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Full Length Research Paper

Activities of sucrose-metabolizing enzymes in grains of two wheat (*Triticum aestivum* L.) cultivars subjected to water stress during grain filling

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Developmental changes in the starch and soluble sugars content of grains and the activities of sucrose metabolism enzymes in drought-tolerant (*Triticum aestivum* L. cv. Zagros) and drought-sensitive with high yielding potential under favorable conditions (cv. Marvdasht) wheat genotypes were investigated under controlled water deficit during grain filling. The two cultivars were grown in pots under well-watered (WW) and water-stressed (WS) starting from anthesis until maturity. Water stress caused a marked reduction in glucose, fructose and sucrose content of grains of sensitive cultivar. These changes were paralleled by sharp decline in the activities of cell wall invertase and soluble invertase in the grains of sensitive cultivar. Whereas in tolerant cultivar as the surge in invertase activity faded; in grains it was replaced by a substantial increase in sucrose synthase activity as seed development proceeds. Notwithstanding the increase in sucrose synthase activity in grains of sensitive cultivar; however, this little raise could not be sufficient to compensate for decreased levels of invertases and might restrict transport efficiency of storage material, and together with insufficient photosynthate may be the main reasons for dramatical reduction in the number of kernel and subsequent grain yield under WS treatment in Marvdasht.

Key words: Invertase activity, sucrose metabolism, sucrose synthase, water stress, wheat (*Triticum aestivum* L.).

INTRODUCTION

Crop plants such as wheat (*Triticum aestivum* L.) are sensitive to soil drought during the grain-filling period (Zinselmeier et al., 1995, 1999). It is generally accepted that grain-filling rate in cereals is mainly determined by sink strength (Venkateswarlu and Visperas, 1987; Liang et al., 2001). During the grain-filling period of wheat, kernels are very strong sinks for carbohydrate (Ho, 1988; Riffkin et al., 1995). The sink strength depends on sink size and sink activity (Venkateswarlu and Visperas, 1987). Sink activity is a physiological restraint that includes multiple factors and key enzymes involved in carbohydrate utilization and storage (Wang et al., 1993; Yang et al., 2004). Water stress during early grain filling has a marked effect on grain yield through reduced endosperm cell number and thus sink strength, which is a product of sink size and the metabolic activity of the sink organ during development (Ho, 1988), therefore grain growth during grain filling are determined mainly by factors that operate within or close to the grain itself (Jenner et al., 1991).

Biochemical conversion and sucrose metabolism is one of the most important components of sink strength and can be determined by the catalytic activities of one or more enzymes involved in this pathway. Uptake in these sinks can occur either directly or through the cleavage of sucrose. Plants contain two types of enzymes capable of this cleavage: (1) sucrose synthase, which catalyzes a readily reversible reaction; the enzyme appears to be largely cytoplasmic, although it has recently been reported that in some cases it may associate with the cell membrane and be responsible for providing substrates for cell wall synthesis (Ruan et al., 1997), and (2) sucrose

invertase, which catalyzes the irreversible hydrolysis of sucrose to glucose and fructose (Copenald, 1990). The invertases of higher plants are classified according to their solubility, localization, and pH optima, and include three types of enzymes: cytoplasmic, vacuolar, and cell wall. Unlike cell wall invertase that is bound to the cell wall, soluble invertase is in the cytosol and/or vacuole and is extractable in the crude supernatant after cell disruption. Xu et al. (1996) reported abundant levels of the two soluble invertases, in developing kernels.

In wheat stems, activities of both sucrose synthase (Wardlaw and Willenbrink, 1994) and acid invertase (Bancal and Triboï, 1993) have been found to be very high at anthesis and to fall sharply during grain filing. The rise in sucrose synthase activity is positively correlated with the onset of starch and storage protein biosynthesis (Obata-Sasamoto and Susuki, 1979), whereas reduced sucrose synthase activity results in a strong decrease of starch accumulation, which is in agreement with the postulated function of sucrose synthase during the storage of sink tissues. In the sink tissues, invertases are associated with developmental processes where their activities are the highest before storage starts (Eschrich, 1980), whereas sucrose synthase is associated with the subsequent storage functions, such as starch synthesis (Heim et al., 1993). While it was originally believed that sucrose synthase was the main determinant of sink strength (Zrenner et al., 1995), measurements on developing tomato fruit suggest that sucrose synthase activity is low in the first week after anthesis, reaching a peak early in development and subsequently declining (Demnitz-King et al., 1997). Sucrose synthase activity is positively related to dry matter accumulation in tomato fruits (Demnitz-King et al., 1997) and the activity of this enzyme was reported to be higher in wheat kernels achieving greater maximum dry weight (Dale and Housley, 1986). The duration of sucrose synthase activity is believed to be important in determining the duration of grain filling (Chevalier and Lingle, 1983). The peak of sucrose synthase activity occurs when it is postulated that import of sugar into the fruit switches from a predominantly symplastic to an apoplastic mechanism (Patrick, 1997).

Evidence is now mounting in favour of such a role for the extracellular wall-bound invertase, especially during the initial stages of sink development (Roitsch et al., 2000; Roitsch, 1999). Various experimental approaches have been used to demonstrate the importance of the latter enzyme for assimilate partitioning and determination of sink strength. These include inhibition of storage tissue development in carrot roots by antisense repression of cell wall invertase (Tang and Sturm, 1999), arrested seed development in maize mutant lacking cell wall invertase (Miller and Chourey, 1992), kernel abortion in maize when cell wall invertase is suppressed by water stress during pollination (Zinselmeier et al., 1995), and specific expression of a cell wall invertase during pre-storage phase in the thin walled parenchyma of faba bean seed coat (Weber et al., 1995). Not surprisingly, several correlative data suggest a major physiological role for cell wall invertase in maintaining source-to-sink unloading of sucrose and ultimately, in determining sink strength. Several studies on the role of cell wall invertase in seed development, especially in establishing the roles of hexoses and sucrose in cell division and storage functions, are reported in *Vicia faba* (Weber et al., 1996, 1997).

In addition to sucrose availability per se, capacity for sucrose use and hexose to sucrose balance may be critically important to zygote development under drought conditions. In general, sucrose levels of stressed ovaries are higher or at least similar to those of non-stressed ovaries (Schussler and Westgate, 1991; Schussler and Westgate, 1995; Zinselmeier et al., 1995; Anderson et al., 2002), indicating that the capacity to use sucrose may be impaired by drought. Drought stress decreases activities of both vacuolar and cell wall-bound acid invertase during kernel development (Zinselmeier et al., 1995), with parallel reductions in ovary growth and concentration of hexoses. Therefore an understanding of how the involved processes are affected is of particular interest for improving drought tolerance (Boyer, 1996). The objective of this study was to investigate isoforms of invertase and sucrose synthase activity on carbon metabolism, in wheat subjected to water deficit throughout the grain filling stage.

MATERIALS AND METHODS

Plant materials

The experiment was carried out in the Agricultural Biotechnology Research Institute of Iran (ABRII), in the growing season of 2009 to 2010. Two contrasting cultivars of *T. aestivum* L. differing in drought tolerance at grain filling stage that is Marvdasht (drought susceptible) and Zagros (drought tolerant) were used. Seeds were sown in porcelain pots (15 cm in height and 16.5 cm in diameter) filled with 2.1 kg of clay-sand-manure 1:1:1(v/v), cultivation was performed in a greenhouse with 16 h supplemental light (300 $\mu molm^{-2}s^{-1}$of photosynthetically active radiation 22°C) and 8 h darkness (15°C), and at 55 to 60% air humidity. Five uniform plants in each pot were retained after seedling establishment and adequately irrigated with tap water. At three true leaves, pots were placed in a field for 40 days, for vernalization. The experiment was 2 x 2 (two cultivars and two water regimes) factorial design in a complete randomized block design, with four treatment.

Each of the treatment had four replication with three subsamples. Half of the plants of both varieties were exposed to water stress (WS), the imposition of water stress commenced at anthesis to maturity to reach 50% FC, and in control treatments were maintained as well watered (WW), and were irrigated to reach FC throughout the experiment (soil water potential, ψsoil, at -0.01 to -0.02 MPa) . Water was withheld from treated pots and the soil water content allowed to fall 50% FC and the pots then weighed every day. Sufficient water was applied on each occasion to return the soil moisture to these original levels. In control treatment, the soil status was maintained at FC by weighing the pots daily and adding sufficient water to bring the soil moisture to its original value.

Sampling

Twenty to fifteen plants from each treatment were sampled at 7 and 21 days after anthesis. The second and third kernel from each spikelet were frozen in liquid nitrogen for 1 min and stored at -80°C for enzymatic assay. From each treatment 20 plants were harvested at maturity for the determination of grain yield. Each measurement was done on plants from four different pots.

Metabolite analyses

All chemicals and enzymes used for enzymatic measurement were from Sigma Chemical Company (St. Louis, MO, USA). All enzyme assays were optimized for substrate concentration and pH and were within the linear phase with respect to incubation time and protein concentration. Protein content was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard. Enzyme activities were expressed as specific activities. Carbohydrate content was measured after grinding approximately 5 mg of frozen samples in Eppendorf vials with acid-washed sand and 500 µl of 80% (v/v) ethanol. Material was subsequently heated to 80°C for 15 min and centrifuged for 5 min at 20,000*g* to pellet insoluble material. Extraction was repeated twice with 500 µl of 80% (v/v) ethanol, and supernatants were pooled for evaporation to dryness in a vacuum centrifuge. Carbohydrates in this fraction were resolubilized in 900 µl of water. Reducing sugars were quantified in an aliquot of 100 µl according to Nelson (1944) with Glc as a standard. Sucrose was quantified by subtraction using the same methods after sucrose inversion by β-fructosidase. Complete sucrose hydrolysis was achieved by adding 20 units of β-fructosidase (Roche Diagnostics, Basel) per sample and incubating for 10 min at 30°C in 50 mM sodium acetate buffer with 15 mM magnesium chloride (pH 4.6).

The Nelson reducing sugar assay (Nelson, 1944) was also used to estimate starch content after digestion of insoluble material. These fractions were dried in a vacuum centrifuge and boiled for 30 min with a thermostable amylase (Termamyl, Novo Nordisk, Glostrup, Denmark) in 1 ml of 5 mm sodium dihydrogen phosphate buffer (pH 6.0). Starch was further hydrolyzed in a 100 µl aliquot with 2.5 units of amyloglucosidase (Roche Diagnostics) in 50 mM sodium acetate buffer with 15 mM magnesium chloride (pH 4.6) at 65°C. Sample blanks, reagent blanks, and samples with known starch content were included. Three samples from each date and pot were analyzed.

Enzymatic activity

Crude enzyme extracts from approximately 50 mg of frozen grain material were further ground in Eppendorf vials with sand and 300 µl of extraction buffer consisting of 50 mM HEPES-NaOH, 1 mM EDTA, and 2.5 mM dithiothreitol, (DTT) pH 7.0. Samples were centrifuged for 10 min at 20,000*g* to pellet insoluble material. The soluble protein extract was removed, and the remaining pellet was washed three times with extraction buffer. Insoluble proteins were then extracted with buffer containing 1 M NaCl (Doehlert and Felker, 1987). Soluble protein extract (200 µl) was dialyzed against extraction buffer for 16 h at 0°C on a 10,000 molecular weight cutoff dialysis membrane (Pierce, Rockford, IL) to remove endogenous soluble carbohydrates. Activities of soluble and insoluble invertase were measured as described by Tsai et al. (1970) with minor modifications.

Soluble (vacuole acid and neutral cytosol) and insoluble invertase extracts (10 or 20 µl) were assayed in a total volume of 300 µl, with an assay buffer containing 50 mM sodium acetate, 15 mM magnesium chloride, and 100 mM sucrose (pH 4.5). Assays were incubated for 0.5 to 2 h at 30°C, with blanks terminated immediately after addition of protein extracts. All reactions were terminated by adding 300 µl of Nelson's no. 1 reagent. Reducing sugars were quantified by spectrometry according to the Nelson-Somogyi's method (Nelson, 1944) with a glucose standard.

A modified method for sucrose synthase extraction was used (Ranwala and Miller, 1998). 50 mg of frozen grain material was homogenized with a mortar and pestle (5 ml buffer per 1 g FW) in 100 mM Hepes (pH 7.5) containing 10 mM isoascorbate, 3 mM $MgCl_2$, 5 ml DTT, 2 ml EDTA, 5% (v/v) glycerol, 3% (w/v) PVPP, and 0.01% Triton X-100. After centrifugation at 15,000 g for 30 min, the supernatant was desalted on a Sephadex G-25 column and the proteins were eluted by the reaction buffer, which contained 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, and 3 mM DTT. SS activities (in the synthesis direction) were determined as described by Wardlaw and Willenbrink (1998) and expressed as l mol sucrose synthesized per mg protein per h.

Statistical analysis

The results were analyzed for variance using the SAS statistical analysis package (version 6.12; SAS Institute, Cary, NC, USA). Data from each sampling were analyzed separately. Means were tested by least significant difference at $P_{0.05}$ level (LSD 0.05). Linear regression was used to evaluate the relationships of starch and sucrose-metabolizing enzyme activities in the grain.

RESULTS

Changes in soluble sugars and starch

The glucose and fructose in grains sharply decreased, during 7 to 21 DAA, under well-watered. Water stress aggravated the reduction of glucose and fructose during the 7 days from anthesis; during the subsequent periods (7 to 21 DAA), both carbohydrates little increased in Zagros and decreased significantly in Marvdasht cultivar as compared with the controls (Figure 1A and B). A similar changing pattern was observed for sucrose concentration in the grains from 7 to 21 DAA (Figure 1C).

During 7 days from anthesis, sucrose concentration in both cultivars was decreased significantly by the water stress, but after 21 days from anthesis, sucrose concentration in water-stressed Marvdasht was reduced to 32% compared to control treatment, whereas no significant difference in sucrose concentration was observed between the water-stressed Zagros cultivar and the controls. During 7 to 21 DAA, starch concentration in the grains was sharply increased in Zagros (2 folds) and Marvdasht (2.5 folds) under well-watered treatment (Figure 1D). Water deficit greatly reduced starch concentration in both cultivars, and the loss was more in Marvdasht cultivar after 21 days from anthesis (Figure 1D).

Enzyme activities

Sucrose synthase activities in the grains under WW and WS treatments were elevated for both cultivars during the anthesis period. The sucrose synthase activity in Zagros

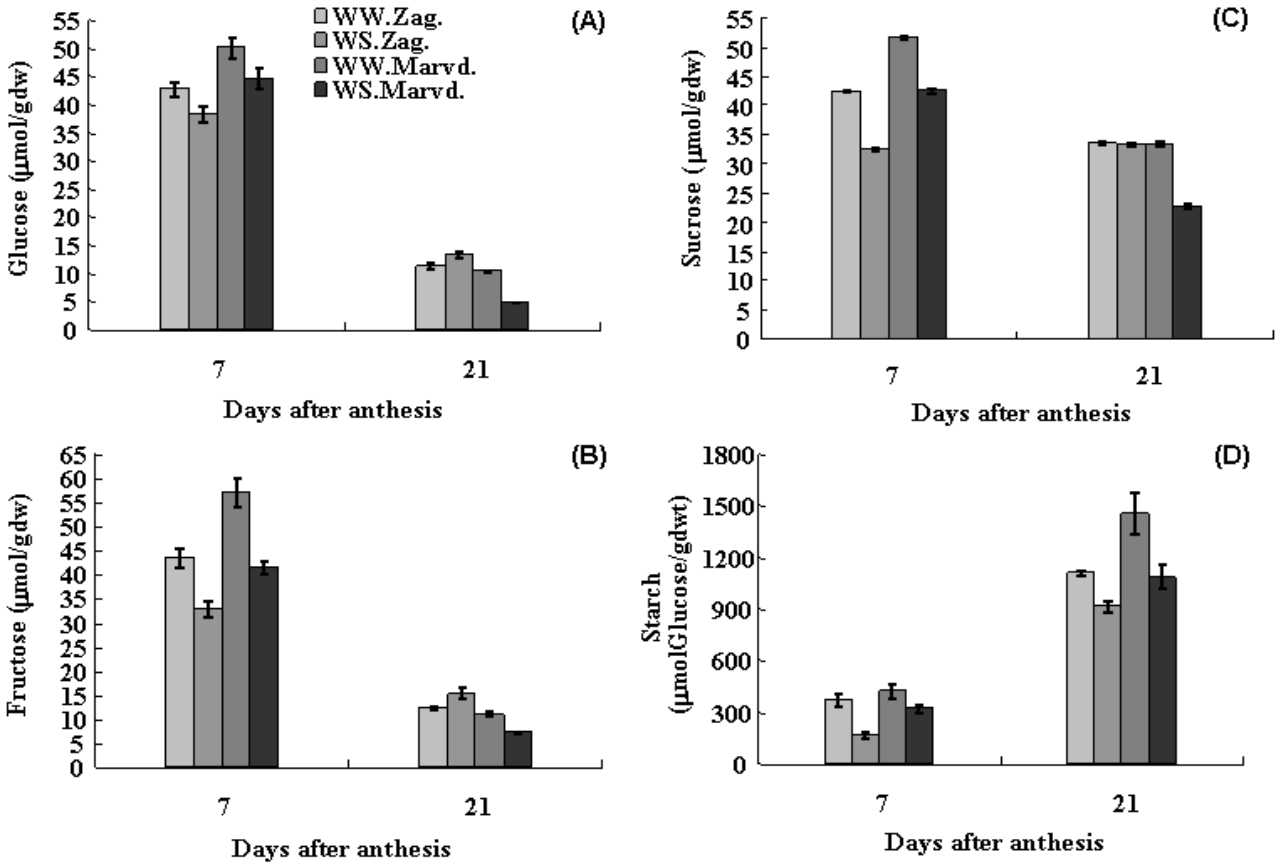

Figure 1. Changes in concentrations of nonstructural carbohydrates Glucose (A), Fructose (B), Sucrose (C), and Starch (D), in well-watered (WW) or water-stressed (WS) in grains during grain filling in two wheat cultivars (Drought Sensitive cv. Marvdasht and Drought Tolerant cv. Zagros). Vertical bars represent ± SE of the mean (n=3).

was enhanced in a higher level than that in Marvdasht, when the water stress was imposed (Figure 2D). Under well-water treatment, there was no significant difference in sucrose synthase activities between the two cultivars during 21 days DAA.

Regardless of water deficit, soluble and insoluble invertase activities were decreased in both cultivars from day 7 onwards, as the reduction in well watered condition achieved to 80.6, 85, 64.7% in Zagros and 75, 97.5 and 55.9% in Marvdasht for acid, alkaline and bound invertase by day 21 respectively (Figures 2A, B and C), however under stress condition the reduction was more pronounced with their respective controls. Sucrose synthase activity, on a per grain basis, was much higher than those of acid invertase during the fast accumulation period of starch in the kernel 7 to 21 DAA (Figures 2A, B, C and D), indicating that sucrose synthase is a predominant enzyme responsible for sucrose cleavage in wheat grain.

Kernel weight and grain yield

For both the cultivars, kernel weight was reduced significantly under water deficits (Table 1); the reduction percentage in Marvdasht (54.4%) was higher than Zagros (15.1%) under stress regime. Similar results were observed for grain yield, aerial biomass and harvest index. In contrast, the reduction percentage of kernel number per Marvdasht spike (13.9%) was lower than that of Zagros (16.3%). These indicate that water deficits during the 7 to 21 days after anthesis control the grain yield mainly by influencing kernel weight rather than the spike number or kernel number per spike.

DISCUSSION

It was hypothesized that high levels of enzymes involved in the breakdown of sucrose in the sink would increase sink capacity by lowering the local concentration of sucrose, thereby generating a gradient that allows further unloading of sucrose from phloem (Wardlaw, 1968; Liang et al., 2001). Since both sucrose synthase and invertase are involved in sucrose cleavage in sink tissue, their activities are regarded as biochemical markers of sink strength (Wang et al., 1993; Ranwala and Miller, 1998).

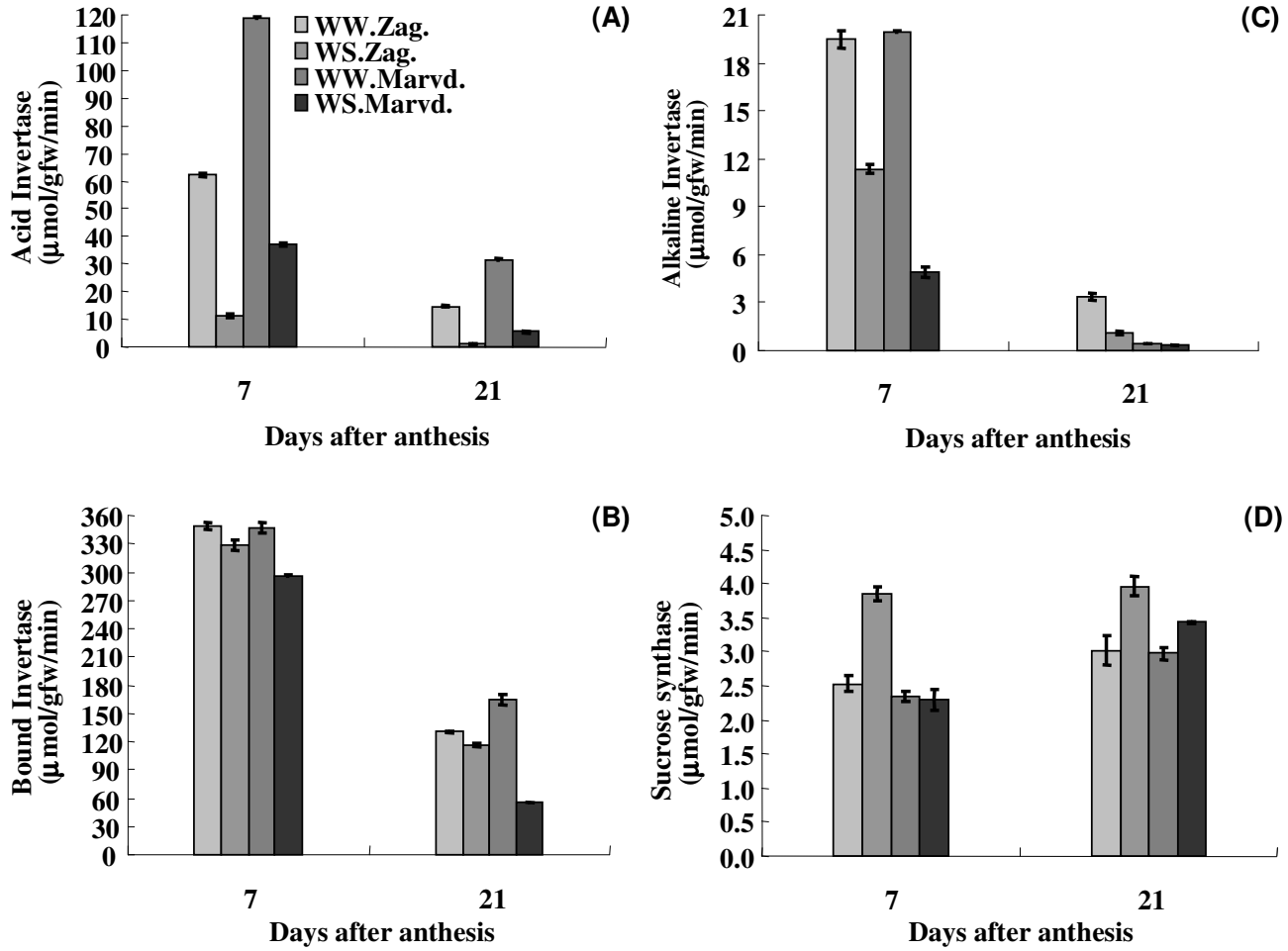

Figure 2. Changes of activities of (INV) Acid Invertase (A), Bound cell Invertase(B), Alkaline Invertase (C) and Sucrose Synthase (D) in well-watered (WW) or water-stressed (WS) in grains during grain filling in two wheat cultivars (Drought sensitive cv. Marvdasht and Drought Tolerant cv. Zagros). Vertical bars represent ± SE of the mean (n=3).

Table 1. Effect of different water treatment, well watered (WW), withholding water from anthesis till maturity (WS) on the final number of kernel per spike, kernel weight per spike, the thousand-kernel weight, aerial biomass of plant and harvest index in two wheat cultivars.

Cultivars	Water-deficit treatment	No. of grains per spike	Grain yield per spike (g)	1000 Grain dry mass (g)	Aerial biomass (g plant^{-1})	Harvest index (HI)
Marvdasht	WW	49.1 a	2.04 a	37.7a	3.55 a	57.9 a
	WS	42.3 c	0.85d	17.2 d	2.22 c	37.4 c
Zagros	WW	36.2 c	1.48 b	23.2 b	2.48 b	55.1 a
	WS	30.3 d	1.12 c	28.2 c	2.23 c	52.1 b
% Reduction compare to control						
Marvdasht	WS1	13.9	58.3	54.4	37.5	35.4
Zagros	WS1	16.3	24.3	15.1	10.1	5.4
LSD (0.05)		2.421	0.102	0.91	0.01	2.426

Letters indicate statistical significance at $p_{0.05}$ within the same cultivar.

Our results showed that the activities of soluble and insoluble invertase in grains were restricted much higher by time and water deficits than well-watered treatment (Figures 2A, B and C), whereas sucrose synthase

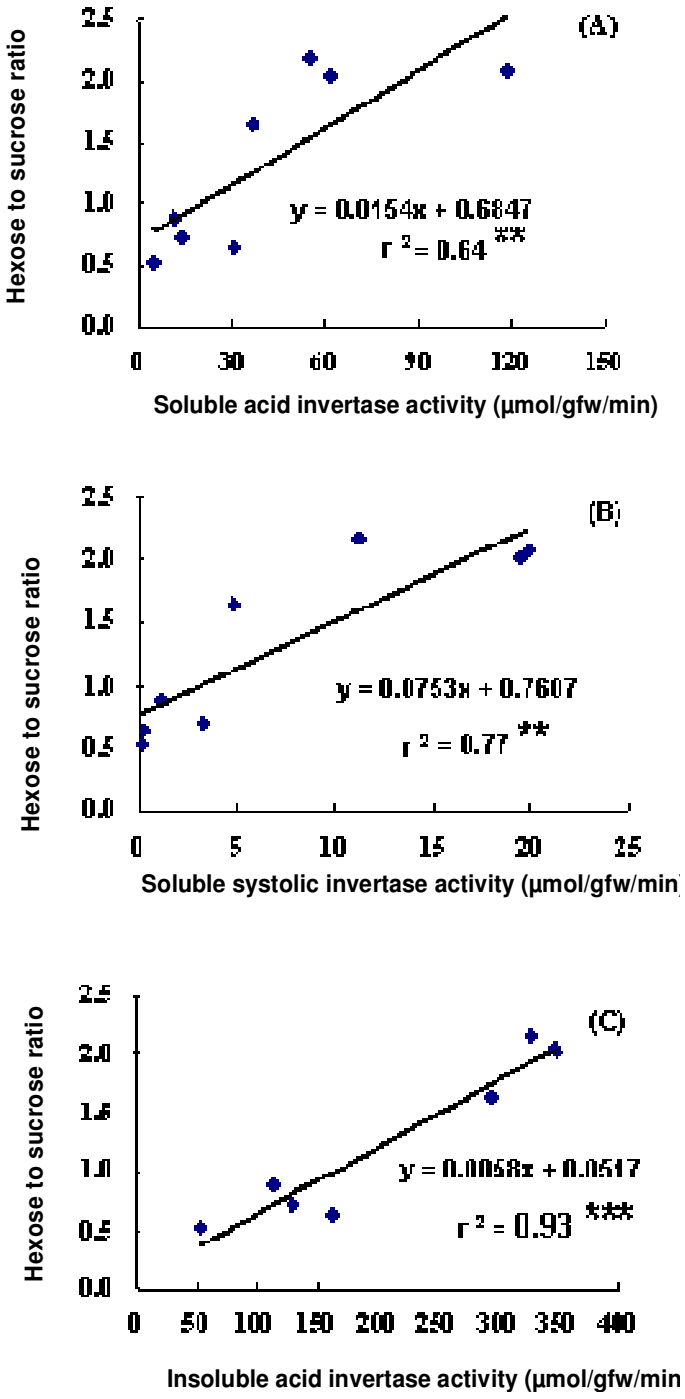

Figure 3. The correlation between the hexose to sucrose ratio (w/w) and acid invertase (A), cytosolic invertase (B) and bound cell Invertase (C). The correlation is significant at the 0.1% level. Each point is the mean of three observations.

activities in grains were higher under water deficits in both cultivars with obviously further activity in Zagros cv. (Figure 2D). Zinselmeier et al. (1995, 1999) also found that drought stress has been to consistently affect sugar metabolism and decrease activities of soluble and insoluble invertases.

We conclude that the enhanced activities of sucrose synthase in the grains of Zagros cv. contributed to the increased assimilates to grains under water deficits. Interestingly, great increases in sucrose synthase were observed in tolerant cultivars than sensitive one by WS which suggests that falls in soluble and insoluble invertases are physiologically compensated for by rises in sucrose synthase in this cultivar. In comparison, water stress led to a larger reduction in grain yield in Marvdasht than that of Zagros, probably due to the higher restriction of the activities of vacuolar invertase and bound invertase (Figures 2A and B). A vacuolar invertase path for sucrose hydrolysis would be especially useful in a symplistically continuous system of phloem-unloading and post-phloem transport, where internal sucrose cleavage could sustain sucrose gradients across plasmodesmata (Duke et al., 1991; Sturm et al., 1995; Fisher and Cash-Clark, 2000; Kim et al., 2000). According to Mathias et al. (2002), sequential expression of first soluble, then insoluble invertases, and activity levels, indicates distinct contributions by these invertases to young ovaries during normal development and under stress and soluble invertase expression in young ovaries is an early target of drought stress, and the response is localized to sites of import and expansion by maternal tissues.

Hence, soluble invertase may contribute to a maternal mechanism for control of kernel number under stress. Long distance transport of assimilate links source output to sink metabolism. Thus, enhanced sink demand associate with grain growth, not only cause an increase photosynthetic reserve mobilization at the source end, it also increases the enzyme activities associated with phloem unloading and sucrose import (Ho, 1988; Patrick, 1997). A central role has also been implicated for hexose to sucrose balance in regulating key aspects of ovary and seed development (Weber et al., 1996, 1998; Wobus and Weber, 1999; Weschke et al., 2000).

In the present study, the hexose to sucrose ratio was correlated to the activity of soluble and very close correlation to insoluble invertase (p=0.92), in grains (Figure 3C). In developing grains, like many other fruits, sucrose transport and metabolism thus occur largely in the symplast of maternal tissues that predominate in the young ovaries. The correlation between insoluble invertase and starch negative and statistically significant ($r = -0.64^{**}$, $p < 0.01$, Table 1). No significant correlation was observed between other enzymes and starch (Table 1). In our study, the correlation between hexose to sucrose ratio and activity of invertases observed here is compatible with a predominantly apoplectic and then symplastic path for sugar movement in kernels (Figures 3A, B and C).

In addition to a drop in sucrose delivery, capacity of endogenous sucrose use could be critically reduced in young ovaries by the observed decreases in soluble

invertase. The importance of this process was initially suggested by Zinselmeier et al. (1995, 1999). Water stress caused a marked reduction in grain starch content at 21 DAA. Since the partitioning of carbon into starch reserves, depends on assimilate supply as well as demand, the decreased starch level could have been due to either photoassimilate supply during the period of intense reserve accumulation or to a direct impairment of the starch synthesis machinery as a result of sink dehydration. The authors suggested that low activity of acid invertases (soluble and bound cell) might contribute to the observed reductions of starch biosynthesis in sensitive cv. (Figure 1D). They further noted that depletion of these starch reserves, together with the reduced sucrose supply under WS (Figure 1C), could be lethal for the newly formed zygote in sensitive cultivar.

Conclusion

The research presented here provides significant insight into our understanding of early stages in grain development, into their marked sensitivity to stress, and also into the regulation and roles of different invertase activity during these formative periods. Our results demonstrate that an accurate assessment of the sensitivity of a genotype to drought stress, the responses of the whole plant, including the vegetative and reproductive organs must be taken into consideration. We found that the continuously high invertases and sucrose synthase activities in grains induced by water deficit, are responsible for yield stability in tolerant cultivar. Hence, acid and bound cell invertase activity levels, before or immediately after pollination indicates distinct contributions to alleviate harmful effects of drought stress.

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