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ORIGINAL ARTICLE

A combination of stomata deregulation and a distinctive modulation of amino acid metabolism are associated with enhanced tolerance of wheat varieties to transient drought

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Abstract
Introduction Mediterranean winter crops are commonly and increasingly exposed to irregular rainfall and high temperatures, which lead to transient drought events of different degrees, adversely affecting growth and yield. Hence, exploring the diverse degrees of tolerance to drought existing in the crop and the molecular strategies behind it is pivotal for the development of ad hoc breeding programs.
Objective We investigated the physiological and metabolic response of six commercial wheat cultivars to transient water stress at the tillering and grain-filling stages.
Methods Drought experiments in lysimeters were set up at two developmental stages including six wheat cultivars. Newly expanded youngest leaves and flag leaves were sampled during the drought and following recovery. Metabolite profiles were generated using a GC–MS based protocol. Data on transpiration were continually acquired by measuring the weight variation of pots using electronic temperature compensated load cells.
Results The tillering stage in wheat is more sensitive to droughts than the grain filling stage. The former stage was characterized by pronounced metabolic alterations also during recovery from the drought, and plants exhibited reduced transpiration. Notably, cultivars varied considerably in their susceptibility to drought. Exceptionally only in cv Zahir was transpiration not reduced at tillering. During recovery, the transpiration rate of Yuval and Zahir was not significantly affected, while except Ruta the other varieties maintained lower values. At grain-filling, a moderate decrease in transpiration in response to drought was evident in Bar-Nir, Yuval and Zahir varieties as compared with the stronger response of Gedera, Galil and Ruta. The transpiration trend during recovery remained lower than the control plants, particularly in Gedera and Zahir, while it reached higher values

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than control plants in Yuval and Ruta varieties. Metabolite profiling of leaves across cultivars showed varietal specific trends of response. Particularly during tillering, amino acid metabolism was differentially regulated across cultivars. For instance, Ruta and Zahir exhibited major changes in central carbon nitrogen metabolism during stress response, accumulating large amounts of proline and threonine during tillering, while in Bar-Nir a general decrease in relative amino acid content was noted. Changes in stress related GABA were common to Galil, Ruta, Yuval and Zahir. Desiccation related raffinose family oligosaccharides were mostly associated with a later stage of grain-filling and recovery stages of response.

Conclusion The results indicate the occurrence of stage-dependent metabolic diversification along with a physiological response during transient droughts among wheat cultivars. It can be concluded that the most tolerant cultivar was Zahir, where a combination of stomatal closure deregulation and a significant accumulation rate of stress-related metabolites were evident.

Keywords GC–MS · Metabolite profiling · Photosynthetic recovery · Transpiration · Water stress

1 Introduction

Wheat (*Triticum aestivum* L.) provides one-fifth of the world's calories, as well as supplying 20% of the total food demand. It is grown on more than 200 million hectares of farmland worldwide, occupying about 17% of areas planted with cereals (FAOSTAT 2011), mostly rain-fed. Several of the major wheat producers fall under the category of semi-arid regions with a Mediterranean-like climate (Iglesias et al. 2011). Annual fluctuations in yield prompt for selection of wheat genotypes with enhanced tolerance to water stress (Rajaram 2001).

Drought stress encompasses limited soil water content and increased temperature, causing a plethora of events in the plant including diminished leaf water content and turgor loss. It also affects photosynthesis, respiration, ion uptake and plant metabolism (Claeys and Inze 2013). Although there are numerous studies on the response of wheat genotypes to drought stress at the molecular level (Krugman et al. 2011), only few studies (Bowne et al. 2012) have tackled the variability in the metabolism and physiology of stress among wheat cultivars.

In spite of the pivotal importance of wheat in human nutrition, increasing demand on the background of widespred desertification, only few studies have been done on the response of primary metabolism in wheat (Bowne et al. 2012) and in its wild relatives, e.g. *Triticum turgidum* ssp. *dicoccoides* (Krugman et al. 2011) to water deficit. It is generally accepted that genotypes with enhanced tolerance to drought stress maintain water more efficiently, with increased accumulation of compatible solutes and enhanced C–N repartitioning (Cramer et al. 2011; Hochberg et al. 2013a, b). Understanding the genetic variance that exists in regulating these metabolic processes during plant growth will aid in the development of breeding programs targeting abiotic stress tolerance in plants (Fernie and Schauer 2009). Here, we employed GC–MS (gas chromatography–mass spectrometry) based metabolite profiling and continuous recording of plant transpiration to explore the link between physiology, water use and leaf metabolism in six commercially cultivated wheat cultivars to transient drought stress under controlled conditions at two developmental stages, tillering and grain filling.

2 Materials and methods

2.1 Plant materials

Six hard spring wheat varieties (Bar-Nir, Gedera, Galil, Zahir, Yuval, and Ruta), bred in Israel, were evaluated in this study. The six genotypes were chosen based on the phenology (heading habit) and genotypic variability between them. Yuval and Zahir are very early maturing phenotypes and present stable yield, Galil and Ruta are late maturing phenotypes, and Gedera and Bar-Nir are intermediate maturing phenotypes (Bonfil 2016).

2.2 Greenhouse drought experiment

Under minimal control greenhouse conditions (temperatures between 28 and 34 °C), two drought experiments were established at two developmental stages: tillering and grain-filling. In order to ensure the same growth stage during the drought treatment, the seeds of the cultivars were sowed at three different times, every 7 days. This enabled to select seedlings at the same growth stage to continue the experiment. Plants were grown under minimal controlled greenhouse conditions at the Faculty of Agriculture, Hebrew University of Jerusalem (HUJI), Rehovot, Israel. Lysimeters mounted with 3.8 L pots with holes at the bottom for drainage filled with peat soil and covered with pellet in the greenhouse were used for both experiments (as described in Wallach et al. 2010). The experimental design consisted of a Randomized Complete Block Design (RCBD) with three blocks consisting of three and eight biological replicates for control and treatments respectively, each block containing 22 pots. Control received 100% irrigation (pot capacity—until drainage) throughout the experiment while drought treatment involved stopping irrigation entirely for 7 days until the plants started wilting. Plants were then irrigated

100% (pot capacity - until drainage), to monitor how the cultivars recovered from water deficit after 7 days.

2.3 Measurement of soil relative volumetric water content and whole-plant transpiration

Soil volumetric water content was measured using the EC-5 soil moisture sensor combined with the 'ProCheck' interface reader (Decagon Devices, Pullman, WA, USA). Transpiration rates were determined using lysimeters, as described in detail in Sade et al. (2009). Pots were placed on electronic temperature compensated load cells, located in a controlled-environment greenhouse. Its weight variation was used as a reference for the temporal variations in potential transpiration rate. The load cell readings, taken every 10 s and averaged over 3-min periods, were recorded by a data logger for further analysis. The whole-plant transpiration was calculated by a numerical derivative of the load cell output following a data smoothing process (Sade et al. 2009). The plant's daily transpiration rate was normalized to the total leaf area (measure by a LI-COR area meter model Li-3100) and the data for a neighboring submerged wick and these data were averaged for a given line over all plants (amount taken up by the wick daily = 100%).

2.4 Metabolic profiling and derivatization

Metabolic profiling was carried out exactly as described in (Lisec et al. 2006). Newly expanded youngest leaf and flag leaf were sampled from the control and drought treatment at the tillering and grain-filling stages experiments, respectively. The sampling was done when soil water contents in the treated pots had dropped significantly (Fig. 1; Additional Table S1) and plants showed signs of wilting and significant reduction in transpiration, after 7 days of stress application (Table 1A, B). Following 7 days, plants were re-watered to control levels and sampled after 7 days as before (but on a different set of plants). Collected samples were flash frozen and kept at −80 °C until further analysis. The samples were then extracted, derivatized and analyzed as described in details in (Aidoo et al. 2017). In brief, tissues were ground under liquid nitrogen in a TissueLyzer (RetschGmbh & Co. KG, Germany) containing pre-chilled holders and beads. Powdered samples (about 100 ± 10 mg, exact weight of each sample was later used for normalization of the chromatography data) were extracted in 1 ml of a pre-chilled (−20 °C) extraction mixture consisting of methanol: chloroform: water (2.5:1:1 v/v), with 8 µl of internal standards ribitol (0.2 mg/ml water). Following incubation on an orbital shaker at room temperature (RT) for 10 min, samples were sonicated for an additional 10 min at RT, and centrifuged for 5 min at 20,817×*g* (microcentrifuge 5417R). The supernatants were transferred to new 2 ml Eppendorf tubes. Phase separation was achieved by adding 300 µl each of water and chloroform to each tube. Following centrifugation (20,817×*g*, 5 min), the upper phase (200 µl) was collected and reduced to dryness under vacuum. Dried residues were redissolved and derivatized for 120 min at 37 °C (in 40 µL of 20-mg/mL methoxyamine hydrochloride in pyridine) followed by a 30-min treatment with 70 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) at 37 °C containing 7 µL of a retention time standard mixture (0.029% v/v *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotracontane, and *n*-hexatriacontane dissolved in pyridine), added prior to trimethylsilylation. The derivatized samples were then transferred to an auto-sampler vial; 1 µl of the sample was injected into the GC–MS (Thermo Scientific) in splitless mode (split/splitless liner with Wool, Restek, USA).

2.5 Data analysis

GC–MS chromatograms were processed using the National Institute of Standards and Technology (NIST, Gaithersburg, USA) algorithm incorporated in the Xcalibur® data system (version 2.0.7) and against standards run together

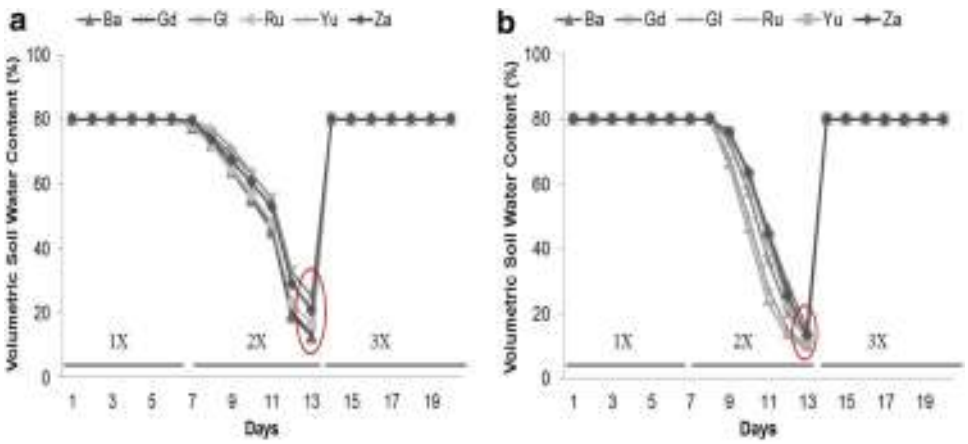


Fig. 1 Volumetric soil water content (%) in the pots for each of the cultivar during 20 days that includes the following treatments: well irrigation (1×), drought (2×) and recovery (3×). Plants at tillering (**a**) and grain-filing (**b**) stages after treatment and recovery. Red cycle denote the water content in the soil at the point of sampling for metabolic analysis. Ba, Bar-Nir; Gd, Gedera; Gl, Galil; Ru, Ruta; Yu, Yuval; and Za, Zahir

Table 1 Effect of water stress and recovery on whole-plant transpiration (mmol m^{-2} s^{-1}) during tillering stage (A) and during grain filling stage (B) of six wheat cultivars at the peak of stress application and recovery where sampling was done for metabolic analysis

Cultivars	Drought treatment			Drought recovery		
	Drought (mmol m^{-2} s^{-1})	Control (mmol m^{-2} s^{-1})	Reduction (%)	Recovery (mmol m^{-2} s^{-1})	Control (mmol m^{-2} s^{-1})	Reduction (%)
(A)						
Bar-Nir	7.20 ± 0.89	12.98 ± 1.05	45*	11.27 ± 0.61	15.27 ± 0.25	26*
Gedera	8.50 ± 0.42	14.87 ± 1.21	43*	10.88 ± 0.49	16.09 ± 0.94	32*
Galil	8.57 ± 0.55	11.87 ± 0.24	28*	9.53 ± 0.45	13.44 ± 0.52	29*
Ruta	8.09 ± 0.37	13.72 ± 0.30	41*	10.72 ± 0.60	15.64 ± 0.87	31*
Yuval	9.19 ± 0.77	11.39 ± 0.65	19*	8.82 ± 1.50	13.25 ± 0.77	33
Zahir	6.55 ± 0.66	8.63 ± 1.16	24	7.80 ± 0.71	10.13 ± 1.72	23
(B)						
Bar-Nir	3.9 ± 1.1	8.9 ± 4.1	41*	7.8 ± 1.3	9.8 ± 2.3	20
Gedera	1.8 ± 0.4	8.1 ± 1.5	78*	7.2 ± 0.9	7.7 ± 1.5	7
Galil	0.8 ± 0.1	8.8 ± 2.0	91*	4.9 ± 0.4	9.6 ± 2.0	49
Ruta	0.6 ± 0.1	6.5 ± 1.1	91*	3.7 ± 0.5	6.2 ± 0.7	40*
Yuval	2.8 ± 0.4	7.5 ± 1.0	62*	11.2 ± 0.9	8.7 ± 0.5	27*
Zahir	2.9 ± 0.7	11.7 ± 4.8	57*	8.4 ± 0.9	13.8 ± 5.6	9

Asterisks indicate significant differences between treatments and control ($P \leq 0.05$, Student's *t* test, n = 3–8)

with experimental samples and retention index (RI) libraries downloadable from the Max-Planck Institute for Plant Physiology in Golm, Germany (http://gmd.mpimp-golm.mpg.de/download/). Peak response values were calculated by dividing each metabolite mass-peak area by that of the internal standard ribitol and by the exact tissue fresh weight. Statistically significant differences in metabolite levels between samples were calculated using Student's *t* test at $P \leq 0.05$ embedded in Microsoft Excel and TMeV statistical software (Saeed et al. 2006). Prior to analysis, data was $\log_{10}$-transformed so as to bring the values closer to a Gaussian distribution. Results of the annotation were elaborated and analyzed by multivariate data analysis approaches using TMeV_4.9 software. Fold change (treatment/control) was calculated to determine the changes in metabolite steady state levels in treated plants compared with control plants. Analysis of variance was conducted using JMP, 2007 to determine the effect of drought on transpiration. The means were separated by the Tukey–Kramer test at $P \leq 0.05$.

3 Results

3.1 Transient drought affected transpiration rate

Relative daily transpiration decreased in treated plants in comparison with their respective control during the stress and recovery at both developmental stages (Fig. 2; Table 1A, B). The extent of reduction in transpiration at tillering varied considerably across cultivars, ranging between 19 and 45% relative to their respective control. Bar-Nir, Gedera, Galil, Ruta and Yuval were all significantly affected (Fig. 2a–f; Table 1A). During recovery, the transpiration rate of Yuval and Zahir decreased by 33 and 23% respectively, but were not significantly different from their controls. In contrast, the transpiration rate of Bar-Nir, Gedera, Galil and Ruta remained lower than control also following stress relief (Fig. 2a–f; Table 1A). During the grain-filling stage, the effect of transient drought stress was significant and greater than at the earlier stage in Galil and Ruta (Fig. 2g–l; Table 1B). The trend of transpiration at recovery remained lower compared with their respective controls than in treated plants at stress especially in Gedera (7%) and Zahir (9%). However, Yuval and Ruta were the only cultivars that increased transpiration significantly in the treated plants by 27 and 40% respectively (Fig. 2g–l; Table 1B).

3.2 Principal component analysis reveals differential and developmental specific metabolic response among cultivars

The metabolite profiles were examined by principal component analysis (PCA; Fig. 3) to estimate the global impact of transient drought on central C-N metabolite composition of the leaves of the six wheat cultivars during stress and recovery at each developmental stage.

At tillering, impact of the treatment on the metabolite composition of leaves of the different cultivars was measured. Only three cultivars (Bar-Nir, Ruta and Zahir) exhibited extensive change when plotting first principal

component (PC1) against second principal component (PC2) (Fig. 3a, b). Threonine, Glucose-6-phosphate, succinate, glycolate, glutarate and phosphate were the metabolites that mostly contributed to the separation on PC1, explaining 61% of the variance. Glucose, sucrose, sorbitol, maltose, GABA and citrate mainly contributed to the separation of the samples on PC2, explaining 12% of the variance. A late response to the applied stress was measured during recovery, when all of the genotypes underwent a change in the metabolite profile visualized on PC1 and PC2. Nevertheless the extent of the change was moderate as it is shown by the variance explained by the first and second component (28 and 14% respectively; Fig. 3b). Metabolites that mainly contributed to the separation of the data-samples on the two components included glucose-6-phosphate, valine, the desiccation related oligosaccharides raffinose and galactinol, mannitol, maltose, arabitol and sorbitol-6-phosphate (Additional file Table S2).

At grain-filling, stressed and control samples neatly separated on the PC plot compared to the earlier stage (Fig. 3c), which was dominated by few outstanding cultivars. At grain-filling the cultivars display a more homogenous distribution on the PC plot, nevertheless changes in the relative metabolite content were overall milder when compared with those at tillering. Proline, lysine, raffinose, asparagine and phenylalanine were the metabolites contributing to the separation of the samples on PC1 (explaining 29% of the variance) and galactose, raffinose, proline, glutamate and phenylalanine contributed to the separation on PC2 (explaining 22% of the variance). During recovery all the samples of the cultivars clustered with their respective control except Galil (Fig. 3d). Metabolites that contributed to the separation on PC1 explaining a variance of 29% included asparagine, serine, methionine, raffinose and phosphate while asparagine, methionine, aconitate, benzoate and phosphate contributed to the separation on PC2 explaining a variance of 13% (Additional file Table S2). It is noteworthy that at both developmental stages the top of the list of metabolites determining the distribution of the samples on the PC plots were enriched by amino acids, emphasizing their pivotal role in drought response.

3.3 Metabolic response to water stress and recovery at the tillering stage

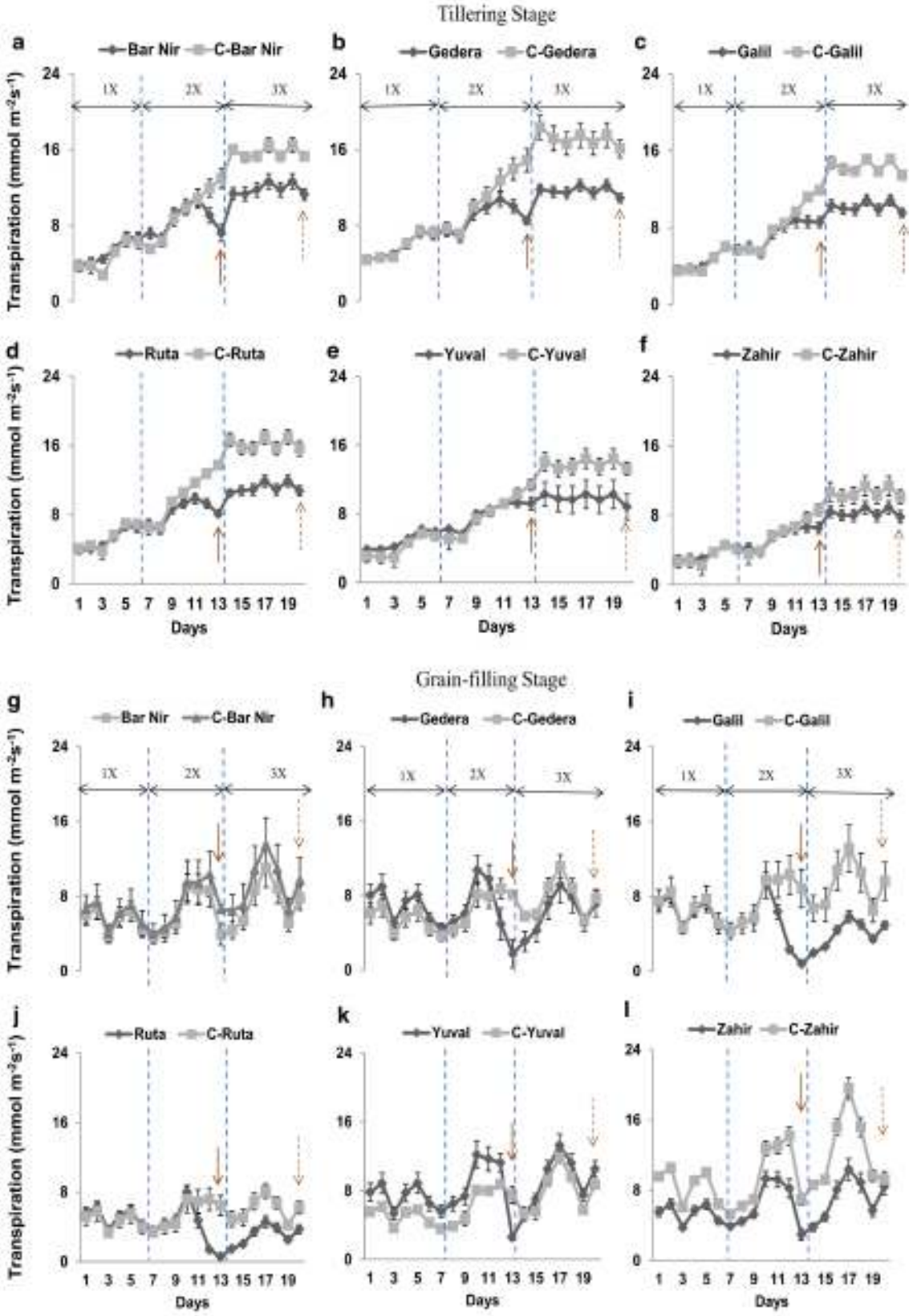
In the newly expanded leaves at tillering stage, 27, 31, 38, 45, and notably 84 and 87% of the metabolites annotated in Gedera, Galil, Yuval, Bar-Nir, and Zahir and Ruta, respectively were significantly ($p < 0.05$) affected by the transient drought (Additional file Table S3). During recovery, among cultivars 52% of the metabolites had altered content in respect to their control. For instance, stress related metabolites such as proline, pyroglutamate, valine, glucose, galactinol, mannitol and sorbitol, myo-inositol that accumulated during stress, declined in content during recovery in the cultivars, especially Ruta and Zahir (Fig. 4). The response to stress was extensively cultivar specific.

In Bar-Nir, all significantly altered amino acids decreased in content under stress and few inverted their patterns of change during recovery. Sugars and polyols showed a tendency to accumulate in response to transient drought, while recovery led to lower content of polyols. A number of sugars significantly accumulated also during recovery; among them galactose, maltose and raffinose. Organic acids aconitate, ascorbate and its oxidated form dehydroascorbate increased significantly during stress. Upon recovery, organic acids returned to their levels, with the exception of malate (Fig. 4; Additional file Tables S3 and S4).

In contrast to Bar-Nir, Gedera significantly accumulated aspartate, glycine and valine in response to drought, while at recovery amino acids were at comparable levels to the control, with the exception of a significant decrease in valine. Gedera exhibited little change in sugar metabolism rates during stress, but significant reductions in most sugars characterized the recovery period. This included fructose, galactose, maltose and raffinose. Melezitose was the only sugar that exhibited significant increase during recovery. A pattern of change similar to the sugars was shown for most polyols. Organic acids were not significantly affected during water stress and recovery. However, mitochondrial electron transport chain associated fumarate significantly decreased in response to water stress and significantly increased upon recovery (Fig. 4; Additional file Tables S3 and S4).

Like Gedera, Galil responded to water stress with very little significant changes in amino acid composition, specifically in stress related GABA and serine. During recovery, proline and valine were the only compounds significantly altered. Transient drought led to a decrease of most sugars in Galil with the outstanding exception of glucopyranose and that of the polyol glycerophosphoglycerol. During recovery few sugars and polyols including galactose, maltose and raffinose accumulated to significant levels (Fig. 4 and Additional file Tables S3 and S4). Stress had little effect on the organic acid content in Galil, also true during recovery.

Few amino acids were significantly altered also in Yuval and they included GABA and glycine. During recovery, alanine, glutamate and pyroglutamate were significantly altered. Among the sugars and polyols only a few decreased. However, during recovery, raffinose, sucrose, mannitol and sorbitol were significantly increased. With the exception of phosphates which increased by 2.3-fold, all significantly affected organic acids decreased in response to water stress. Glycolate, gulonate, itaconate and threonate were significantly increased during recovery. Fumarate was not affected while the other annotated TCA cycle intermediates except succinate (aconitate, citrate and malate) decreased during


Tillering Stage
Grain-filling Stage
a
b
c
d
e
f
g
h
i
j
k
l
Bar Nir
C-Bar Nir
Gedera
C-Gedera
Galil
C-Galil
Ruta
C-Ruta
Yuval
C-Yuval
Zahir
C-Zahir
1X
2X
3X
Transpiration (mmol m-2s-1)
Days
0
8
16
24
1 3 5 7 9 11 13 15 17 19

◂**Fig. 2** Effect of water stress and recovery on whole-plant transpiration rate (mmol m^{-2} s^{-1}) of six wheat cultivars during tillering stage (**a–f**) and grain-filling stage (**g–l**) during pretreatment (1×), treatment (2×) and recovery (3×). Values are means ± standard error bars of 3–8 replicates. Solid arrows indicate a time point of the treatment and broking arrows indicate recovery where sampling was done for metabolic analysis

stress. During recovery, citrate and malate were significantly increased (Fig. 4; Additional file Tables S3 and S4).

Ruta exhibited an exceptional alteration in its metabolic phenotype during the transient drought, characterized by the accumulation of most compounds. Among the significantly increased metabolites to note are proline and threonine. During recovery, most of the amino acids returned to levels comparable to those of the control plants. Sugars and polyols significantly accumulated during stress and maintained their increased levels in stressed plants well into recovery. In contrast with the other cultivars, organic acids in Ruta significantly increased during stress, including ascorbate and its degradation products (dehydroascorbate and threonate; Fig. 4; Additional file Tables S3 and S4).

Similarly to Ruta, Zahir responded to water stress with greater metabolic changes. For instance, 80% of the annotated amino acids were significantly changed during stress and 50% changed during recovery, also exhibiting an increased content, except aspartate and glutamate. Several sugars and polyols were also altered in response to stress and the trend continued into recovery. Organic acids also significantly accumulated during stress, however, during recovery all returned to the levels comparable to those of the control with the exception of Malate (Fig. 4; Additional file Tables S3 and S4).

3.4 Metabolic response to water stress and recovery at the grain-filling stage

The relative metabolite content of flag leaves during grain-filling showed a significant alteration of 52, 48, 37, 35, 30 and 26% of the metabolite profile in Ruta, Gedera, Bar-Nir, Zahir, Galil and Yuval, respectively in response to stress. During recovery 42, 23, 21, 8, 8 and 0% of the metabolite profile changed in Bar-Nir, Gedera, Ruta, Zahir, Galil and Yuval respectively. The data show that Bar-Nir, Gedera and Ruta exhibited increased alteration in metabolism during recovery, while the metabolic response of the other cultivars decreased in magnitude, similar to response during tillering (Figs. 4, 5; Additional file Tables S4 and S6).

In Bar-Nir, most amino acids increased in content during water stress and the most pronounced change in amino acid was that of proline. Desiccation associated sugar galactinol and glycerophosphoglycerol significantly accumulated in response to water stress, while ascorbate and dehydroascorbate decreased. Tricarboxylic acid cycle (TCA) intermediates showed a general pattern of decrease in content during stress. Recovery induced a significant accumulation of GABA, glutamate, phenylalanine, proline and serine and a concomitant increase in sugars and polyol and ascorbate. TCA intermediates fumarate and succinate significantly increased during recovery (Fig. 5; Additional file Tables S5 and S6).

Similarly to Bar-Nir, Gedera accumulated amino acids at a rate of between 1.7 and 6.6-fold in response to water stress. The magnitude of phenylalanine accumulation was highest, in line with a concomitant increase in shikimate. Glycerophosphoglycerol significantly accumulated similarly to Bar-Nir, but galactose, sucrose and galactinol significantly decreased in response to stress. Ascorbate metabolism showed a general mild trend of increase. Itaconate and maleate significantly increased in response to water stress. In contrast to Bar-Nir, TCA cycle intermediates increased significantly except for citrate and fumarate. During recovery, amino acids glutamate, leucine and proline increased. Glucose and maltose were the only sugars that significantly changed. Aconitate, maleate, phosphate and shikimate increased as well (Fig. 5; Additional file Tables S5 and S6).

In Galil, the general increase of amino acids during grain-filling in response to water stress was also measured, with the greatest increase being in proline. Galactinol showed a salient accumulation but most sugars and polyols showed a tendency to decrease. Threonate, glycerate, and shikimate significantly decreased in response to water stress, while TCA intermediates displayed a mixed pattern of change. During recovery, levels of several amino acids decreased, however none of them showing a significant difference in content (e.g. alanine, glycine, isoleucine and valine). Sugars and polyols followed a similar pattern, except raffinose which significantly increased. TCA cycle intermediates did not show significant alterations in recovered plants compared to their control (Fig. 5; Additional files S5 and S6).

A greater magnitude of metabolic change in response to water stress was found in Ruta. The accumulation of amino acids in the flag leaves ranged significantly from 2.0 to 66-fold compared to the control. Sugars and polyols decreased in content except for glycerophosphoglycerol. Most of the organic acids including TCA cycle intermediates, ascorbate and its degradation products also decreased significantly. During recovery, lysine increased while GABA and valine decreased. In contrast, sugars and polyols were lower in content compared to control plants. Most of the organic acid levels were lower compared to control also during recovery, except for phosphate and threonate, the levels of which increased. TCA cycle intermediate aconitate decreased during recovery (Fig. 5; Additional file Tables S5 and S6).

Yuval displayed a mixed response to water stress in amino acid changes. Asparagine, glutamate and serine

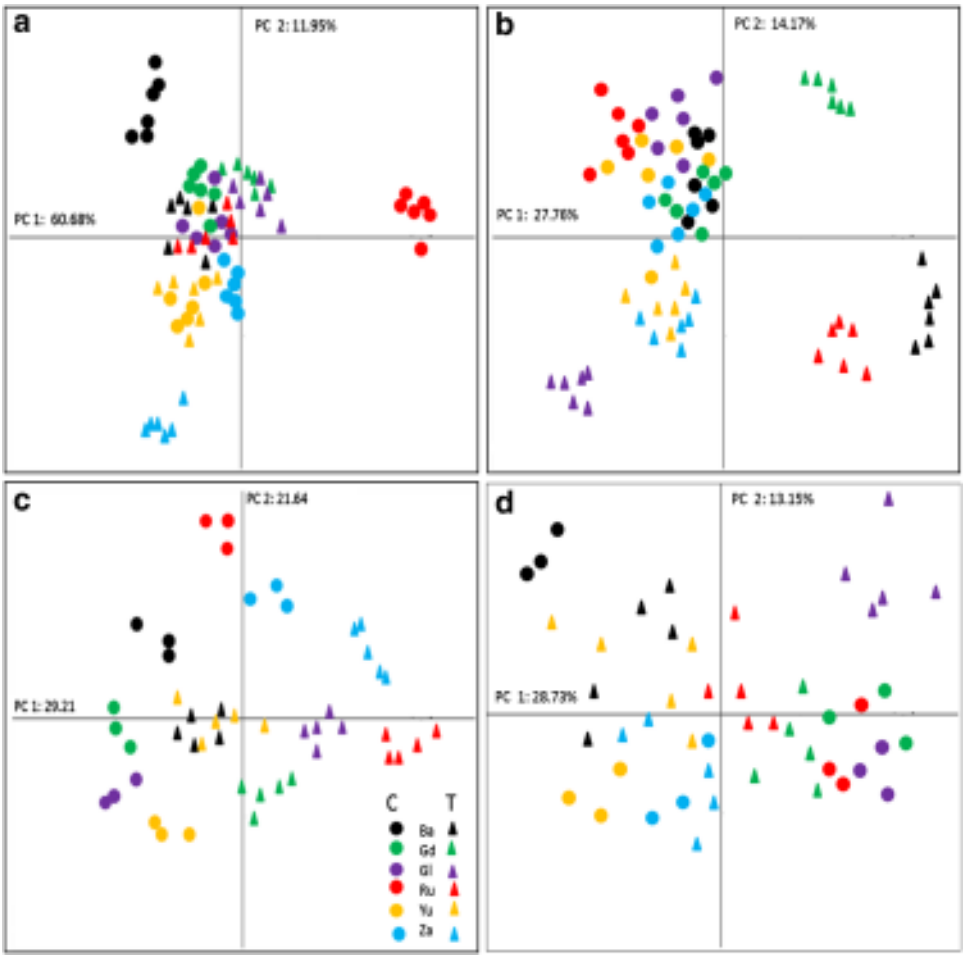


Fig. 3 PCA analysis based on annotated metabolites of leaf samples from plants at tillering (**a, b**) and grain filling (**c, d**) subjected to stress (**a, c**) and recovery (**b, d**). Shapes, circles denote control samples while triangle represents stressed and recovered samples. C and T represent control and treated samples, respectively. Ba, Bar-Nir; Gd, Gedera; Gl, Galil; Ru, Ruta; Yu, Yuval; and Za, Zahir. Values are relative abundance of each replicates (n = 3–6) of the treatments and control plants

accumulated significantly. Several sugars and polyols decreased drastically but a few increased, including galactose, glucose, sucrose and myo-inositol. Likewise, TCA cycle associated aconitate, citrate and succinate significantly increased during water stress. In contrast to the period of stress, amino acid, sugar, polyol and organic acid relative contents were similar to the control plants when compared during recovery (Fig. 5; Additional file Tables S5 and S6).

Zahir displayed a high variation of metabolite changes in response to water stress. Most of the patterns of change were similar to the other cultivars; that is an increase in some amino acids including asparagine, glutamate, lysine, phenylalanine, proline and serine during stress. Also the majority of the sugars and polyols which significantly increased included galactose, glucose, raffinose, galactinol and myo-inositol as well as ascorbate. In contrast to these changes, TCA intermediates were not significantly altered during water stress except for the accumulation of aconitate. During recovery, lysine was the only metabolite that was significantly increased. GABA, succinate and glycerate significantly decreased. The content of the rest of the metabolites in the recovery plants was as in control plants (Fig. 5; Additional file Tables S5 and S6).

4 Discussion

In a field evaluation study of six commercial wheat cultivars by RapidScan (NDVI and NDRE), Bonfil (2016) reported that each of the cultivars has a unique yield production potential as related to biomass and concluded that Zahir presented a stable production rate, while Ruta presented an unstable production pattern that was highly affected by the environment due to its latest phenology. In the present study we explored the physiology and metabolic response to transient water stress in the same six commercial wheat cultivars (Bar-Nir, Gedera, Galil, Ruta, Yuval and Zahir). Water stress caused a general reduction in transpiration rate among the cultivars, a common strategy to avoid drought damages by reducing growth and conserving water (Chaves et al. 2009; Jones 2004) and in agreement with previous reports (Izanloo et al. 2008). Notably, cultivar Ruta significantly continued to reduce transpiration during the recovery from stress in both

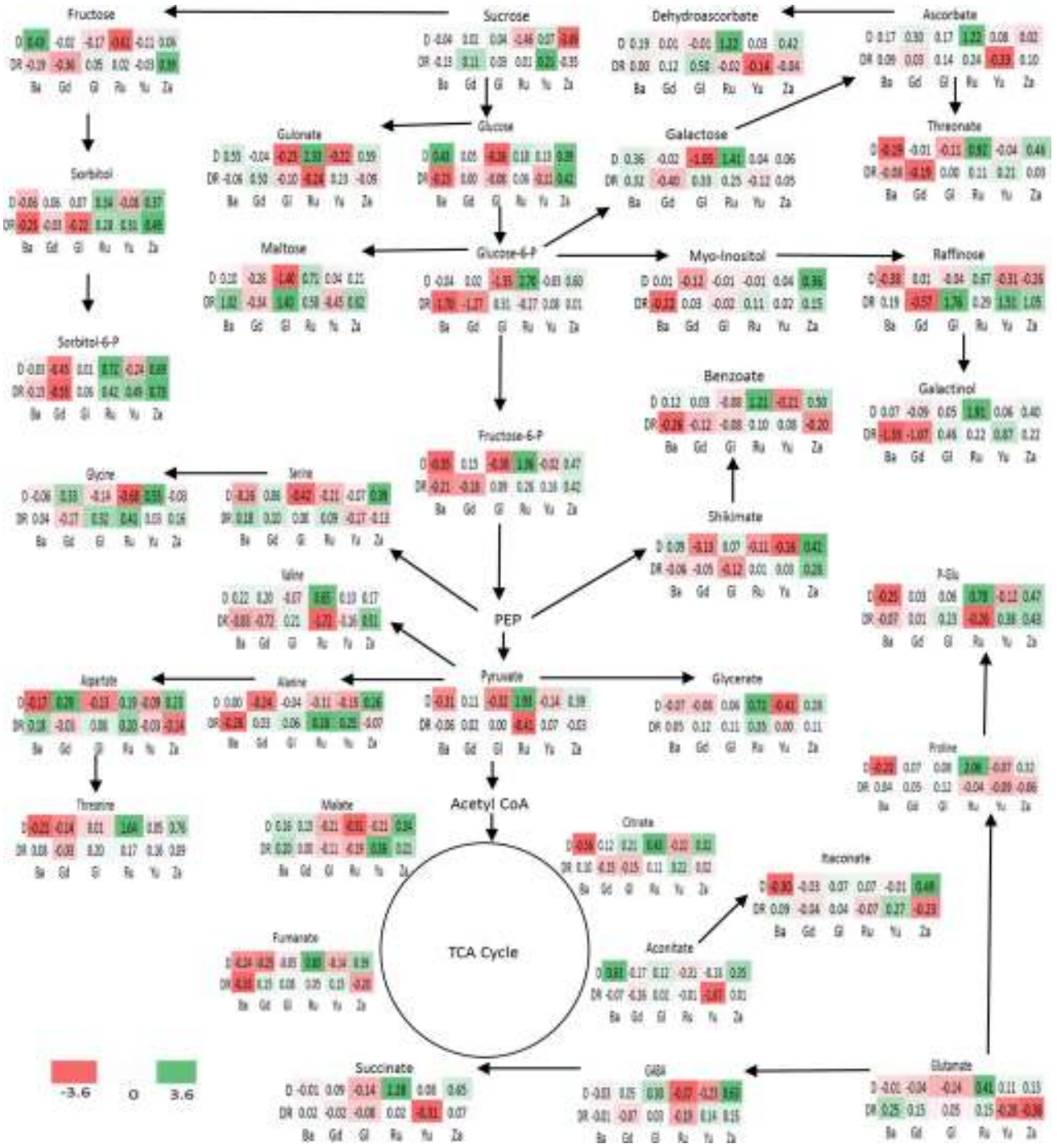


Fig. 4 Metabolic map showing the shift in stress related metabolites during stress (D) and recovery (DR) in Ba-Bar-Nir, Gd-Gedera, Gl-Galil, Ru-Ruta, Yu-Yuval and Za-Zahir at the tillering stage. Values are fold change ($\log_{10}$ transformed) of each condition over its own control of relative metabolite content in newest expanded leaf with 3–6 replicates. Color legend shows the gradient of increase and/or decrease in the metabolite content

developmental stages (Table 1A, B), suggesting a possible "over" regulation of stomata, with potentially detrimental effects on photosynthesis, growth and development. Outstandingly, Zahir was the only cultivar that did not reduce transpiration (at a significant level) during tillering stage in response to stress. This lessened stomata regulation was accompanied by a metabolic response of the central leaf metabolism (Table 2A, B). In addition, Zahir variety had

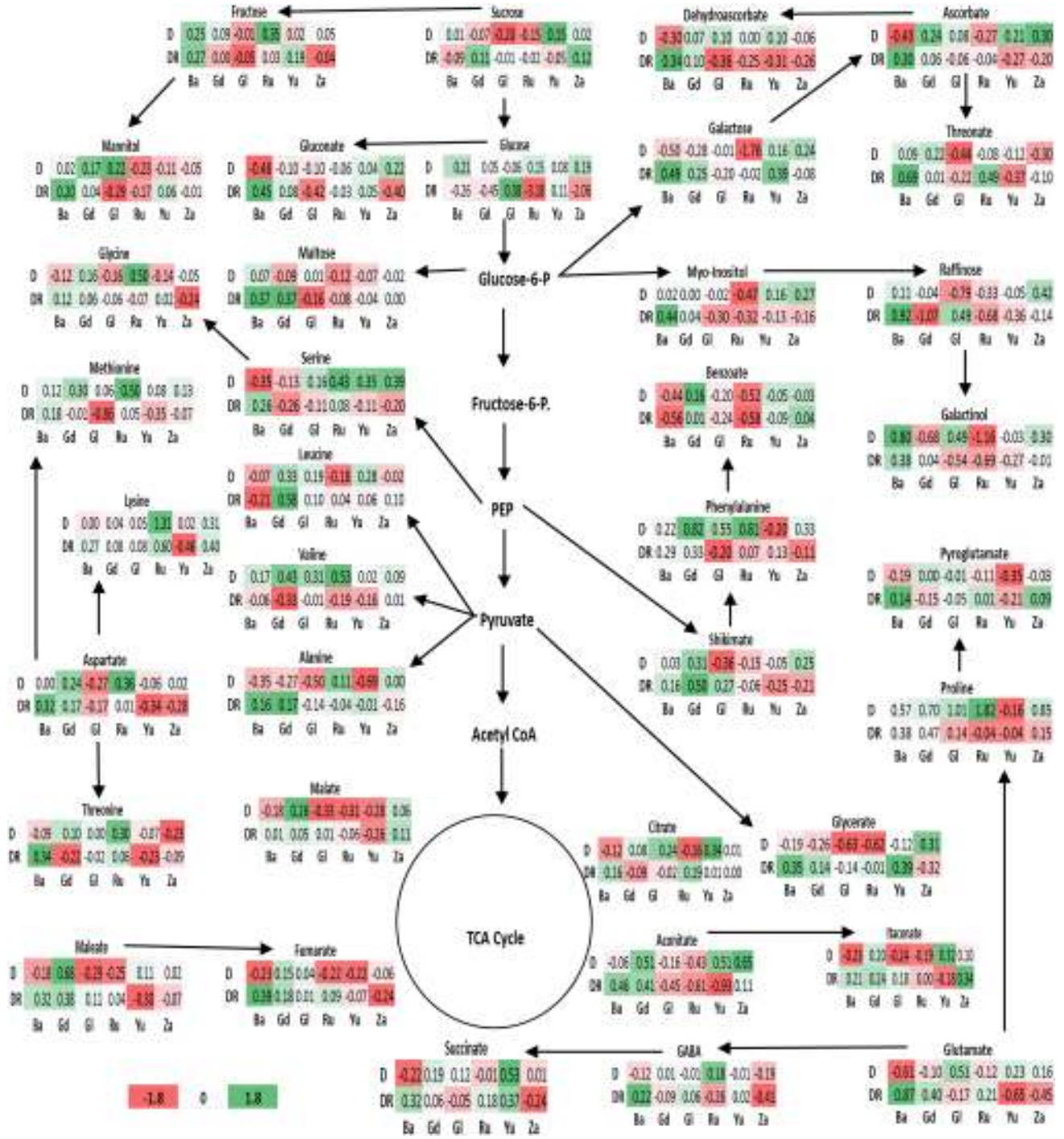


Fig. 5 Metabolic map showing the shift in stress related metabolites during stress (D) and recovery (DR) in Ba-Bar-Nir, Gd-Gedera, Gl-Galil, Ru-Ruta, Yu-Yuval and Za-Zahir at the grain filling stage. Values are fold change (log_{10} transformed) of each condition over its own control of relative metabolite content in flag leaf with 3–6 replicates. Color legend shows the gradient of increase and/or decrease in the metabolite content

high 100 seed mass (kg) compared to the rest of the cultivars (Additional File Fig. 1). Taking together these findings indicates that the singled combination of metabolic and physiological response make Zahir a potentially highly promising yielding variety in environments suffering from transient drought events.

A varying development-specific metabolic response exists among the cultivars in response to water stress. At

Table 2 Metabolites that significantly ($p < 0.05$) accumulated in cultivar Zahir during stress at tillering stage (A) and grain-filling stage (B) and their relative content across all cultivars. Values are fold change of normalized response values in the treatment dataset over their respective control. Metabolites significantly changed between treatment and control is emphasized (bold)

Metabolites	Bar-Nir	Gedera	Galil	Ruta	Yuval	Zahir
(A)						
GABA	0.94	1.13	2.01	0.43	0.59	**4.27**
Proline	**0.59**	1.17	1.21	**114.29**	0.86	**2.08**
Threonine	0.62	0.72	1.03	**44.00**	1.12	**5.77**
Valine	**1.65**	**1.57**	0.85	**4.50**	1.27	**1.48**
Glucopyranose	**0.39**	**0.55**	**17.47**	**1.91**	**0.49**	**1.54**
Melezitose	**2.34**	0.88	1.31	**11.75**	1.22	**1.32**
Raffinose	**0.42**	1.02	0.90	**4.73**	**0.49**	**0.54**
Galactinol	1.18	0.82	1.13	81.97	1.15	**2.54**
Glycerophosphoglycerol	**0.52**	1.02	**2.29**	**81.62**	1.12	**2.49**
Sorbitol	0.86	1.16	1.18	**2.18**	0.84	**2.36**
Citrate	**0.28**	1.31	**1.64**	**2.67**	**0.61**	**2.07**
Ascorbate	**1.50**	**1.98**	**1.49**	**16.51**	1.20	1.04
Glycerate	0.85	0.83	1.15	**5.21**	**0.39**	**1.92**
Gulonate	**0.62**	0.80	1.07	**5.20**	1.14	**1.85**
Itaconate	**0.50**	0.94	1.19	1.17	0.97	**3.08**
Threonate	0.64	0.97	0.78	8.23	0.91	**2.91**
Uracil	**0.50**	1.07	1.19	**17.01**	1.03	**3.41**
(B)						
Asparagine	0.66	**1.84**	**3.71**	**2.80**	**2.50**	**6.80**
Lysine	1.01	1.10	1.13	**20.64**	1.05	**2.04**
Phenylalanine	**1.68**	**6.59**	**3.56**	**6.41**	0.63	**2.15**
Proline	**3.70**	**5.05**	**10.14**	**65.67**	0.70	**7.16**
Serine	**0.45**	0.73	**1.45**	**2.69**	**2.24**	**2.44**
Valine	1.48	**2.70**	**2.03**	**3.39**	1.05	1.24
Raffinose	1.28	0.91	0.16	0.47	0.89	**2.61**
Galactinol	**6.25**	**0.21**	**3.06**	**0.07**	0.93	**2.00**
Glycerophosphoglycerol	**3.12**	**5.61**	**1.51**	**10.41**	**1.40**	0.84
Ascorbate	0.37	1.75	1.19	**0.54**	1.64	**2.00**

tillering, when the response was greater, two cultivars (Ruta and Zahir) displayed outstanding changes in relative metabolite content. Amino acids and few sugars showed highest response to drought events. Similar results were shown by Sanchez et al. (2012) in *Lotus japonicus* species and Alvarez et al. (2008) in Maize plants, where most changes observed were in the levels of amino acids, organic acids, sugars and polyols.

The variance in metabolic response of the cultivars was genotype specific and in some cases also associated with soil moisture content. For instance Ruta which depleted the soil profile faster than the other cultivars during the grain-filling stage and among the top three cultivars at tillering stage significantly accumulated stress related metabolites. The differences in soil water and metabolic status at sampling may be due to the cultivars different physiological response to water stress as reported in Bowne et al. (2012). It should be mentioned that analogous studies pose a methodological challenge in respect to the timing of sampling. Here all genotypes were sampled the same day, generally having a similar phenology. Having said that, sampling according to the volumetric soil water content can be an alternative strategy, nevertheless different genotypes might reach a similar degree of soil water content on different days due to the differential regulation of stomata and stress response. By a similar approach one would likely sample plants at different developmental stages and under different environmental conditions characterizing the subsequent days; a challenging situation for data analysis that would be very likely reflected in the metabolic analysis.

The tillering stage seems to be more sensitive to drought events, displaying pronounced metabolic alterations also during recovery, e.g. in the cultivar Galil, raffinose accumulated 58-fold in treated plants during recovery compared with control plants. This was also reflected by the higher percentage of metabolites that significantly changed in four of the six cultivars compared to recovery at the grain-filling stage. These results are similar to the finding of Zhao et al. (2015) in their evaluation of metabolic and physiology of rice introgression line under the field conditions.

A common response to drought was the accumulation of amino acids such as proline (in Ruta and Zahir at tillering), and in all cultivars with the exception of Yuval at grain-filling stage (Figs. 4, 5). Proline has been identified to increase under drought stress in numerous plant species (Sanchez et al. 2012; Hong-Bo et al. 2006) likely providing an osmoprotective function (Chen and Murata 2011). Another amino acid found to increase significantly in the present study is valine. Valine accumulation was reported also in barley plants exposed to drought (Malatrasi et al. 2006) and may serve as protectant against denaturation of several enzymes in response to water stress (Vierstra 1996). Another possible role of valine may be that of an alternative electron donor for the mitochondrial electron transport chain (Obata and Fernie 2012). The accumulation of γ-aminobutyric acid (GABA) during the tillering stage was cultivar specific. GABA mediates nitrogen and carbohydrate metabolism, is an osmoregulator and possibly acts as a signaling molecule (Bouche and Fromm 2004; Fait et al. 2008). GABA accumulated in Galil, which also exhibited the highest accumulation rate of raffinose during recovery, suggesting a higher sensitivity to drought events when compared to other cultivars. A general increase of phenylalanine at the grain-filling stage in all cultivars with the exception of Yuval may suggest the induction of the phenylpropanoid pathway (Singh et al. 2009). Relatedly, phenylalanine-precursor shikimate accumulated in Zahir in response to water stress at the tilling stage and in Gedera at the grain-filling stage.

The observed accumulation of sugars is a common response to drought as reported by Kameli and Losel (1996), which may provide an initial defensive state against further water loss (Obata and Fernie 2012) acting as osmoprotectants, stabilizing macromolecules and providing immediate energy source to plants following stress relief (Yancey 2005). Soluble sugars can also act as signaling molecules (Chaves and Oliveira 2004) and interact with hormones as part of the sugar sensing and signaling network in plants (Rolland et al. 2006). The detected decreased levels of tricarboxylic acid (TCA) cycle intermediates can be a result of a lower TCA cycle activity rate due to possible drought induced ROS accumulation and thus a down regulation of the key enzyme of the cycle (Fait et al. 2008). Relatedly, ascorbate, a major antioxidant (Smirnoff and Pallanca 1996), was significantly increased under stress, in a developmental and cultivar-dependent manner.

The shift in metabolism during recovery was characterized by a decrease of most amino acids and sugars. However, the change in Raffinose Family Oligosaccharides such as raffinose and galactinol and other sugars and polyols during recovery at tilling stage, likely reflects an acclimation process to environmental stress and possibly acquired drought tolerance as shown in various plant species (Xu et al. 2008). From the metabolic perspective, the results presented here suggest that following recovery from water stress, wheat cultivars display a cultivar distinct rate of recovery. The concurrent increase of organic acid content in Yuval, Ruta and Zahir may be an indication of growth metabolism resumption from stress and a recycling of stress metabolites and this might have been influenced by the high temperature conditions in the greenhouse (Du et al. 2011; Aidoo et al. 2016). At the tillering stage leaf metabolism was less prone to return to its control metabolic homeostasis compared to grain-filling stage, indicating the enhanced responsiveness of the younger plants to environmental stress and the increased tendency to acclimation. This conclusion is in agreement with the report by Pecio and Wach, (2015) showing that spring barley was more tolerant to the drought stress at tillering stage compared to grain-filling stage.

When comparing the diversity in the stress response it is worth highlighting two contrasting cultivars, Gedera with a relatively mild metabolic alteration but tight regulation of transpiration (even during recovery) and Zahir, which displayed extensive changes in central metabolism and a lessened regulation of transpiration. Would then a preferred strategy for improved crop tolerance to transient drought events aim to maintain transpiration and growth coupled with the biosynthesis and accumulation of protective metabolites and a relative fast recovery from stress? Whilst tempting, a clear-cut answer cannot be given as it might very likely depend on the length of the drought event. Having that said, it can be concluded that Zahir exhibited improved tolerance to water stress, based on a combined deregulation of stomatal closure and accumulation of stress-related metabolites at both developmental stages tested, which likely contributed to a stable high yielding of Zahir irrespective of season, water and nitrogen treatments as reported by the findings of Bonfil (2016).

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest related to this manuscript.

Ethical approval All authors declare that our study does not involve the use of animals and humans in research.

Informed consent Informed written consent was obtained from all individuals for being included in this study.

References

Aidoo, M. K., Bdolach, E., Fait, A., Lazarovitch, N., & Rachmilevitch, S. (2016). Tolerance to high soil temperature in foxtail millet

(*Setaria italica* L.) is related to shoot and root growth and metabolism. *Plant Physiology and Biochemistry, 106*, 73–81.

Aidoo, M. K., Sherman, T., Lazarovitch, N., Fait, A., & Rachmilevitch, S. (2017). A bell pepper cultivar tolerant to chilling enhanced nitrogen allocation and stress-related metabolite accumulation in the roots in response to low root zone temperature. *Physiologia Plantarum*. doi:10.1111/ppl.12584.

Alvarez, S., Marsh, E. L., Schroeder, S. G., & Schachtman, D. P. (2008). Metabolomic and proteomic changes in the xylem sap of maize under drought. *The Plant Cell, 31*, 325–340.

Bonfil, D. J. (2016). Wheat phenomics in the field by RapidScan: NDVI vs. NDRE. *Israel Journal of Plant Sciences* 1–14.

Bouche, N., & Fromm, H. (2004) GABA in plants: Just a metabolite? *Trends in Plant Science, 9*, 110–115.

Bowne, J. B., Erwin, T. A., Juttner, J., Schnurbusch, T., Langridge, P., Bacic, A., & Roessner, U. (2012). Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at the metabolite level. *Molecular Plant, 5*, 418–429.

Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Annals of Botany, 103*, 551–560.

Chaves, M. M., & Oliveira, M. M. (2004) Mechanisms underlying plant resilience to water deficits: Prospects for water-saving agriculture. *Journal of Experimental Botany, 55*, 2365–2384.

Chen, T. H. H., & Murata, N. (2011). Glycine betaine protects plants against abiotic stress: Mechanisms and biotechnological applications. *Plant Cell Environment, 34*, 1–20.

Claeys, H., & Inze, D. (2013). The agony of choice: How plants balance growth and survival under water-limiting conditions. *Plant Physiology, 162*, 1768–1779.

Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biology, 11*, 163.

Du, H., Wanga, Z., Yub, W., Liub, Y., & Huang, B. (2011).) to heat stress. *Physiologia Plantarum, 141*, 251–264.

Fait, A., Fromm, H., Walter, D., Galili, G., & Fernie, A. R. (2008). Highway or byway: The metabolic role of the GABA shunt in plants. *Trends in Plant Science, 13*, 14–19.

FAOSTAT. (2011) The FAO (Food and Agriculture Organization of the United Nations) Statistical Database. Retrieved September 20, 2013, from, http://faostat.fao.org.

Fernie, A. R., & Schauer, N. (2009). Metabolomics-assisted breeding: A viable option for crop improvement? *Trends in Genetics, 25*, 39–48.

Hochberg, U., Degu, A., Fait, A., & Rachmilevitch, S. (2013a). Near isohydric grapevine cultivar displays higher photosynthetic efficiency and photorespiration rates under drought stress as compared with near anisohydric grapevine cultivar. *Physiologia Plantarum, 147*, 443–452.

Hochberg, U., Degu, A., Toubiana, D., Gendler, T., Nikoloski, Z., Rachmilevitch, S., & Aaron, F. (2013b). Metabolite profiling and network analysis reveal coordinated changes in grapevine water stress response. *BMC Plant Biology, 13*, 184.

Hong-Bo, S., Xiao-Yan, C., Li-Ye, C., Xi-Ning, Z., Gang, W., Yong-Bing, Y., Chang-Xing, Z., & Zan-Min, H. (2006).) Investigation on the relationship of proline with wheat anti-drought under soil water deficits. *Colloids and surfaces. B, Biointerfaces, 1*, 113–119.

Iglesias, A., Mougou, R., Moneo, M., & Quiroga, S. (2011). Towards adaptation of agriculture to climate change in the Mediterranean. *Regional Environmental Change, 11*, 159–166.

Izanloo, A., Condon, A. G., Langridge, P., Tester, M., & Schnurbusch, T. (2008). Different mechanisms of adaptation to cyclic water stress in two South Australian bread wheat cultivars. *Journal of Experimental Botany, 59*, 3327–3346.

Jones, H. (2004) What is water use efficiency?. In: M. A. Bacon (Ed.), Water use efficiency in plant biology. Oxford: Blackwell 27–41.

Kameli, A., & Losel, D. M. (1996) Growth and sugar accumulation in durum wheat plants under water stress. New Phytologist 132.

Krugman, T., Peleg, Z., Quansah, L., Chagué, V., Korol, A. B., Nevo, E., Saranga, Y., Fait, A., Chalhoub, B., & Fahima, T. (2011). Drought adaptation mechanisms in roots of wild emmer wheat. *Functional and Integrative Genomics, 11*, 565–583.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., & Fernie, A. R. (2006). Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols, 1*, 387–396.

Malatrasi, M., Corradi, M., Svensson, J. T., Close, T. J., Gulli, M., & Marmiroli, N. (2006). A branched-chain amino acid aminotransferase gene isolated from Hordeum vulgare is differentially regulated by drought stress. *Theoretical and Applied Genetics, 113*, 965–976.

Obata, T., & Fernie, A. R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. *Cell, Molecular, Life Science, 69*, 3225–3243.

Pecio, A., & Wach, D. (2015). Grain yield and yield components of spring barley genotypes as the indicators of their tolerance to temporal drought stress. *Polish Journal of Agronomy, 21*, 19–27.

Rajaram, S. (2001). Prospects and promise of wheat breeding in the 21 first centuries. *Euphytica, 119*, 3–15.

Rolland, F., Baena-Gonzalez, E., & Sheen, J. (2006) Sugar sensing and signaling in plants: Conserved and novel mechanisms. *Annual Review of Plant Biology, 57*, 675–709.

Sade, N., Vinocur, B. J., Diber, A., Shatil, A., Ronen, G., Nissan, H., Wallach, R., Karchi, H., & Moshelion, M. (2009). Improving plant stress tolerance and yield production: Is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion? *New Phytologist, 181*, 651–661.

Saeed, A.I., Hagabati, N.K., Braisted, J.C., Liang, W., Sharov, V., Howe, E.A., Li, J.W., Thiagarajan, M., White, J.A., & Quackenbush, J. (2006). TM4 microarray software suite. In A. Kimmel & B.Oliver (Eds.), *DNA Microarrays, Part B: Databases and Statistics* (Vol. 411, pp. 134–193). San Diego: Elsevier Academic Press.

Sanchez, D. H., Schwabe, F., Erban, A., Udvardi, M. K., & Kopka, J. (2012). Comparative metabolomics of drought acclimation in model and forage legumes. *Plant Cell and Environment, 35*, 136–149.

Singh, K., Kumar, S., Rani, A., Gulati, A., & Ahuja, P. S. (2009). Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. *Functional and Integrative Genomics, 9*, 125–134.

Smirnoff, N., & Pallanca, J. E. (1996). Ascorbate metabolism in relation to oxidative stress. *Biochemical Society Transactions, 24*, 472–478.

Vierstra, R. D. (1996). Proteolysis in plants: Mechanisms and functions. *Plant Molecular Biology, 32*, 275–302.

Wallach, R., Da-Costa, N., Raviv, M., & Moshelion, M. (2010). Development of synchronized, autonomous, and self-regulated oscillations in transpiration rate of a whole tomato plant under water stress. *Journal of Experimental Botany, 61*, 3439–3449.

Xu, P., Chen, F., Mannas, J. P., Feldman, T., Sumner, L. W., & Roossinck, M. J. (2008). Virus infection improves drought tolerance. *New Phytologist, 180*, 911–921.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology, 208*, 2819–2830.

Zhao, X., Zhang, G., Wang, Y., Zhang, F., Wang, W., Zhang, W., Fu, B., Xu, J., & Li, Z. (2015). Metabolic profiling and physiological analysis of a novel rice introgression line with broad leaf size. *PLoS ONE, 10*, e0145646.