and Jenner 1986). Supply of assimilates is never the limiting factor rather its utilization within the grain is the controlling factor of sink activity (Cheikh and Jones 1995; Wallwork et al. 1998). Sucrose upon entering into the grain is metabolized by invertases, sucrose synthases and sucrose – phosphate synthase (Ho 1988; Yang et al. 2004). Activity of these enzymes has been associated with the developmental processes of sinks (Lovell et al. 1989; Xu et al. 1989; Tian et al. 2006) and they appear to be potential targets of regulation in improving grain filling processes and yield in wheat under heat stress (Wahid et al. 2007).

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Phytohormones might play a significant role in imparting thermotolerance to cereal grains. For instance, the exogenous application of GA₃ (Vettakkorumakankav et al. 1999) and ABA (Larkindale and Huang 2005) have been shown to reverse the effects of heat stress and impart thermotolerance. A shift in hormone balance of kernels is one mechanism by which heat stress disrupts maize kernel development (Wahid et al. 2007). Hormonal homeostasis, stability, content, biosynthesis and compartmentalization are altered under heat stress (Maestri et al. 2002). Higher concentrations of ABA that occur under elevated temperatures help plants to cope with heat stress (Kurepin et al. 2008). Chandrasekar et al. (2000) have reported a higher accumulation of ABA in drought tolerant wheat cultivar C 306 than susceptible Hira in response to water stress.

Information regarding phytohormone application on carbon and nitrogen utilization in a near *in vivo* condition remains poorly understood. Alternative way of studying these effects is through the use of liquid culturing technique where detached ears of wheat were cultured in complete liquid medium that provides a near *in vivo* conditions. This technique has been successfully employed in our laboratory for cereals (Asthir et al. 1998b). The study was facilitated by using three wheat cultivars PBW 343, C 306 (heat tolerant) and WH 542 (heat susceptible).

Materials and methods

Three wheat (*Triticum aestivum* L.) cultivars namely, PBW 343, C 306 and WH 542 were raised in fields of Punjab Agricultural University, Ludhiana (Punjab), India under recommended agronomic practices. Ears of uniformly developing plants were tagged at anthesis. All the chemicals used were of analytical grade.

Culturing of detached ear heads in liquid medium

Ears at mid-milky stage, i.e. 12–15 days post anthesis (DPA) were cut under water below penultimate node and cultured according to the method of Bhatia and Singh (2002) keeping twelve replications for each treatment. The concentrations in the culture medium of sucrose and L-glutamine were 117 mM and 17 mM, respectively. Phytohormones namely GA₃ and ABA (100 µM each) were added in the culture media. Culture solution devoid of hormones was kept as control. After adjusting the pH of the culture solution to 5.5, the medium was ultra filtered through 0.22 µM Millipore membrane. Before culturing, the flag leaf and its sheath were removed and stems were surface sterilized with 40% EtOH followed by quick washing with distilled water. Ear-heads carrying 20 cm peduncle length from the cut end were placed (one ear-head

per tube) in culture tube containing 35 ml cold-sterilized liquid medium. These cultured ear-heads were then transferred to water bath maintained at 2–4 °C. In the growth chamber, a photosynthetic active radiation (400 µmol m⁻² s⁻¹) measured with Licor 188B quantum meter (Li-cor, Inc. Lincoln, NE, USA) was provided with cool white fluorescent tubes (TL 40 W/54, Philips India) and incandescent lamps (60 W, Sylvania, India) for a day length of 14 h. Ear heads were treated at 45 °C for 2 h daily for 5 days and non-treated ears were kept as control. After required culturing for 5 days, the grains were separated and used for analysis.

Extraction and estimation of free sugars, starch and protein

Free sugars were extracted sequentially with 80% and 70% ethanol. The extracts containing sugars were concentrated by evaporating off the ethanol under vacuum. The total free sugars were determined colorimetrically using the reaction with phenol (Asthir et al. 1999). From the sugar free residue so obtained, starch was extracted with cold (2–4 °C) perchloric (52%), purified with iodine precipitation and estimated as described above. Soluble proteins were extracted in 0.1 M NaOH and precipitated with trichloroacetic acid (TCA) and estimated by the method of Lowry et al. (1951).

Extraction and assay of enzymes

From freshly collected samples soluble acid invertase (EC 3.2.1.26; pH 4.8), soluble neutral invertase (EC 3.2.1.27; pH 7.5) and sucrose synthase (synthesis, EC 2.4.1.13), were extracted by the procedure employed by Asthir et al. (1999). Grain samples (4 g) homogenized at 0–4 °C in 50 mM Hepes- NaOH buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM Sodium EDTA, 2.5 mM DTT, 0.5 mg ml⁻¹ BSA and 0.05% (v/v) Triton X100. Homogenates were centrifuged at 10,000 g for 15 min and the pellets resuspended in extraction buffer and centrifuged as before. The supernatants were pooled and passed through Sephadex G-25 column (17 cm) equilibrated with the above buffer without EDTA and Triton X-100. For extraction of glutamate-oxaloacetate/ glutamate pyruvate transaminase (GOT/GPT, EC 2.6.1/2.6.1.2 L-aspartate/ L-alanine :2 oxaloglutarate aminotransferase), 50 mM Tris -HCl buffer (pH 7.5) containing 100 mM β-mercaptoethanol, 2 mM MgCl₂, 2 mM EDTA and 10 mM cysteine was used in place of Hepes buffer.

Enzyme assays

Soluble acid and soluble neutral invertases were assayed from the test extracts according to Asthir et al. (1999). The reaction mixture (1 ml) consisted of 0.6 ml of 0.2 M Na-

Table 1 Effect of temperature and phytohormones (ABA and GA₃) on the free sugar content ($\mu\text{g grain}^{-1}$), starch (mg grain^{-1}) and protein (mg grain^{-1}) in developing grains of wheat

Temp	Phytohormone in culture medium	C-306			PBW-343			WH 542		
		Free sugar	Starch	Protein	Free sugar	Starch	Protein	Free sugar	Starch	Protein
25 °C	Control	368.2±6.21	10.19±0.46	756±8.01	389.8±20.02	10.74±0.60	804±7.61	171.9±13.68	8.17±0.52	
	ABA	330.8±15.30	9.35±0.44	865±9.62	250.8±14.26	9.35±0.50	920±7.94	157.8±13.52	7.53±0.49	683±7.32
	GA ₃	451.9±29.10	10.71±0.62	929±10.12	429.2±27.24	12.32±0.8	952±9.56	442.9±28.08	8.50±0.61	767±8.01
45 °C	Control	600.0±27.20	8.74±0.38	777±7.92	417.6±26.24	9.02±0.76	859±9.56	414.1±26.98	6.14±0.55	730±8.61
	ABA	259.3±14.20	9.16±0.42	852±9.16	159.4±12.20	9.15±0.82	888±8.86	293.9±15.21	6.87±0.68	732±8.61
	GA ₃	387.3±19.60	9.62±0.56	840±8.96	236.0±17.6	10.19±0.66	897±9.37	301.5±15.98	7.31±0.66	779±9.20
CD at 5%	Temperature	20.74	0.52	9.24	20.69	0.72	9.10	20.43	0.61	8.64
	Hormones	25.40	0.61	11.32	25.34	0.88	11.14	20.02	NS	10.58
	Temp × Horm	35.92	NS	16.01	35.85	NS	15.76	35.39	NS	14.96

Table 2 Effect of temperature and phytohormones (ABA and GA₃) on the activities of soluble invertases (μg of sucrose hydrolysed /min/ g) and sucrose synthase (μg of sucrose formed /min/g) in developing grains of wheat

Temp.	Phytohormone in culture medium	C 306			PBW 343			WH 542		
		Neutral invertase	Acidic invertase	Sucrose synthase	Neutral invertase	Acidic invertase	Sucrose synthase	Neutral invertase	Acidic invertase	Sucrose synthase
25 °C	Control	135.0±4.43	74.9±3.93	199.1±6.31	147.8±4.32	179.44±5.02	150.8±3.46	259.7±3.66	179.4±3.97	203.1±5.72
	ABA	110.3±1.92	67.7±2.00	184.6±5.92	143.7±5.22	173.4±4.60	147.7±4.04	229.8±5.21	160.9±2.94	177.5±4.68
	GA ₃	206.4±3.92	104.6±3.02	211.8±5.32	172.4±4.32	192.1±5.14	159.2±3.59	260.2±6.32	189.2±3.08	142.8±3.02
45 °C	Control	213.1±6.29	102.9±4.68	198.9±5.92	148.6±3.61	209.3±6.20	155.4±4.18	122.5±3.92	83.7±1.76	119.5±2.98
	ABA	269.2±5.06	130.3±4.16	255.0±8.61	170.7±6.88	256.8±5.52	188.3±4.46	145.4±4.00	122.7±2.86	131.5±2.76
	GA ₃	213.0±4.76	122.1±3.10	222.2±4.52	219.8±4.06	253.3±7.30	162.2±3.69	179.0±5.06	119.9±3.21	137.8±2.49
CD at 5%	Temperature	4.72	3.69	6.40	4.99	5.86	4.03	4.92	3.31	3.90
	Hormones	5.78	4.52	7.84	6.11	7.17	4.93	6.02	4.06	4.78
	Temp × Horm	8.17	6.39	11.08	8.64	10.14	7.01	8.52	5.73	6.75

acetate, pH 4.8 (for soluble acid invertase) or 0.2 M Na-phosphate, pH 7.5 (for soluble –neutral invertase), 0.2 ml of 250 mM sucrose and 0.2 ml of enzyme extract. The contents were incubated at 37 °C for 20 min and the reaction was terminated by addition of 1 ml of Nelson reagent C. The amount of reducing sugars were measured (Nelson 1944) and the concentration of hydrolysed sucrose was calculated by multiplying the reducing sugar concentration by the factor of 0.95. Sucrose synthase was assayed from the test extracts as described by Asthir et al. (1999). The reaction mixture (0.5 ml) for sucrose synthase (synthesis) contained 3 mM UDPG, 10 mM fructose, 5 mM MgSO₄, 40 mM Tris-HCl buffer (pH 8.2) and 0.2 ml enzyme preparation. After incubation at 37 °C for 30 min, 0.1 ml of 30% KOH was added. The contents were heated for 20 min in boiling water bath to destroy free fructose. One ml of resorcinol (0.15 g glacial acetic acid + 0.25 g thiourea) and 3 ml of 30% HCl were added. The contents were mixed thoroughly and incubated at 80 °C for 10 min and absorbance was read at 490 nm.

GOT was assayed according to Tonhazy (1960a). 0.2 ml of 0.1 M 2-oxoglutarate solution was added to a mixture of 0.5 ml of 0.1 M L-aspartate in 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml 2 mM pyridoxal phosphate and 0.2 ml of enzyme extract. The incubation was done at 37 °C for 10 min. The reaction was terminated by the addition of 0.1 ml of TCA solution, shaken vigorously and then 0.2 ml of aniline citrate was added. The contents were mixed and allowed to stand for 10 min. Pyruvate produced, together with excess of 2-oxoglutarate was converted to their hydrazones with the addition of 1 ml of chromogen solution (5 mM 2,4-dinitrophenyl hydrazine). The contents were shaken with 2 ml of water saturated toluene and then centrifuged. One ml of upper layer was drawn into another test tube and to this 5 ml of alcoholic KOH was added. After 5 min, 1 ml of distilled water was

added and the absorbance of the colored solution was measured at 520 nm and the enzyme activity was calculated from the standard curve using oxaloacetate (0.3–1.8 µmol). The procedure employed for assaying the activity of GPT was same as described for GOT except that in case the addition of aniline citrate step was omitted (Tonhazy 1960b). The absorbance of colored solution containing pyruvate hydrazone, selectively extracted with water saturated toluene, was measured at 520 nm. For enzyme activity, the amount of pyruvate formed was calculated from the curve of authentic pyruvate standards (0.3–1.8 µmol) run simultaneously.

Statistical analysis

All the values reported in this paper were the mean of three replicates. All data obtained was subjected to analysis of factorial experiment in CRD at 5% level of CD. In all the tables ± values representing standard error of the means.

Results and discussion

Culturing of detached tillers for 5 days in presence of GA₃ and ABA under heat shock conditions showed variable effects on carbon and nitrogen metabolism in grains of heat tolerant (PBW 343, C 306) and susceptible cultivars (WH 542). Elevated temperature (45 °C) during grain development significantly increased total free sugars but decreased starch content as compared to grains maintained at ambient temperature (25 °C) without phytohormones (Table 1). Decrease in starch content of grains was more pronounced in susceptible cultivar WH 542 (22.44%) as compared to tolerant cultivars PBW 343 (8.33%) and C 306 (14.25%). Similar reduction in starch content in grains under heat stress has earlier been reported (Bhullar and Jenner 1986; Cheikh

Table 3 Effect of temperature and phytohormones (ABA and GA₃) on the activities of GOT (µM of oxaloacetate formed /min/g) and GPT (µM of pyruvate formed /h / g) in developing grains of wheat

Temp.	Phyto hormone in culture medium	C 306		PBW 343		WH 542	
		GOT	GPT	GOT	GPT	GOT	GPT
25 °C	Control	4.47±0.37	4.11±0.60	4.62±0.43	5.32±0.52	5.10±0.56	2.85±0.32
	ABA	5.23±0.52	4.86±0.33	5.33±0.52	8.24±1.05	5.45±0.54	5.52±0.66
	GA ₃	4.67±0.27	6.58±0.93	5.21±0.48	7.56±0.90	6.45±0.68	4.43±0.54
45 °C	Control	5.18±0.18	4.32±0.42	4.72±0.52	5.60±0.38	6.06±0.58	4.40±0.48
	ABA	5.25±0.19	4.56±0.61	5.47±0.62	7.92±0.84	6.23±0.72	8.10±0.70
	GA ₃	4.92±0.20	7.26±0.98	5.61±0.74	9.41±1.02	5.33±0.54	6.72±0.72
CD at 5%	Temperature	0.322	NS	NS	NS	NS	0.603
	Hormones	NS	0.87	NS	1.04	NS	0.739
	Temp × Horm	NS	NS	NS	NS	1.08	NS

and Jones 1995). A higher build-up of sugars in wheat grains under heat shock was probably due to their reduced conversion of sugars to starch. Sumesh et al. (2008) associated high temperature tolerance of a cultivar with higher catalytic efficiency ($V_{\max}/K_m$) of soluble starch synthase at elevated temperature and higher content of heat shock protein 100.

Application of GA_3 stimulated free sugar content while ABA inhibited this effect in grains maintained at normal temperature in all three cultivars (Table 1). Wang et al. (2006) also reported that the assimilate transport into the ears after treatment with phytohormones was promoted by GA_3 and markedly inhibited in the presence of ABA as compared to control (Table 1). The hormonal stimulation of grain sink activity in this way may thus stimulate grain filling metabolism (Bhatia and Singh 2002) leading to increased dry matter accumulation, as also reflected in starch content (Table 1). GA and ABA application under heat shock conditions resulted in higher accumulation of starch content in grains as compared to control in correspondence with a decline in free sugar content. It is interesting to note that ABA application inhibited starch accumulation under normal temperature but it showed a positive effect under stress conditions.

Heat stress-induced increases in endogenous ABA concentration are known for several plant systems (Nilsen and Orcutt 1996), and these increases occur very rapidly. Thus, while applied ABA had no effect on endogenous ABA concentration grown under normal temperatures, ABA application to ears that were subsequently given a high temperature stress of 45 °C effectively doubled grain ABA concentration. This increase in ABA occurred within the period when one would expect to observe increased synthesis of heat shock proteins, e.g. after 4 h of high temperatures according to Dhaubhadel et al. (2002).

An appreciable activity of invertases (acid, neutral) was recorded in developing grains and invariably, the activity of acid invertase was higher than that of neutral invertase indicating the predominant role of acid invertase. Acid and neutral invertase activities increased under high temperature stress in tolerant cultivars but declined in susceptible cultivar (Table 2) indicating its superior tolerance mechanism in the former in terms of carbon utilization over susceptible one. Significance of invertases in temperature stress tolerance has earlier been reported by Asthir et al. (1998a). Differential behaviour in kinetic properties of invertases in tolerant and susceptible cultivars of wheat grains was earlier reported by Asthir et al. (1998a, b). Activity of sucrose synthase (synthesis) remained unaffected under normal and stress conditions of wheat grains.

GA_3 increased invertase activities both under normal and stress conditions. Positive response of GA_3 on starch accumulation as well as on activities of invertases has earlier been reported (Ranwala and Miller 2008; Asthir et

al. 1998c). Wu et al. (1993) showed an increase in acid invertase mRNA levels within 4 h after gibberellin treatment in dwarf pea seedlings indicating that enhanced invertase expression is an initial response to gibberellins causing increased sugar availability and elongation growth.

As in case of free sugars, cytosolic amino acid synthesis is also affected by high temperature as reflected in increased activities of aminotransferases. For example, glutamate-oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities were enhanced by HT (Table 3), correlating with an increase in soluble protein content (Table 3). Enzymes of nitrogen metabolism are often up-regulated by elevated sugar content in plant tissues (Lam et al. 1996). The resulting increase in amino acids would in turn affect protein synthesis. Protein content as well as activities of GOT and GPT of grains increased in all the cultivars with both hormones (Tables 1 and 3). Increase in soluble protein in pollen of rice under high temperature stress was observed by Tang et al. (2008) which contributes to maintain cell structure and function under abiotic stresses.

In conclusion, disruption of starch biosynthesis under the influence of high temperature resulted in increased accumulation of free sugars that favoured nitrogen metabolism. At normal temperature (25 °C), GA_3 act as a positive and ABA as a negative modulator of grain sink activity. However, under stressed conditions at high temperature (45 °C), both the hormones were able to impart some thermotolerance in wheat grains by modulating activities of sucrolytic and aminotransferases in relation to starch and protein accumulation.

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