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A view on the role of metabolites in enhanced stem reserves remobilization in wheat under drought during grain filling

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Abstract

Terminal drought is one of the major factors limiting the yield of wheat (*Triticum aestivum L.*). Remobilization of pre-stored carbohydrates from stem to grain during grain filling is one of the drought tolerance mechanisms. To investigate the enhanced stem reserves remobilization under drought condition, we compared metabolome pattern of two contrasting wheat landraces (NO.49 and NO.14) during days after anthesis (DAA) under drought stress. Wheat genotypes were cultivated in a greenhouse and drought stress initiated just after anthesis and peduncle, penultimate and lower internodes samples were collected from well-watered and drought-stressed plants at 0, 10, 20 and 30 DAA. The peduncle of NO.49 showed remarkably higher stem reserves remobilization efficiency compared with NO.14 during this period. The higher content of fructan in peduncle of NO.49 genotype under drought stress suggesting a higher oxidative stress defense response and a faster fructan remobilization supports grain filling under stress. A positive correlation observed between the fructan and sucrose:sucrose fructosyltransferase (SST) activity (r^2=0.77) in No.49, whereas this was not the case in NO.14 genotype. A higher Invertase (INV) activity in NO.49 genotype under drought stress compared with control during 20 DAA revealed an increase in sucrose degradation during fructan remobilization. The decrease in arginine and increase in proline content in NO.49 under drought stress only during 10 DAA could be considered as the index of senescence initiation. Increase accumulation of arginine and ACC amino acids in NO.49 peduncle in after anthesis period, suggesting enhancement of senescence during grain filling. Our results indicate that carbon remobilization in NO.49 increases from the stem to the grains by a good coordination between enhancing senescence and oxidative stress defense to protect stem cells from premature cell death.

Keywords: Acid invertase, Amino acids, Drought, Fructan, Soluble sugars, Stem reserves remobilization, Sucrose:sucrose fructosyltransferase, Wheat.
Abbreviations: DAA: Days after anthesis, INV: Invertase, SST: Sucrose:sucrose fructosyltransferase.

Introduction

Wheat (*Triticum aestivum* L.) is an important crop in Mediterranean environments, where yield potential is usually constrained by drought stress. In areas of the world with a Mediterranean climate, rainfall decreases and soil evaporation increases in spring when bread wheat (*Triticum aestivum* L.) enters the grain-filling period. Wheat crops often experience water deficit and heat stress during grain growth and development, which limits productivity (Ehdaie and Waines 1989; Ehdaie et al., 1988). Grain filling and development in wheat depends on carbon from three sources: current assimilation, remobilization of pre-anthesis assimilates stored mainly in the stem, and redistribution of assimilates stored temporarily in the stem after anthesis (Daniels et al., 1982; Gallagher et al., 1975; Kobata et al., 1992). Under drought, there is a rapid decline in photo-synthesis after anthesis, limiting the contribution of current assimilates to the grain. Drought increases the portion of the grain matter originating from stem reserves, with values ranging from near 10 % under normal conditions to greater than 40 % when drought or heat stress occurs (Aggarwal and Sinha 1984; Austin et al., 1977; Bidinger et al., 1977; Davidson and Chevalier 1992; Ehdaie and Waines 1989; Palta et al., 1994; Rawson and Evans 1971; Yang et al., 2000). Genotypic variation exists for various aspects of grain filling from stem reserves. The estimated contribution of stored assimilates to grain yield in wheat depends on the genotype, experimental conditions, and the method of measuring stored carbohydrates. Stored reserves and their contribution to grain can be estimated by measuring post-anthesis changes in internode dry matter (Borrell et al., 1993; Cruz-Aguado et al., 2000; Hunt 1979; Pheloung and Siddique 1991; Shakiba et al., 1996) and/or changes in internode water- soluble carbohydrate (WSC) content during grain

filling period (Blum et al., 1994; Davidson and Chevalier 1992; Kiniry 1993; Shakiba et al., 1996). Water soluble carbohydrates (WSCs) in wheat stems are mainly composed of fructan, sucrose, glucose and fructose. The main storage forms of WSCs in the stem (culm + sheath) of wheat are fructans and sucrose (Wardlaw and Willenbrink 1994; Yukawa et al., 1995). This storage peaks well into the period of grain filling under adequate moisture conditions and declines during the latter stages of kernel development as a result of supporting a high proportion of the concurrent kernel development (Wardlaw and Willenbrink 1994). Most plants store starch or sucrose as reserve carbohydrates, but about 15% of all flowering plant species store fructans, which are linear and branched polymers of fructose. Among the plants that store fructans are many of significant economic importance, such as cereals (e.g. barley, wheat, and oat) vegetables (e.g. chicory, onion, and lettuce), ornamentals (e.g. dahlia and tulip), and forage grasses (e.g. Lolium and Festuca) (Hendry and Wallace 1993). In plants, fructans may have functions other than carbon storage; they have been implicated in protecting plants against water deficit caused by drought or low temperatures (Hendry and Wallace 1993; Pilon-Smits et al., 1995). Drought stress after anthesis causes accelerated mobilization of stored carbohydrate reserves to the grain and a shorter but more intensive period of grain filling (Yang et al., 2000). The substrate for fructan synthesis is sucrose, and like sucrose, fructans are stored in the vacuole. Although sucrose is synthesized in the cytoplasm, fructans are produced in the vacuole by the action of specific enzyme sucrose: sucrose fructosyltransferase that transfer fructose from sucrose to the growing fructan chain. Fructan synthesis is modulated by light, which changes the availability of sucrose in the cell (Fig. 1). The biosynthetic enzymes are evolutionarily related to invertases which hydrolyze sucrose. During growth of the wheat crop, in addition to sucrose nitrogen and sulfur are accumulated in the vegetative tissues and are then redistributed to the developing seed during the concurrent processes of vegetative tissue senescence and grain development (Dalling 1985). Amino acids are the major form in which nitrogen is remobilized from the leaf and stem to the grain during grain filling. During senescence, amino acids for remobilization are provided by the proteolysis of leaf proteins such as Rubisco which are degraded by developmentally regulated cysteine endopeptidases and peptide hydrolases (Buchanan-Wollaston et al., 2003; Chandlee 2001; Feller et al., 2008). Import of sulfur into the grain is largely in the form of glutathione,S-methyl-methionine and sulfate (Anderson and Fitzgerald 2001). The amino acid cysteine which is used to synthesize a wide range of sulfur-containing organic molecules such as methionine and glutathione (GSH) (Hawkesford and Wray 2000; Kopriva and Rennenberg 2004; Leustek et al., 2000). Even the magnitude of mobilized dry matter and the mobilization efficiency in different internodes of the main stem differed between genotypes. Genotypic variation for stem reserves and mobilization under drought stress at the late booting stage in wheat has been studied by (Ehdaie et al., (2006). They found that several genotypes including NO.14 exhibited a reduction for mobilized dry matter in the peduncle internode under drought stress, whereas genotypes including NO.49 showed a high level of mobilized dry matter under drought treatment. Two tall landrace genotypes NO.14 and NO.49 from southwestern and central eastern regions of Iran, respectively differed extremely in stem remobilization under drought stress exposed at late booting stage. (Mohammadi-Bazargani et al., (2011) studied the stem reserves and remobilization of these genotypes under a progressive post-anthesis drought stress. They found that during this period, peduncle intrnode in NO.49 had remarkably higher stem reserves remobilization efficiency compared to NO.14. They also conducted a proteomics analysis to understand molecular events associated with or leading to differential stem reserve remobilization in these genotypes under stress. The purpose of this study is metabolomics analysis of stem reserves remobilization potential of two wheat contrasting genotypes (NO.49 and NO14) under a progressive post-anthesis drought stress. In this respect, we studied the changes in soluble sugar, fructan, amino acids, SST and INV activity during grain filling in the peduncle and their relationships with stem reserves and remobilization.

Results

Stem reserves remobilization

The ANOVA indicated significant main effects for both treatment and harvest date (DAA) only for peduncle dry weight of both genotypes; whereas significant main effect only for harvest date was observed for dry weight of the penultimate and the lower internodes (Table 1). In addition the treatment×harvest date interaction only for peduncle of two landraces and lower internodes of N14 landrace was significant (Supplementary Table 1). Different patterns of post-anthesis changes in peduncle dry weight were observed between two landraces (see Fig 2 adopted from Mohammadi-Bazargani et al., 2011). Under both well-watered and droughted conditions, peduncle of N14 reached its maximum weight at 20 DAA, whereas N49 reached its maximum weight at 10 DAA. MDM varied in two genotypes under well-watered and droughted conditions (Table 2). Under well-watered condition, MDM was greater in N14 52.5mg than in N49 (19.8 mg), whereas under drought stress, it was lower in N14 37.2mg than in N49 (75.3 mg). ME was greater for N49 under droughted (30.2%) than under well-watered (8.1%) conditions, whereas ME for N14, was lower under droughted (15.1%) than underwell-watered (19.3%) conditions (Table 2).

Spike grain yield

In both genotypes grain number per spike, grain weight and spike grain yield did not significantly change under drought stress at maturity stage (Table 3).

Soluble sugar in the stem (Peduncle)

Drought stress increased significantly the content of fructose, glucose and fructan in peduncle of NO.49 genotype as compared to control condition but not for NO.14. Sucrose content increased significantly in peduncle of both genotypes under drought stress (Supplementary Fig 1). Fructose and glucose content had the highest concentration at 0 DAA under well-watered condition in both genotypes and decreased afterwards (Fig 3), In well-watered treatment, fructan and sucrose contents were low at 0 DAA for both genotypes and increased gradually until 20 DAA (their attained maximum content at 20 DAA for NO.14 and at 10 DAA for NO.49) and decreased afterwards (Fig 3). Under drought stress, glucose, fructose, sucrose and fructan had the highest content at 10 DAA in NO.49 and decreased subsequently (remobilized), while for NO.14, glucose and fructose had the highest content at 0 DAA, Sucrose and fructan had the highest contents at 20 DAA and declined afterwards (Fig 3).

Table 1. Weight of main stem internodes of wheat genotypes including peduncle, penultimate and lower internodes subjected to well-watered and drought treatments.

Genotype	Moisture treatment	Dry weight (mg)		
		Peduncle	Penultimate	Lower internodes
NO.49	Well-watered	232.19 a §	138.00 a	339.77 a
	Droughted	217.19 b	136.28 a	343.75 a
NO.14	Well-watered	231.18 a	116.00 a	298.28 a
	Droughted	222.99 b	117.83 a	305.47 a

§ In each section, means followed different letters within the same column indicate statistical significance at $P<0.05$ level

Table 2. Postanthesis maximum and minimum mean for peduncle weight, mobilized dry matter (MDM) for and mobilization efficiency (ME) for three internodes in two genotypes under well-watered and drought conditions

Internodes		Well-watered				Drought			
	Genotype	Max	Min (mg)	MDM *	ME ‡ %	Max	Min (mg)	MDM	ME %
Peduncle	NO.49	244.2	224.4	19.8	8.1±6 #	248.3	173	75.3	30.3±6 #
	NO.14	270.7	218.2	52.5	19.3±1	244.8	207.6	37.2	15.2±4
Penultimate	NO.49	149	130.4	18.6	12.5±10	150.8	115.1	35.7	23.7±11
	NO.14	130	108.8	21.2	16±10	123	116	7	5.7±4
Lower internodes	NO.49	374	290.2	83.8	22±6	382.5	285.4	97.1	25±12
	NO.14	344.1	274.3	69.8	20.3±3	322.7	274	48.7	15±2

* Maximum weight- minimum weight. ‡(mobilized dry matter/maximum weight)*100. # Standard deviation

Changes in enzyme activities in peduncle

Sucrose: sucrose fructosyltransferase (SST)

The activity of SST examined for two genotypes in relation to fructan synthesis (accumulation) and mobilization in the peduncle indicated different responses under well-watered and drought during grain filling. SST activity in peduncle of NO.49 increased under drought compared to well-watered condition whereas for NO.14 no significant change was observed under drought stress (Supplementary Fig 2). The activity of SST in the peduncle of NO.49 under drought stress increased during 0 to 10 DAA and reached the maximum activity at 10 DAA, which was 2.8 times higher than control condition and declined sharply thereafter (Fig 4). However, under well-watered condition the SST activity for NO.49 was high at anthesis initiation (0 DAA) and did not show any significant changes during 0 to 10 DAA and decreased slowly afterwards (Fig 4). Under control condition the SST activity for NO.14 increased rapidly from 0 to 10 DAA, and declined sharply until harvesting (Fig 4). The most prominent change of SST activity was observed for the genotype NO. 49 during 10 DAA under drought stress (Fig 4). In contrast to the increase in SST activity of NO.49 under drought stress, the SST activity of NO.14 was 1.5 times reduced during 0 DAA to 10 DAA. The reduction was gently from 0 to 10 DAA, and sharply increased from 10 to 20 DAA (Fig 4). Under drought SST activity of NO.49 had positive and moderate correlation with fructan in peduncle (r^2=0.77), whereas in NO.14 SST activity had no significant correlation with fructan.

Acid invertase activity (INV)

INV activity in peduncle of NO.49 increased under drought as compared to well-watered condition, but did not show any significant changes for NO.14 genotype (Supplementary Fig 2). Interestingly, activity of acid invertase (INV) in the peduncles gradually decreased in both genotypes until 20 DAA and then increased dramatically. Under drought condition the INV activity of NO.14 had similar pattern to control condition, but during 20 to 30 DAA INV activity was only slightly higher than control that was not significant (Fig 4). The INV activity of NO.49 also declined until 20 DAA,

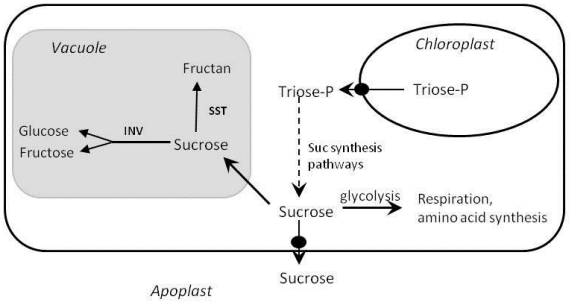

Fig 1. Simplified pathway of sugar metabolism in wheat stem cells. Sucrose synthesis occurs in the cytoplasm and is either exported the vacuole to be stored or it is used in glycolytic metabolism to produce energy or it is transported into apoplast towards sink tissues for further metabolism. In the vacuole, sucrose can be either converted to fructan by the activity of sucrose:sucrose fructosyl transferase (SST) or hydrolyzed to glucose and fructose by the activity of invertase (INV).

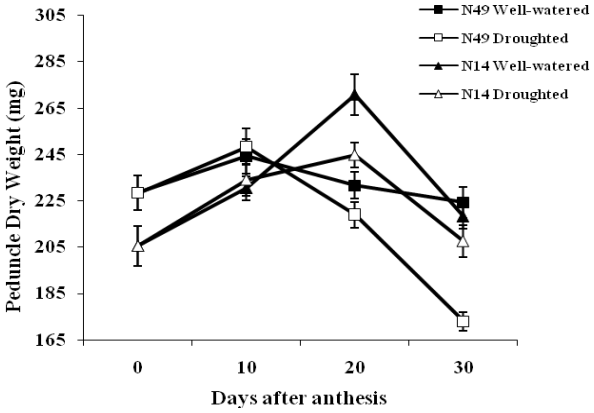

Fig 2. Changes in the weight of main stem peduncle during grain filling in 2 bread wheat genotypes under well-watered & drought treatments (adopted from Mohammadi-Bazargani et al. 2011).

and increased afterwards in both conditions (Fig 4). In contrast to well-watered condition, the INV activity of NO.49 increased more dramatically under drought condition (Fig 4).

Amino acids

The changing pattern of 20 different amino acids was studied in peduncle internode for two genotypes at different stages after anthesis under drought and well-watered conditions. Study of relative contribution of every amino acid in their total content in peduncle internode showed that contribution of proline was greatest in both genotypes and water treatments and the contribution of methionin, cyclopropan carboxylic acid (ACC), γ −amino butyric-acid (GABA) and tyrosin was the least (Fig 5). The changing pattern of total concentration of amino acids at different times after anthesis showed that under control condition, total concentration of amino acids in peduncle of NO.49 genotype was low in the first stage of anthesis (0 DAA), increased during 10 DAA and declined until 30 DAA. This changing pattern was enhanced under drought stress condition (Supplementary Table 2, Fig 6). In contrast to NO.49, total concentration of amino acids in NO.14 genotype was high under control condition in the first stage (0 DAA) and declined during 30 DAA. But under drought it was low at 0 DAA and increased during 10 DAA and declined afterwards (Supplementary Table 3, Fig 6). The decreasing level of amino acids was less in NO.49 compared to NO.14 under drought stress, so that the total content of amino acids was greater during 30 DAA in NO.49 under drought than control condition, and was greater as compared with control and stress conditions of NO.14 (Fig 6). Drought stress significantly increased the total concentration of amino acid in peduncle internode for both genotypes (Supplementary Table 4). Drought stress for NO.49 genotypes had no significant effect on concentration of Asp, Ser, Asn, Thr, Ala, GABA and Leu, but led to a decreased level of Glu and in a significant increase of Gly, Gln, His, Arg, ACC, Met, Pro, Tyr, Val, Ile, Lys and Phe (Supplementary Table 4). Drought stress for NO.14 had no significant effect on the concentration of Asp, Glu, Ser, Asn, Gln, Arg, Ala, GABA, ACC and Met, but resulted in an increase in concentration of Gly, His, Thr, Pro, Tyr, Val, Ile, Lys, Leu and Phe (Supplementary Table 4). Interestingly, the level of increase differed in two genotypes. The amount of increase in His, Pro, Tyr, Lys, Val and Phe was greater under drought in peduncle of NO.49 compared to NO.14 genotype (Supplementary Table 4). It is notable that drought stress led to a reduction of Glu concentration and an increase of concentration in Asn, Gln, Arg and ACC amino acids in NO.49 genotype whereas no significant change was observed in these amino acids in NO.14 genotype.

Discussion

Stem reserves remobilization

Grain filling in cereals depends on carbon from current assimilates and from carbohydrate stored in vegetative tissues either pre- or post-anthesis (Schnyder 1993). Current assimilation normally diminishes due to natural senescence and the effect of various stresses (Aggarwal and Sinha 1984; Blum 1998; Yang and Zhang 2006). Therefore, the demand of the growing kernel increases, in addition to the demand due to maintenance and respiration of the plant, thus stem reserves become an essential carbohydrate source for completed grain filling (Mohammadi-Bazargani et al., 2011). Remobilization of stored assimilate in vegetative tissue such as stem to fill the grain in monocarpic plants such as wheat require the initiation of whole-plant senescence under drought (Yang and Zhang 2006). Our results indicated that drought during 30 DAA in NO.49 plants increased dry matter mobilization efficiency 3.7, 1.9, and 1.13-fold in peduncle, penultimate and lower internodes respectively, whereas in NO.14 decreased by 1.6, 2.8, and 1.3-fold respectively (Table 2). Therefore, further metabolites analysis were performed on peduncles collected from the two landraces under well-watered and droughted conditions at four stages of plant growth (0, 10, 20, and 30 DAA). Remobilization of assimilate stored in peduncle initiated at 10 and 20 DAA for NO.49 and NO.14 genotypes respectively under drought (see Fig 2 adopted from Mohammadi-Bazargani et al. 2011)

Fructan and soluble sugars in wheat peduncle

Based on the results in this study we could show that drought stress increased significantly fructose, glucose and fructan concentrations in peduncle of NO.49 genotype as compared to control condition while there was no significant change of the mentioned sugars in the line NO.14 (Supplementary Figure 1). The sucrose concentration increased significantly in peduncle of both genotypes under drought stress. The most important role of soluble carbohydrates such as glucose and fructose is the supply of required substrates for the respiration. The accumulation of these carbohydrates in the peduncle internode of the NO.49 genotype under drought stress showed that these hexoses are less consumed in the respiration chain. In general, a part of these photosynthetic substances are stored temporarily in the stems of the plant, so that they can be used in stem mobilization. Fructans are fructose-based polymers synthesized from sucrose by fuctosyltransferases (SST). Sucrose is the major form of those carbohydrates which is transported into the stem and can be either stored as sucrose or converted to fructan (Blum 1998). In our study, fructan was significantly higher in the peduncle of NO.49 plants under drought but not for NO.14. This has also been previously observed in wheat experiencing water stress (Conocono 2002; Goggin and Setter 2004; Kerepesi and Galiba 2000). Fructans fulfil various protective physiological and functional roles in plant metabolism (Hendry 1993; Le Roy et al., 2007; Morvan-Bertrand et al., 2001). They have been increasingly recognized as protective agents against abiotic stresses (Valluru and Van den Ende 2008) and have been suggested to have a more direct role in conferring tolerance to drought (Goggin and Setter 2004). Fructan can protect plants against cold and drought stress through membrane stabilization and is known as important membrane protectors in plants (Goggin and Setter 2004; Hincha et al., 2003; Valluru and Van den Ende 2008; Van den Ende and Valluru 2009). It might either directly detoxify reactive oxygen species (ROS) in chloroplasts and vacuoles or indirectly stimulate the classic antioxidative defence systems (Van den Ende and Valluru 2009). Production of ROS often increases in plant under environmental stress that can cause oxidative damage. Therefore, the higher content of fructan in peduncle of NO.49 genotype under drought stress might indicate the more oxidative stress defense response against the toxic stress metabolites. Protection against oxidative damage protects stem cells from premature cell death important for successful stem reserves remobilization in NO.49 genotype. Our results showed that the changes in fructan content are coincided with changes in peduncle dry weight in two genotypes. Fructan

Table 3. Spike grain yield, grain number per spike (GN) and grain weight per spike (GW) in two genotypes under well-watered and drought conditions.

Genotype	Well-watered			Drought		
	GN (no)	GW (mg)	Spike grain yield (mg)	GN (no)	GW (mg)	Spike grain yield (mg)
NO.49	20.30 a §	0.72 a	34.80 a	18.53 a	0.61 a	32.83 a
NO.14	24.87 a	0.80 a	30.97 a	24.60 a	0.71 a	29.10 a

§ In each section, means followed by letters within the same row indicate statistical significance at P=0.05 level

remobilization started 10 DAA for NO.49 and 20 DAA for NO.14 under drought (see Fig 2 adopted from Mohammadi-Bazargani et al., 2011; Fig 4). Edelman and Jefford suggested that fructan accumulated in the vacuole and provided a sink within the cell that allowed photosynthesis to continue. Investigations have indicated that fructan accumulation continues during stem growth, flowering, and anthesis and falls during the later stages of grain filling when flag leaf photosynthesis is limited (Archbold 1940; Blacklow et al., 1984; Borrell et al., 1989). In our study according to these results we assume that under drought, photosynthesis was limited on 10 and 20 DAA for NO.49 and NO.14, respectively. Therefore the faster fructan remobilization in NO.49 supports grain filling under drought condition as one of drought tolerance mechanisms (see Fig 2 adopted from Mohammadi-Bazargani et al., 2011; Fig 4 and Table 3).

SST activity in peduncle

SST is considered to be the most important enzyme for fructan synthesis as it increases concomitantly with fructan accumulation (Dubois et al., 1990; Wagner et al., 1986; Yang and Zhang 2006; Yukawa et al., 1995). In our study, increasing in SST activity (Supplementary Fig 2) was accompanied by an increase in the amount of fructan in No.49 genotype under drought stress (Supplementary Fig 1) demonstrating a positive correlation between the fructan and related enzyme in peduncle (r^2=0.77) whereas this was not the case in NO.14 genotype. Previous studies have shown that conditions which lead to an increase of sucrose and the initiation of fructan synthesis are simultaneous with increasing of SST activity (Cairns and Pollock 1988; Wagner et al., 1983). There are convincing evidences that sucrose is the major substrate for fructan metabolism (Edelman and Jefford 1968; Pollock 1986a; Pollock 1986b; Pontis and del Campillo 1985). These relations were seen clearly in sucrose, fructan and SST enzyme of NO.49 genotype (Fig 3 and 6). Sucrose is not only the substrate for fructan synthesis but may also have a regulatory effect by inducing SST activity (Dubois et al., 1990). Our results showed that increased activity of SST in NO.49 might have been induced by sucrose. Induction of SST activity by sucrose has been shown in barley (Wagner et al., 1986), while under water stress despite of high content of sucrose and fructan, such a SST activity in the NO.14 was not observed especially during 20 DAA. Similar observations were on leaves of barley which accumulated fructan markedly in the lower leaf segments although this tissue possess low extractable SST activity (Cairns et al., 1989). Another similar observation has also found that a weak positive correlation (r^2=0.35-0.38) between fructan concentration and SST activity across development in the stem of both rainfed and irrigated wheat, by which fructan concentration was on average 2.5-fold higher in the stems of rainfed wheat compared with irrigated plants. The average SST activity was similar in both (Goggin and Setter 2004). However, in our study the correlation between fructan concentration in peduncle and SST activity varied from strong (r^2=0.77) in NO.49 to no

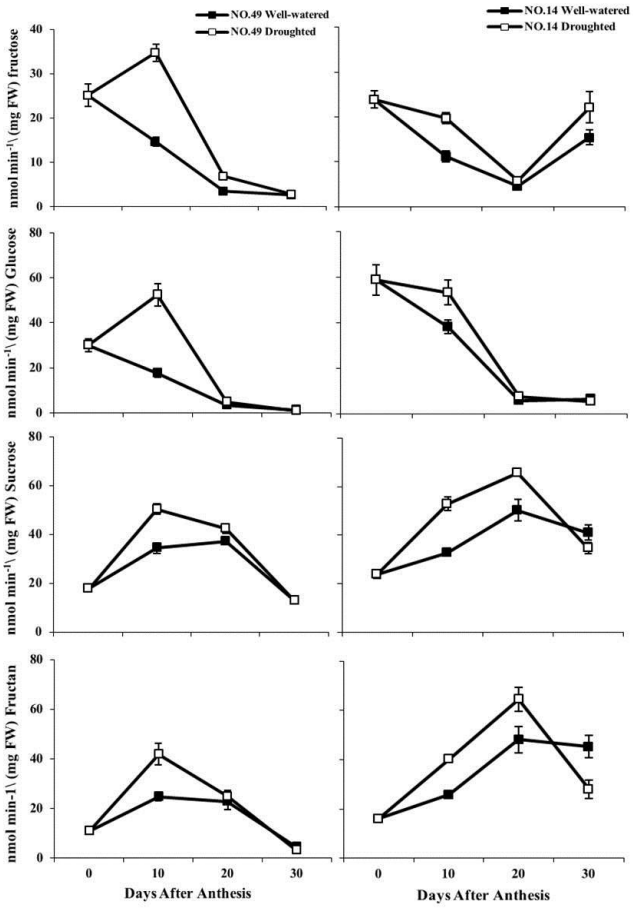

Fig 3. The content of glucose, fructose, sucrose and fructan in peduncle of wheat genotypes during grain filling under well-watered and drought treatment.

association in NO.14. Studies conducted by Saijo et al. (2000) and Noël et al. (2001) indicated that the rate of fructan accumulation is dependent on several different factors regulated by sugar-mediated induction of fructan synthesis and breakdown. We could illustrate that in NO.49 genotype under drought stress, the activity of SST enzyme decreased, while the remobilization of dry material increased (at 10 DAA). Yang et al. (2004) have been reported that under drought stress, the activity of STT enzyme and the remobilization were opposite to each other and the activity of this enzyme decreased contemporary to the beginning of stem remobilization. However, SST activity of NO.14 under stress before the beginning of stem remobilization (during 20 DAA) has followed a decreasing trend. This may suggest that during 20 DAA, current photosynthesis in NO.14 is high enough for grain filling, and plant use SST for remobilization of stored carbohydrates from stems to the grains only after senescing of the flag leaf. Yang et al. (2004) reported that SST activity has been inhibited by water stress and negatively correlated

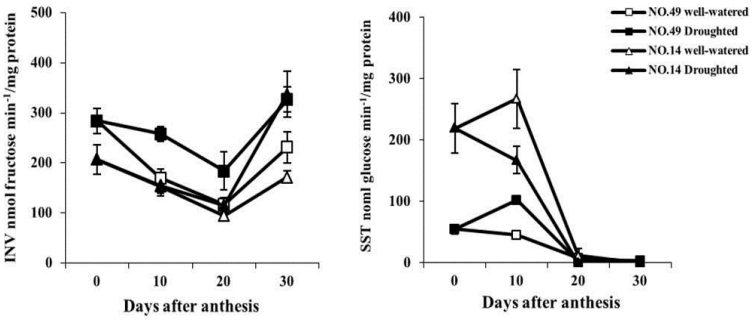

Fig 4. Changes in the activity of SST and INV for two genotypes (NO.14 and NO.49) during grain filling under well-watered and drought conditions. Each point is the mean of three replicates (3 observations per replicate).

with mobilization of stem reserves. Therefore, based on higher activity of SST, and remobilization of stem reserves in NO.49, the source activity in the peduncles of NO.49 was higher than it was the case in NO.14 genotype.

INV activity in peduncle

A higher INV activity in NO.49 genotype under drought stress compared with control at 10 and 20 DAA indicates an increase in sucrose degradation during fructan remobilization in NO.49. A similar results has been observed in potato (Wang et al., 2000). There was, however, no significant change in INV activity in NO.14 genotype during 20 DAA under drought stress as compared with well-watered condition. Unlike in NO.49, INV activity was higher under drought stress after 20 DAA in NO.14 genotype. Koch et al. (Koch 2004) reported that only two pathways exist for sucrose degradation catalysed either by INV or sucrose synthase. Plant cells contain three kinds of invertases including vacuolar, apoplastic and cytosolic (Koch 2004). Vacuolar INV is more specific for sucrose (Sturm et al., 1999) which is a hydrolase, cleaving sucrose irreversibly into glucose and fructose (Sturm and Tang 1999; Wang et al., 2000). The increased amount of glucose and fructose in NO.49 genotype during 10 days drought stress compared to the control condition was due to the high INV activity (Fig 3 and 6). Our results indicated that INV activity decreased during 20 DAA in peduncle of two genotypes under well-watered and drought conditions and increased afterwards. These changes were higher under drought than well-watered conditions for the NO.49 plants (Fig 4). The behavior of invertase in Geramineae is controversial. Several studies have shown that soluble invertase activity in Geramineae decreases under condition of fructan synthesis and increases during fructan remobilization (Prud'Homme et al., 1992; Simmen et al., 1993; Simpson et al., 1991). However, other reports indicated that invertase activity during fructan accumulation were neglectible (Gallagher et al., 2004; Gurrend et al., 1996; Wagner and Wiemken 1987).The results in our study in both genotypes were in agreement with the first suggestion that INV activity decreases with fructan accumulation and increases with fructan remobilization. However, in NO.49 genotype decreasing in INV activity was observed until 20 days while fructan synthesis continued until 30 days (Fig 3 and 6). Wardlaw and Willenbrink (2000) also observed that a decrease of fructan content under water stress was accompanied by a rise in both fructan exohydrolase and acid invertase in peduncle and penultimate internodes. In addition, Simpson and Bonnett (1993) showed that the induction of fructan hydrolyses and remobilization towards growing grains of wheat under drought stress requires an increase in INV activity which is coinciding with our observations on wheat peduncle. Because sucrose is the major substrate for fructan synthesis, a decrease of INV activity during 20 DAA result in the accumulation of fructan, probably due to increase in sucrose availability for SST to synthesize fructan. Dubois et al (1990) also reported fructan accumulation is greater when sucrose is high in the penultimate internode of wheat. On the other hand, with fructan remobilization initiation, INV activity increases, in which in turn increases sucrose degradation. This may decrease substrate content for SST that result in fructan remobilization instead of accumulation.

Grain number per spike, weight and spike grain yield

We could demonstrate that grain number per spike did not change in both genotypes under drought conditions (Table 3) because water stress was imposed at the anthesis initiation, while grain number is determined before anthesis. Early report also indicated that water stress during DAA had no effect on the grain number (Yang et al., 2004). Grain weight of wheat usually reduces under drought during grain filling, mostly because of reduction in grain filling period (Palta et al., 1994; Wardlaw and Willenbrink 2000; Zhang et al., 1998). This reduction usually resulted in limitation or reduction of photosynthesis due to water deficiency (Yang et al., 2004). We observed that grain weight and spike grain yield were not significantly reduced under water stress (Table 3), and it seems that NO.49 genotypes benefited mostly from stem reserves remobilization for grain filling (Table 2 and see Fig 2 adopted from Mohammadi-Bazargani et al., 2011**).** This strategy is supposed to be the major drought tolerance mechanism of this genotype whereas NO.14 genotype was not able to perform the same tolerance activity (Table 2 and see Fig 2 adopted from Mohammadi-Bazargani et al., 2011). We, therefore, postulate that NO.14 genotype might use other drought tolerance mechanisms (perhaps maintains higher photosynthesis under drought and/or senesce more slowly) to fill the grains under drought condition. According to the results of fructan and dry matter mobilization (see Fig 2 adopted from Mohammadi-Bazargani et al., 2011; Fig 4), it seems that photosynthesis was limited lately (20 DAA) for NO.14. However this requires further investigation. Generally speaking NO.14 genotypes could not use stem reserves properly like NO.49 genotypes during grain filling under drought.

Amino acids

Amino acids play a major role in regulation of most metabolic pathways in the plants such as protein synthesis. Some amino acids act as nitrogen source while other amino acids act as biosynthetic substrates for secondary compounds (Rampino et al., 2006). According to the different roles of amino acid in the plant biosynthetic pathways, difference of their concentration in different stages of growth is expected (Silveira et al., 2004). Our results showed that the concentration of Pro increased in peduncle internode of two genotypes under drought stress condition in which it was observed only during 10 DAA (Supplementary Table 2 and 3). However the rate of increase was significantly higher in NO.14 than NO.49. The accumulation of Pro is a common physiological response in many plants in response to a wide

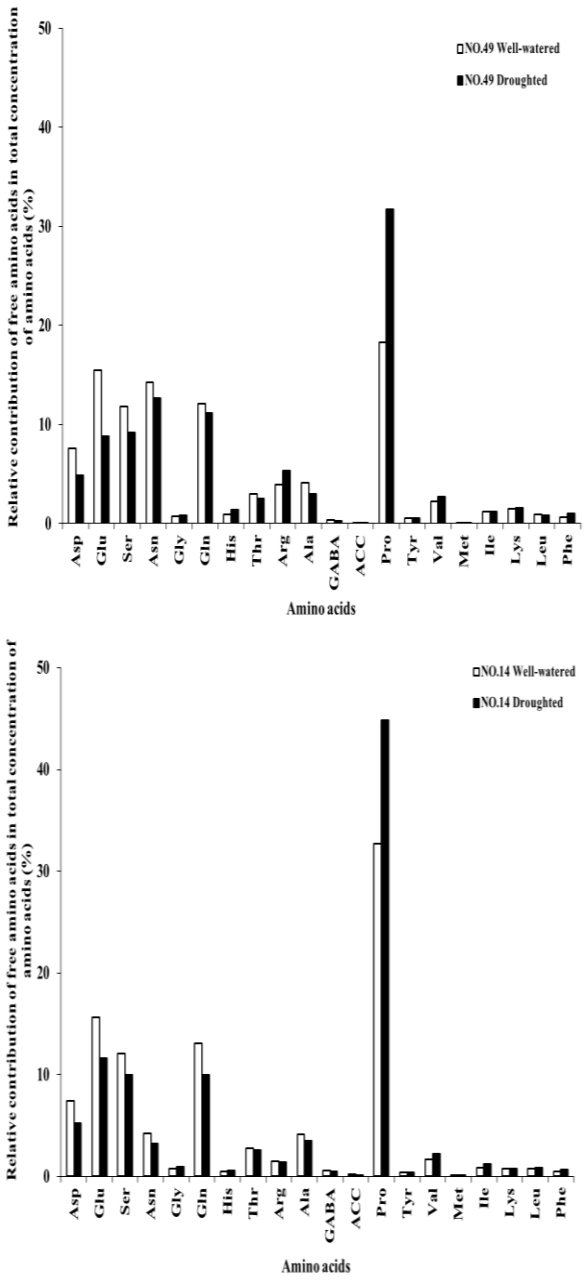

Fig 5. Relative contribution of amino acids in peduncle internode of two wheat genotypes NO.49 and NO.14 under drought and well-watered conditions.

range of biotic and abiotic stresses. Pandey et al. (1974) suggested that low arginine/proline ratio may be attributed to initiation of senescence in grape. They observed senescence in the stage coincide with the rapid decline in arginine and increase in proline content (Pandey et al., 1974). We also observed the same pattern in NO.49 only during 10 DAA that it could be considered as the index of senescence initiation (Supplementary Table 2 and 3). The concentration of Arg in NO.49 increased gradually at different times after anthesis under drought as compared to well-watered conditions (Fig 7) while in NO.14 Arg concentration did not change significantly under drought as compared with control condition (Fig 7). Accumulation of Arg in stems of poplar during senescence has also been reported by Couturier et al. (2010). It is notable that utilization of assimilates stored in vegetative tissues to fill the grain in monocarpic plants such as wheat require the initiation of whole-plant senescence which is an adaptive response to remobilize stores assimilates from stem to fill the grain (Yang and Zhang 2006).Therefore our results suggest that high concentration of Arg in NO.49 genotype under drought stress enhance senescence in peduncle which is necessary to stem reserves remobilization. Taken together, our results suggest that senescence in NO.49 initiates with low arginine/proline ratio during 10DAA and enhance by Arg accumulation after wards (Fig 7). Mohammadi-Bazargani et al. (2011) observed the up-regulation of several senescence-associated proteins and breakdown of photosynthetic proteins in peduncle of NO.49 (at 10 and 20 DAA) and suggested that the carbon remobilization from stem to the grains in NO.49 increases due to enhanced senescence. Methionine and ACC are substrates of ethylene biosynthesis in plants (Hoeberichts et al., 2007). Concentration of these amino acids increased in peduncle of NO.49 genotype under drought stress. These two amino acids, among the other amino acids had the least concentration in peduncle of both genotypes. There is a positive relationship between the increase of ABA concentration in plant tissues with an increase in ethylene production from methionine and ACC and probably ABA influences stimulation of senescence process in the plants by ethylene (Yang et al., 2003). Despite of low concentration of methionine and ACC in normal conditions and drought stress in peduncle relative to other amino acids, they are playing a major role in inducing senescence in the plant under drought stress. It is important to note that the concentration of those amino acids increased in NO.49 genotype under drought stress during 10 DAA, and decreased later (Supplementary Table 2). An increased in level of ACC may induce senescence in which improve stem reseves remobilization. Glutamate decreased during 30 DAA under drought stress as compared to the control condition in both genotypes. The reduction of glutamate leads to an increase in glutamine concentration which is a direct product of glutamate and can be transported across the stem and supplied to the grains for further remobilization. However, the increase of glutamine was observed in our study up to 10 DAA and declined afterwards indicating that it might have been remobilized and used in the grains during the grain development. Interestingly, for NO.14 genotype no significant difference was observed in these two amino acids, glutamate and glutamine (Supplementary Table 4). It is notable that during growth of the wheat, nitrogen and sulfur are accumulated in the vegetative tissues and are then redistributed to the developing seed during the concurrent processes of vegetative tissue senescence and grain development (Dalling 1985). Nitrogen in the form of amino acids is remobilized from vegetative tissues to the grain. However, with regard to drought stress and senescence, a tide regulation resulted in the production of glutamine which can be remobilized to the grain as a major form of amino acid remobilization during grain filling period (Howarth et al., 2008).

Materials and methods

Plant growth

The study was conducted in the experimental greenhouse of Agriculture Faculty of Tehran University on two landraces, NO.14 and NO.49, originating from southwestern and central

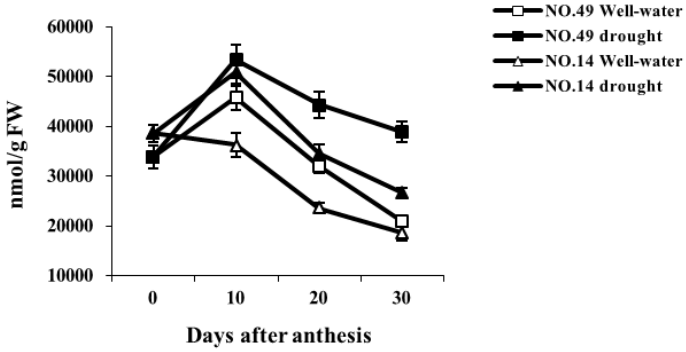

Fig 6. The changing pattern of total concentration of amino acids in peduncle internode during grain filling in NO.49 and NO.14 wheat genotypes under drought and well-watered conditions.

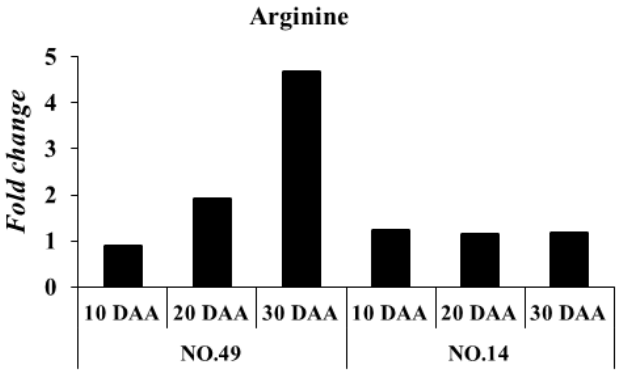

Fig 7. The ratio (stress over control) of Arg concentration for both genotypes in different times after anthesis.

eastern regions of Iran, respectively. These landraces showed significant differences in their stem reserves remobilization potential when drought stress exposes at late booting stage in field condition (Ehdaie et al., 2006). PVC pots with 20-cm height and 20-cm diameter were used as experimental units. Each pot was filled with 3-kg soil containing mix of clay: loamy sand: animal fertilizer in a ratio of 1:2:1. The sowing date was 28 August 2007. Plants were grown in a temperature-controlled greenhouse under 24/16 ºC day/night cycle and under well water condition until anthesis (Mohammadi-Bazargani et al., 2011).

Treatment and sampling

The experiment was 2×2×4 (two genotypes, two moisture treatments: (well-watered and drought) and four times of sampling. FactoriaA factorial design with 15 treatments in three replicates was used. Drought treatment initiated at anthesis when 50% of the spikes had extruded anthers. The soil moisture was maintained at 80–90% and 50% field capacity (FC) in well-watered and droughted conditions, respectively, until physiological maturity.. Pots were weighed daily to record the mass of water loss by evaporation. Based on the evaporation water loss, the amount of water needed to bring each pot to appropriate FC was added to each pot. In each pot, main tillers were tagged as spikes emerged from the flag leaf sheaths. Six main tillers were harvested randomly, in triplicate from both stressed plants and well-watered controls at starting anthesis: 0 DAA (0 day after anthesis) and 3 times after anthesis: (a) 10 DAA, (b) 20 DAA and (c) 30 DAA. The main tillers were harvested from the soil surface. After each harvest, leaf blades were removed. Samples selected for changes in dry matter analysis were immediately dried in a forced-air drier at 80 ºC for 48 h. Then, each main tiller was divided into spike and stem and each stem into three segments including peduncle (first internode below the spike including the distal node), penultimate internode (the internode below the peduncle including the distal node) and the lower internodes. These samples were later used to determine dry matter weight and stem reserve remobilization (Mohammadi-Bazargani et al., 2011). The spike grain yield, grain weight and the number of grain per spike were measured at maturity. Samples for soluble sugars, fructan and enzymes analysis were frozen immediately in liquid nitrogen and stored at -80 ºC until analysis. Date of anthesis was determined, when 50% of the spikes had extruded anthers.

Stem reserve remobilization analysis (Post anthesis changes in internodes dry matter analysis)

The weight of each segment was measured. The magnitude of mobilized dry matter in each internode segment was estimated as the difference between post anthesis maximum and minimum weight. Mobilization efficiency of dry matter in each internode segment was estimated by the proportion (%) of mobilized dry matter relative to post anthesis maximum weight of that segment (Ehdaie et al., 2006; Mohammadi-Bazargani et al., 2011).

Soluble sugars and Fructan analysis

Sugars were determined photometrically in enzyme-coupled assay. Stems (peduncle) were freeze-dried and ground to a fine powder. Samples (50 mg) of plant material were incubated for 60 min at 80 ºC in 0.7 ml 80% ethanol. After centrifugation of homogenate for 10 min at 14,000 rpm at 4 ºC, the supernatant was collected. The extracts were dried under reduced pressure at 40 ºC for around 60-90 min and resolved in 250 µl distilled water. Produced glucose, fructose, sucrose were measured as described in (Hajirezaei et al., 2000). For fructan assay an aliquot of solution was hydrolyzed with an equal amount of 0.4 M $HClO_4$ for 1 h at 60 ºC (Dubois et al., 1990). Glucose and fructose in the hydrolyzed sample were determined as described in Hajirezaei et al., (2000). Fructan concentration was estimated by the determination of polymerized fructose. Concentration of polymerized fructose was calculated from the increase in fructose due to fructan hydrolysis in the hydrolyzed sample as compared to non-hydrolyzed sample, taking into account the fructose released by sucrose hydrolysis.

Enzyme extraction and assays

All chemicals and enzymes used for enzymatic measurement were from Sigma chemical company (St.Louis, Mo, USA). Protein content was determined according to Bradford (1976).

Acid invertase (INV) and sucrose:sucrose fructosyltransferase (SST)

SST and acid INV were assayed according to (Savitch et al., 2000). The freeze-dried plant material (peduncle) was ground to a fine powder. The powder was homogenized with extraction buffer containing 50 mM sodium acetate pH 5.0, 20 mM EDTA, 5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.04% (w/v) polyvinylpolypyrrolidone (PVPP) and 20 mM b-mercaptoethanol. After centrifugation of the homogenate for

10 min at 14,000 rpm at 4ºC, The supernatant was desalted by centrifugal filtration on Sephadex G-25 columns equilibrated with extraction buffer minus PVPP and ß-mercaptoethanol. The reaction mixture (final volume 100 µl) containing enzyme solution and 0.1 M sucrose, was incubated at 30 ºC for 40 min. The reaction was incubated at 95 ºC for 4 min. Glucose and fructose present in the reaction mixture were quantified using an enzyme-coupled assay described by Housley et al., (1989). The excess amount of glucose over fructose was used to estimate SST activity. The acid INV was determined by measuring the increase in fructose in the reaction mixture.

Amino acids analysis

Peduncle samples that were extracted in 80% ethanol for soluble sugar assay were used for amino acid analysis. For the determination of amino acid contents, primary and secondary amino acids were derivatized using the fluoropohore 6-aminoquinolyl- N-hydroxysuccimidyl carbamate (AccQ Taq) and separated at a flow rate of 1 ml/min at 37 ˚C on a Waters Alliance HPLC system essentially as described by Zurbriggen et al., (2009) using the eluents A (140 mM sodium acetate, pH 5.8; 7 mM triethanolamine), B (acetonitrile) and C (purest water) and fluorescence detection (excitation at 300 nm and emission at 400 nm).

Statistical procedures

Analysis of variance (ANOVA) was performed for each character and genotype separately using the MSTAT statistical analysis system. Means were tested by least significant difference at $p_{0.05}$ level ($LSD_{0.05}$) (Steel et al., 1997). The results were analyzed for correlation analysis using the SPSS statistical analysis package (version 13) to evaluate the association of SST activity with fructan concentration and dry matter remobilization.

Conclusion

Metabolomics analysis was applied to study the effects of drought stress on stem metabolome pattern during grain filling. We compared some metabolite changing pattern of genotype NO.14 with genotype N49. Our results demonstrate that NO.49 genotype has a higher remobilization capacity of stem reserves compared to the NO.14 genotype under drought stress. The results provide evidence that drought stress causes a change in metabolites pattern to enhance the stem senescence and stem reserve remobilization interestingly in NO.49 genotype leads to an effective use of pre-stored carbon for grain development delivered from peduncle internode. Furthermore some metabolites under dought stress cause to increase oxidative stress defense response in NO.49 in which lead to decrease or control oxidative damages during stem senescence and survival, more stability of stem cells and successful mobilization of storage material in NO.49 genotype. We, therefore, suggest that NO.49 genotype might be useful for wheat yield production in breeding programs via using selection of stem reserves remobilization. Future investigates should focus on the identification of genes involved in the remobilization of stem reserves and thus are responsible for drought tolerance.

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