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Pivotal metabolic pathways related to water deficit tolerance and growth recovery of whole maize plant

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Abstract

Tolerance to water deficit as well as growth recovery from water deficit by re-watering are two important and inseparable processes for maize survival. However, details of metabolic mechanisms are unknown and still need to be filled. Leaf water potential and photosynthetic parameters of three-leaf-stage seedlings of maize inbred line Huangzao 4 under progressive water deficit and re-watering were measured, and metabolites of the seedlings under the corresponding treatments were identified and subjected to analyses of hierarchical cluster, principal component and self-organizing mapping. A total of 142 polar metabolites, 127 in leaves and 125 in roots, were identified, including 13 amino acids, 17 organic acids, 8 sugars, 2 sugar alcohols, and 4 others, of which 85 in leaves and 53 in roots showed a significant change in metabolic level under water deficit. Fifty-nine metabolites had a significant change in metabolic level under re-watering, the majority of which were organic acids and anmino acids. In conclusion, there is a significant crosstalk of metabolisms between water deficit tolerance and growth recovery under re-watering, with glycolysis, starch and protein degradation as pivotal pathways; metabolic level changes in the roots are to maximize water uptake whereas the changes in the leaves are to prevent water loss and to increase water-use efficiency; water deficit tolerance depends on metabolic level-controlled balance between the networks; and maize uses an emergency repair mechanism for growth recovery. The results give informative clues to address the mechanisms of maize water deficit tolerance and growth recovery at the metabolic level.

Keywords: Maize, Metabolites, Metabolic profiling, Water deficit, Re-watering.
Abbreviations: GC-MS_gas chromatograph–mass spectrometry; HC_hierarchical cluster; HZ4_Huangzao 4; MPa_megapascals; PC_principal component; PEG_polyethylene glycol; RI_retention index; RW_re-watering; SOM_self-organizing mapping; WD_ water deficit; WL_WD-treated leaves; WP_water potential; WR_WD-treated roots.

Introduction

Plants are frequently subjected to different degrees of environmental stresses. Drought is indisputably the most important type of the abiotic stress that leads to larger crop productivity losses than any other environmental factor (Mittler, 2006; Wilson et al., 2009). Maize (*Zea mays* L.) is one of the world's most important food crops but very susceptible to drought stress (Heinigre, 2000). The three-leaf stage is important to maize because it is a transition for the cells from autotrophy to heterotrophy (Greaves et al., 1996). Once drought occurs at this stage, it has a direct effect on growth and development of subsequent reproductive stage of maize. However, metabolic mechanisms of drought tolerance at this growth stage failed to arouse much attention. In the field, soil drought and rainfall often occur alternately, implying that drought tolerance and growth recovery ability from the drought are equally important for maize survival. Additionally, the soil drought often occurs in a progressive manner whereas soil re-watering (RW) after prolonged drought often occurs in a flood irrigation-like or sudden rainfall-like manner. In view of this, the progressive drought stress and growth recovery from drough should be jointly considered in conducting studies on maize metabolomics under drought. Studies on transcriptomics and proteomics have pointed to that drought stress can led to great alteration of metabolisms in maize (Zinselmeier et al., 2002; Yu and Setter, 2003; Bassani et al., 2004; Riccardi et al., 2004; Zheng et al., 2004; Hajheidari et al., 2005; Poroyko et al., 2005; Jia et al., 2006; Roche et al., 2007; Zhuang et al., 2007; Spollen et al., 2008; Hayano-Kanashiro et al., 2009; Li et al., 2009; Cohen et al., 2010; Moumeni et al., 2011; Benešová et al., 2012), but true metabolic changes need further verification because neither transcriptomics nor proteomics can completely and accurately exhibit how plants respond to the stresses (Mendes, 2002; Sumner et al., 2003). Metabolomics is one of powerful tools to address metabolisms (Fiehn et al., 2000a,b; Field and Lake, 2011; Rasmussen et al., 2012). A recent study on maize metabolic responses of greenhouse-grown maize hybrids to experimentally controlled drought stress has provided a paradigm, identifying some drought stress-responsive metabolites of leaves, ear, husk, sheath and silks (Witt et al., 2012). However, the existing built-up knowledge of metabolisms falls far short of requirements for addressing drought tolerance of the whole maize plant because the different species of living organisms has their own unique metabolic level matching with their biological activities upon specific conditions (Glazier, 2008; Glazier, 2010; Debnath et al., 2011). .Polyethylene glycol (PEG; $M_r \geq 6000$) consists of inert, non-ionic and virtually impermeable chains (Hohl and Peter, 1991; Lu and Neumann, 1998). PEG solution can maintain a

uniform water potential (WP) throughout the experimental period and is always used to mimic soil drying (Van den Berg and Zeng, 2006). More important is that water deficit (WD) is controlled more easily and exactly by quantifying PEG concentrations than by soil drying approach. To provide an outline of responsive metabolic network and to find the key metabolic pathways, we comparatively analyzed the metabolites of leaves and roots of a maize inbred line Huangzao 4 (HZ4) at the three-leaf stage under progressive WD imposed by PEG and during growth recovery. HZ4 is the foundation genotype germplasm in maize breeding in China (Zeng et al., 1996).

Results

Phenotyping HZ4 grown under progressive WD and during RW

In this study, WD treatment of seedlings of maize HZ4 was conducted in a progressive manner in 0.5× Hoagland's nutrient solution (Hoagland and Arnon, 1938) supplemented progressively with PEG. Consequently, the WP of maize leaves was almost constant under control condition (non-stressed, i.e. with the nutrient solution without PEG), but dramatically decreased as WD progressed. However, the leaf WP of the stressed seedlings returned to control level 72 h after RW treatment (Fig 1). Photosynthesis of WD-stressed seedlings was significantly affected when compared to that of control seedlings (Fig 2), consisting with the acknowledged conclusion that drought can inhibit plant photosynthesis (Chaves et al., 2009; Pinheiro and Chaves, 2011). After RW treatment, all parameters tested tended to, but not fully reached, the control levels. These results indicated that the addition of PEG effectively made a notable impact of WD on maize seedlings.

Polar metabolites

A total of 142 polar metabolites were identified, including 127 in leaves and 125 in roots, of which 110 were regulated in tissues of both roots and leaves. Of the metabolites detected, 44 were known metabolites, including 13 amino acids, 17 organic acids, 8 sugars, 2 sugar alcohols, and 4 other metabolites (Table 1). The number and metabolic categories of the metabolites identified in this study were similar to results from maize hybrids and parental inbred lines naturally grown in the nursery (Lisec et al., 2011), maize hybrids grown under experimental controlled drought stress (Witt et al., 2012) and droughted wheat (Bowne et al., 2012). The gas chromatograph (GC)–mass spectrometry (MS) traces of part of the metabolites were shown in Suppl. Fig 1. The original response ratios of 142 metabolites detected by MS were supplied in Suppl. Table 1.

WD-responsive metabolites in leaves

Of 127 metabolites identified in the leaves, 85 (66.9%) showed significant change in metabolic level under WD (Fig 3A; Suppl. Table 2). The number of regulated metabolites increased as WD progressed, correlating with the continuous decline in leaf WP (Fig 1) and also consisting with change in the number of WD-regulated genes in the leaves of this maize line at the three-leaf stage with the time course of WD (Li et al., 2009). There were 99 metabolite with an notably enhanced metabolic level and 44 metabolites with a significantly decreased metabolic level, which showed metabolic changes at least at two or more time points of WD treatment, such as homocysteine, succinic acid, trehalose, P1165.9, P1157.8, P1190.6, P1471.2, P1779.8, P1877.1, P2154.2, P2179.8, and P3081.4 (Fig 3A; Suppl. Table 2).

WD-responsive metabolites in roots

Fifty-three (42.4%) out of 125 metabolites identified in the roots showed significant change in metabolic levels under WD (Fig 3B; Suppl. Table 3). Unlike in the leaves (Fig 3A; Suppl. Table 2), the number of the affected metabolites in the roots was relatively constant and did not undulate greatly with the time course of WD treatment (Fig 3B) such as maltose, P1222.0, P2821.0, P2863.4, and P3421.2, which all remained a relatively constant metabolic level throughout WD treatment. The number of the metabolites with a significant increase in metabolic level was much greater than that of the metabolites with a significant decrease in metabolic level regardless of the type of the tissues. Overall, change in the number of the regulated metabolites was also similar to change in the number of WD-regulated genes in the roots of this maize line at the three-leaf stage with the time course of WD (Li et al., 2009).

RW-responsive metabolites

In nature, plants grown in the soil often undergo a process of growth recovery under RW following the withdrawal of WD. To identify the RW-responsive metabolites, the seedlings were subjected to the 24-h WD treatment at -0.5 WP followed by a treatment of 72-h RW (Fig 2). Such seedlings after RW treatment were then analyzed by GC-MS and compared to parallel-treated control seedlings. As a result, a total of 59 metabolites were found to be RW-responsive (Fig 3C; Suppl. Table 4). Of these responsive metabolites, 43 were found in the leaves, of which 27 (62.7%) and 16 (27.3%) showed significant increase and decrease in metabolic level, respectively. Among metabolites with an increased metabolic level, metabolic levels of 14 increased 5 to 318.4 times when compared with counterparts in the control seedlings (Suppl. Table 4). There were 20 RW-responsive metabolites in the roots, of which 7 (35%) and 13 (65%) showed significant increase and decrease in metabolic level, respectively. In these metabolites, 2 had 5.99-fold and 12.32-fold increase in metabolic level, respectively (Suppl. Table 4). Sorbitol, isoleucine, threonine and P1559.5 presented significant change in metabolic level in both leaves and roots (Suppl. Table 4). Change of the metabolites in metabolic level under WD or RW could be classed into three dynamic patterns with the time course from WD to RW (Suppl. Tables 2, 3 and 4): pattern 1 was characterized by continued increase or decrease, pattern 2 by increase or decrease only at one or more time-point of the treatments, and pattern 3 by change in increase at one time point but in decrease at another time point.

Metabolites responsive specifically to either WD or RW, and commonly to both WD and RW

The gene expression analysis has indicated that numerous genes are of RW-specific response (Li et al., 2009). As shown in Fig 3D, there were 22 metabolites of WD-specific response in the leaves, and 8 metabolites of WD-specific response in the roots. Undoubtedly, metabolites commonly responsive to both WD and RW or specific to RW should be important for maize to tolerate WD and recover from WD under RW. It was found that a total of 19 metabolites in leaves and roots were responsive to both WD and RW, including 9 known metabolites that were asparagine, threonine, glycine, isoleucine, proline, adenosine, fructose, spermine, and valine (Suppl. Tables 5, 6 and 7).

Table 1. Known metabolites identified by GC-MS in hydrophilic extracts of leaves and roots of maize seedlings at the three-leaf stage.

Amino acids	Sugars	Sugar alcohols	Organic acids	Other
Alanine	Fructose	myo-inositol	Benzoic acid	Adenosine
Asparagine	Fructose-6-phosphate	Sorbitol	Cinnamic acid	Altrose
Aspartic acid	Galactose		Citric acid	Phosphate
Glutamine	Glucose		Galactonic acid	Spermine
Glycine	Glucose-6-phosphate		Galacturonic acid	
Homocysteine	Maltose		Glucaric acid	
Isoleucine	Sucrose		Gluconic acid	
Proline	Trehalose		Glyceric acid	
Serine			Glycolic acid	
Threonine			Hexadecanoic acid	
Tryptamine			α-Ketoglutarate	
Tyrosine			Lactic acid	
Valine			Octdecenoic acid	
			Succinic acid	
			Malic acid	
			Pentonic acid	
			Tetradecanoic acid	

GC-MS, gas chromatograph–mass spectrometry.

Experimental reproducibility under WD

Both hierarchical cluster (HC) and principal component (PC) analyses are bioinformatic tools used to evaluate the reproducibility of large data sets. Three groups of the data from each treatment time point either in leaves or in roots could be clearly clustered by HC analysis (Suppl. Fig 2A), indicating that the experimental reproducibility was very well. On the other hand, HC analysis indicated that regardless of treatment conditions (stress or control) and the kind of maize tissues, the metabolism also obviously varied with time course of the growth. Analysis also presented three ranks of PCs (Suppl. Fig 2B): PC1, PC2, and PC3. PC1 clearly depicted a panoramic view of change of the whole maize plant in metabolism either under WD or under the control condition, indicating the greatest total variance of 48.2% and therefore suggesting that the whole maize plant had the greatest difference in metabolome upon the treatment. PC2 indicated that the metabolism of a specific tissue (leaves or roots) also varied greatly with the treatment. PC3 indicated a significant difference between leaves and roots in metabolic level under a specific condition of WD or control.

Metabolic loading capacity of the metabolites under WD

To evaluate the contribution of individual metabolites to maize tolerance to WD, metabolic loading capacity of the individual metabolites for each PC group was further analyzed through PC approach (Suppl. Fig 2B), indicating significant differences in metabolic loading capacity between metabolites (Suppl. Fig 3). For PC1, 56 (39%) of total metabolites showed a loading capacity score of ≥ 0.5 (Suppl. Fig 3A), including fructose-6-phosphate, glyceric acid, malicacid, *cis*-coniticacid, altrose, *myo*-inositol, sucrose, fructose, pentonic acid, tyrosine, trehalose, tetradecanoic acid, lactic acid, gluconic acid, and glucose. For PC2, there were 17 (12%) metabolites that showed a loading capacity score of more than 0.5 (Suppl. Fig 3B), including α-ketoglutarate, succinic acid, adenosine, tryptamine, and phosphoric acid. For PC3, 12 (8.4%) metabolites were of a loading capacity score of 0.5 (Suppl. Fig 3C), including spermine, glycine, glutamine, citric acid, and octdecenoic acid. The loading capacity score of each metabolite was listed in Suppl. Table 8.

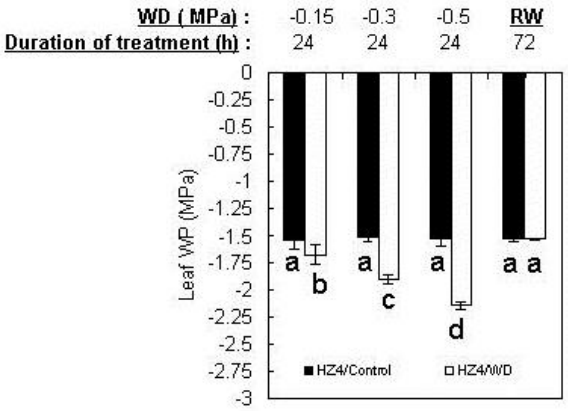

Fig 1. Changes of leaves of maize seedlings in WP under WD and RW conditions. WP datum at each time point is the mean of data from the second leaves of 4 to 6 individual seedlings at the three-leaf stage. WP values shown on the horizontal axis indicate water potentials of Hoagland's nutrient solution. RW was conducted for 72 h on seedlings treated for 24 h at -0.5 MPa. The error bars indicate the standard errors of the means. HZ4, Huangzao 4; MPa: megapascals. RW, re-watering; WD, water deficit; WP, water potential. The different lower case letters above columns inside the same Figure represent the statistically significant difference between data by multiple statistical analyses at a level of $P < 0.05$. The sample number (n) for each datum was from 4 to 6.

Self-organizing mapping (SOM) of regulated metabolites

Metabolites with the same metabolic profiling patterns are likely to be functionally related to each other (Kim et al., 2007). SOM is an unsupervised neural network algorithm, which can cluster high-dimensional complex data into visualization (Abe and Ikemura, 2007). SOM analysis indicated that a total of 42 known metabolites could be categorized into 6 expression clusters (Fig 4). Obviously, clusters 1, 2, 4, 5 and 6 included metabolites resulted from different metabolic pathways, suggesting that the interplay between the involved pathways exists. Maltose was categorized into a separate cluster 3,

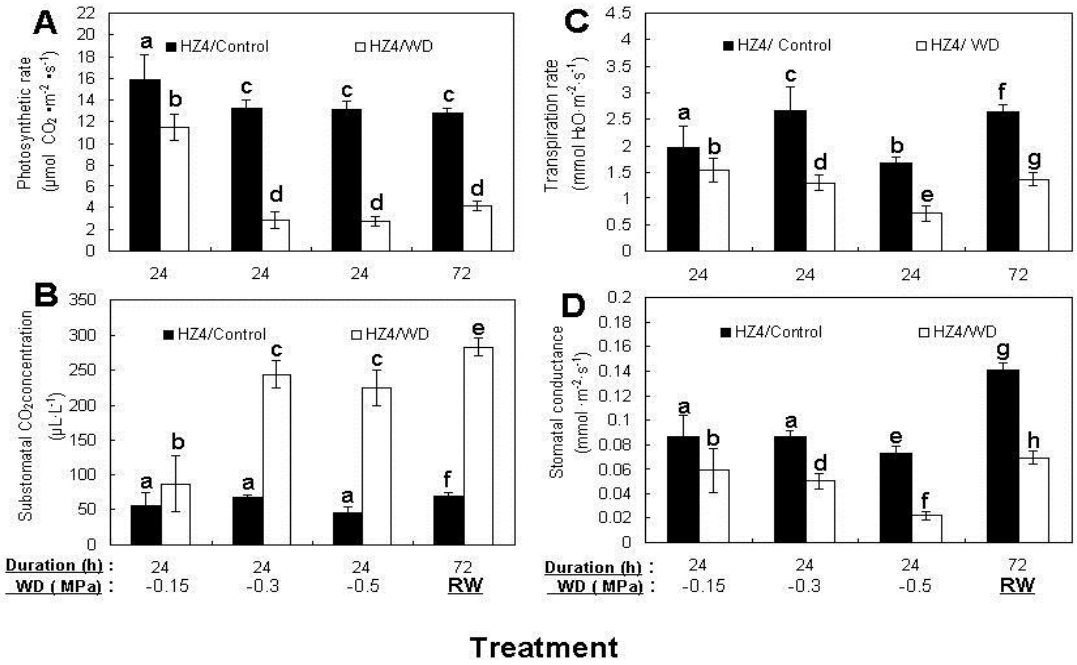

Fig 2. Changes of photosynthetic parameters in leaves of maize seedlings under WD and RW. RW conditions. A: Photosynthetic rate. B: Substomatal CO_2 concentration. C: Transpiration rate. D: Stomatal conductance. The datum at each point in time is the mean of data from the second leaves of 4 to 6 individual seedlings at the three-leaf stage. WP values shown on the horizontal axes indicate WPs of Hoagland's nutrient solution. RW was conducted for 72 h on the seedlings treated for 24 h at -0.5 MPa. The error bars indicate the standard errors of the means. HZ4, Huangzao 4; MPa, megapascals; RW, re-watering; WD, water deficit; WP, water potential. The different lower case letters above columns inside the same Figure represent the statistically significant difference between data by multiple statistical analyses at a level of $P < 0.05$. The sample number (n) for each datum was from 4 to 6.

suggesting that its metabolic actions are relatively independent of other metabolites in maize response to WD.

Discussion

GC-MS technology-based identification of the metabolites

GC-MS technology is a very rapid and robust technique for high throughput and comprehensive analysis of soluble and polar metabolic metabolites/compounds of low-to-moderate molecular weight (Roessner et al., 2000; Fiehn et al., 2000a), resulting in fairly comprehensive coverage of the central pathways of primary metabolism (Obata and Fernie, 2012) but the number and type of the polar metabolites identified by this technique are limited because of problematic derivatisation (Callahan et al., 2009) and/or when concentrations of metabolites are at lower concentrations (Rojo et al., 2012). In this study, the polar metabolites identified were in agreement with those identified droughted maize hybrids (Witt et al., 2012) and droughted wheat (Bowne et al., 2012).

Responses of maize to both WD and RW are associated with fluctuation of carbohydrate in metabolic level

Changes of metabolites of WD-stressed maize seedlings in metabolic level were associated at least with WD effects on photosynthesis because photosynthesis changes under WD could lead to fluctuation of carbohydrate in metabolic level (Pinheiro and Chaves, 2011). The fluctuation of carbohydrate can act as an important WD signal which then triggers a cascade of metabolic changes through some secondary signals such as reactive oxygen species (Pinheiro and Chaves, 2011). By the same token, changes in metabolism of maize growth recovery under RW following WD treatment should be also explained partly by changes in photosynthesis (Fig 1).

Tolerance of maize to WD depends on balance and harmony of metabolome of whole plant instead of on changes of individual tissues in metabolites

PC analysis showed variance of whole maize plant in metabolic level was much higher than that occurring in any single tissues (leaves or roots) either under WD or under control conditions (Suppl. Fig 3B). This indicated that maize WD tolerance must be comprehended at the level of whole maize plant rather than individual tissues. Changes of the same metabolites in metabolic levels varied not only with tissues but also with time course of the treatment (Suppl. Tables 2, 3 and 4). WD-responsive metabolites with different metabolic levels resulted from different pathways (Tables S2 and S3), suggesting an interplay and the necessity of balance among metabolic the pathways for maize WD tolerance. Therefore, the concept of tolerance of whole maize plant to WD at the metabolic level may be proposed with three aspects: (1) balance of metabolic levels among different metabolites in the same tissues and/or among different tissues with the time course of WD stress; (2) harmony among different metabolic pathways in the same tissues and/or in different tissues; and (3) coordination of metabolome through controlled levels of metabolites among tissues of maize.

Tolerance of maize to WD is a dynamic cycle from WD sense, WD adaptation to WD tolerance at the metabolic level

In our study, the number of the significantly affected metabolites at the first stress time point was significantly less

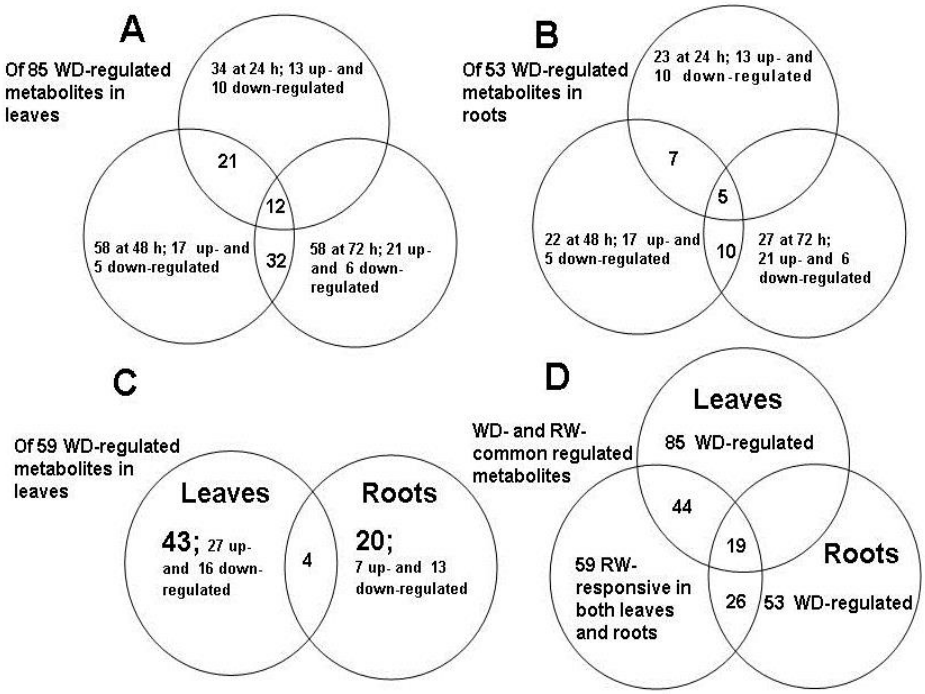

Fig 3. Regulated metabolites in maize seedlings under WD and RW conditions at the three-leaf stage. A: Metabolites in leaves under WD. B: Metabolites in roots under WD. C: Metabolites regulated in both leaves and roots under WD. D: Metabolites regulated in both leaves and roots under both WD and RW. RW, re-watering; WD, water deficit.

than those at the last two time points (Fig 3). Some metabolites showed a significant alteration in metabolic level at several time points likely because of production of transient signals (Obata and Fernie, 2012), but the others changed their metabolic level at one time point (Suppl. Tables 2 and 3). This strongly indicates that the metabolic mechanism for maize WD tolerance should follow a distinct process from WD sense at the early stage of WD, WD adaptation via adjustment or reprogramming of the metabolic systems at the middle stage of WD then to WD tolerance by establishment of entire WD tolerance-related metabolic networks. There have been indications that metabolic pathways in plants do sequentially act in a differential time period (Obata and Fernie, 2012). Additionally, tissues (leaves or roots)-specific metabolites under WD maybe constitute the basis of the tissue-specific WD tolerance metabolic mechanism.

Glycolytic pathway is a key pathway conferring maize WD tolerance by production of building blocks and production of ATP energy

The type of known metabolites included amino acids, sugars, sugar alcohols, and organic acids (Table 1). It has been acknowledged that sugars are the second messengers inside the cells of droughted plants, which do interact with drought tolerance-related plant hormones such as ABA for long-distance signaling from roots to shoots (Obata and Fernie, 2012). Sugars are as biosynthetic precursors for almost all other organic compounds (Valluru and Van den Ende, 2008) and usually play central roles in structure and metabolism of plants at the cellular and whole-organism levels (Couée et al., 2006). Levels of fructose-6-phosphate and glucose-6-phosphate positioning on the glycolytic pathway were decreased levels only in the stressed maize roots (Suppl. Table 4) at the later stress time point(s), accompanied by significant increases in metabolic levels of drought tolerance-related metabolites such as *myo*-inositol, sorbitol, trehalose and galactose at the later stress time point (s) (Suppl. Tables 2, 3 and 4). This indirectly supports that the glucose is as an initial substance for biosynthesis of these metabolites (Plaxton, 1996; Hare et al., 1998; Dennis et al., 2000; Schwender et al., 2004; Lu et al., 2006; Merchant and Richter, 2011; Krasensky and Jonak, 2012). According to the latest view, the glycolytic pathway aims at production of building blocks such some amino acids as alanine, glycine, isoleucine, serine, tyrosine and valine, and is responsible for production of ATP energy (Bar-Even et al., 2012). Therefore, all above clues of maize WD tolerance were directed to glycolytic pathway (Fig 5).

Glucose: the first drought- or water status-signaling molecule, which would be associated with starch degradation in plastid under WD conditions

Glucose is indeed the first drought- or water status-signaling molecule in the stressed plants (Chaves et al., 2009). Generally, glucose and fructose are produced through the degradation of sucrose (Koch, 2004). Sucrose could be largely produced through starch degradation in plastid under stress conditions (Geigenberger et al., 1997; Sparla et al., 2006; Harb et al., 2010). These two sugars showed an increased metabolic level in leaves of the WD-stressed maize seedlings, however, glucose showed an earlier changes in metabolic level than fructose (Suppl. Table 2), complying with the order of their metabolism in the glycolytic pathway. Most importantly, interconversions between sucrose, glucose, and fructose are very rapid, with half-lives of interconversions ranging between 0.3 and 0.8 h (Lattanzi er al., 2012). Rapid response of the metabolic pathways is central to maize tolerance to WD because it makes maize timely produce energy and implement re-construct of cell structure when the stress occurs.

Roots and leaves employ distinct metabolic networks for coping with WD

Patterns of the metabolic profiling in both leaves and roots showed obviously dynamic differences, strongly implying that

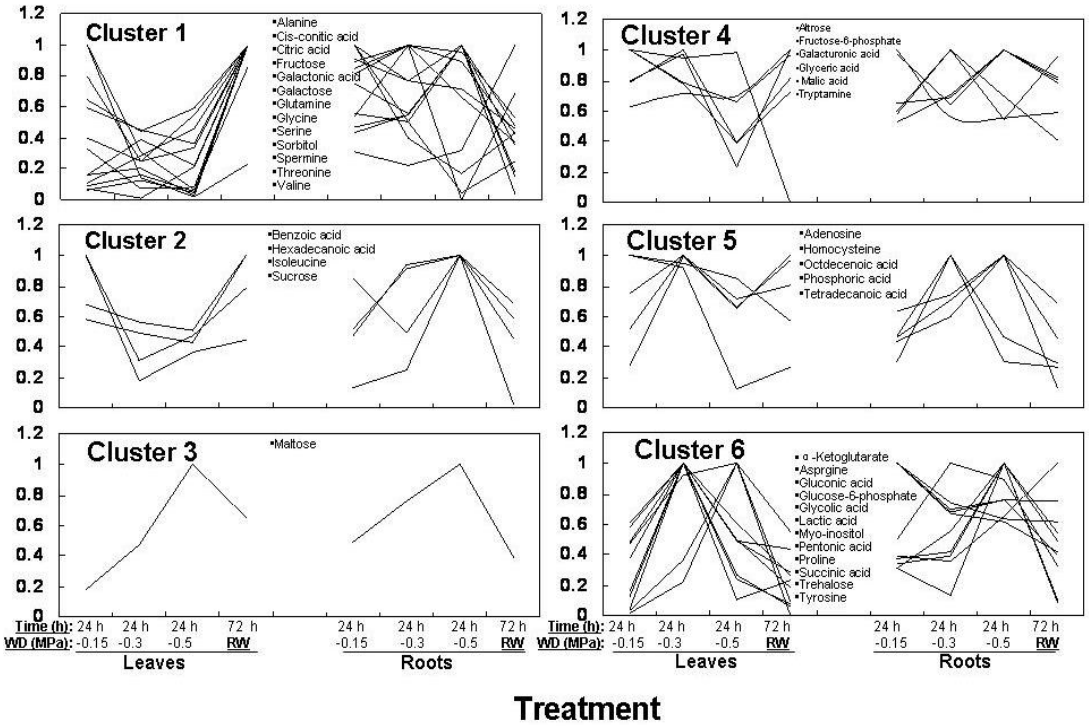

Fig 4. SOM-based metabolic profiling of detected and known regulated metabolites. Duration of treatment and WP in the solution are indicated on the horizontal axes. Relative variance is shown on the vertical axes. SOM analysis was performed using default values. MPa: megapascals; RW, re-watering; SOM, self-organizing mapping; WD, water deficit; WP, water potential.

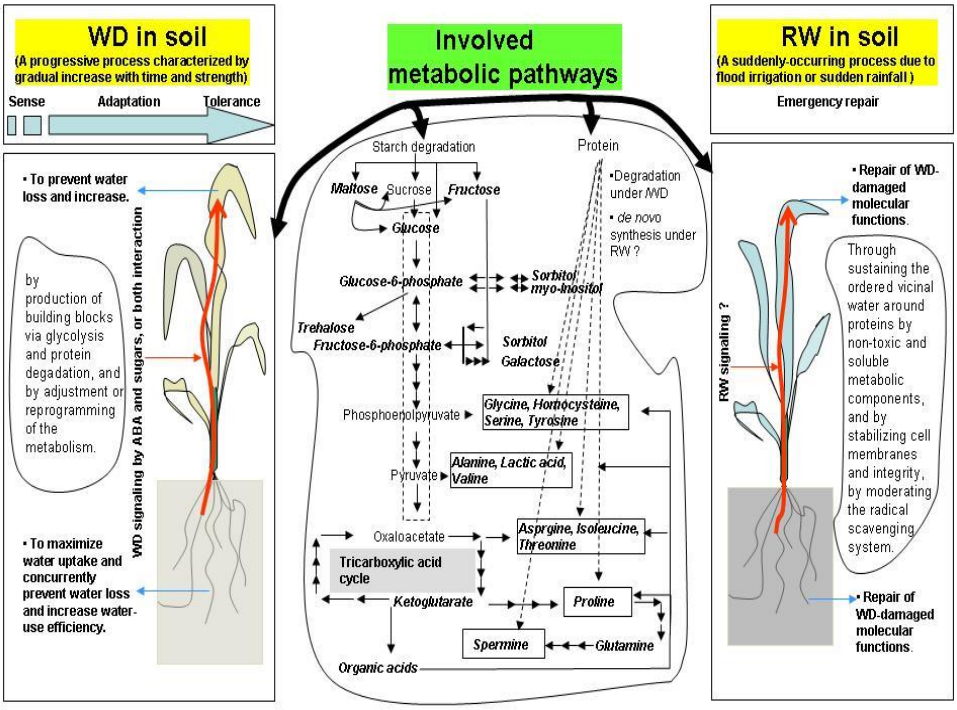

Fig 5. The schematic overview of mechanisms of WD tolerance and growth recovery from WD upon the known metabolites identified in this study. The map was drawn based on the data of this study and also referred to Venekamp et al. (1989), Ingram and Bartels (1996), Plaxton (1996), Hare et al. (1998); Dennis et al. (2000), Schwender et al. (2004), Lu et al. (2006), Sparla et al.(2006), Fettke et al. (2009), Merchant and Richter (2011), Krasensky and Jonak (2012), and Obata and Fernie (2012). The metabolites identified by this study are highlighted with italics. In this functional metabolic network, pivotal pathways are marked with the dotted line rectangular frame for glycolytic pathway or the lines for protein degradation. The detailed discussion was given in the text. RW, re-watering; WD, water deficit.

two tissues employ different metabolic networks for coping with WD. For water use, the water lost (95-99%) from the plants, even grown under well watered conditions, can occur through the pores of stomata (Bowne et al., 2012). Reportedly, leaves and roots of the plants perform very differently under water-limited conditions (Chaves et al., 2003). Leaves are mainly to prevent water loss and increase water-use efficiency via metabolic adjustments (Asch et al., 2009) predominantly by some drought signals produced by dehydrating roots (Benešová et al., 2012) or in part through morphological adaptations (Bowne et al., 2012). Under drought, growth of the leaves is usually inhibited whereas roots may continue to grow to mainly maximize water uptake (Parent et al., 2010). Therefore, in terms of water use, changes of the roots in metabolic level endow maize roots with the active and defensive mechanisms against WD which maximize water uptake, whereas the changes in the leaves enable maize leaves to adopt the passive mechanism against WD which is to prevent water loss and increase water-use efficiency in leaves (Fig 5).

Roles of metabolites in WD tolerance were determined by their metabolic loading capacities not by the absolute metabolic levels

Although the metabolite levels of some metabolites such as WD tolerance-related proline (Yordanov et al., 2003) were highly increased (Suppl. Table 2), they had lower loading capacity scores than those of the others (Suppl. Table 8). These may warn that evaluation of effects of a specific metabolite on WD tolerance should not only focus on its metabolic levels but also pay more attention to the importance of his positioning-on metabolic pathways. In general, the specific biological roles of the metabolic networks depend on their good balance between metabolite channeling, which is greatly affected by amounts of the inputs and outputs of the internal metabolites in the metabolic nods under the specific conditions such as drought stress, namely metabolic balance (Provost and Bastin, 2006). This is could be indirectly evidenced by some researches. For example, moderate accumulation of sorbitol induces dwarfism in Japanese persimmon (Deguchi et al., 2004). In contrast, a minor accumulation of trehalose enhances drought tolerance of some drought-sensitive plants (Karim et al., 2007). Inappropriate metabolic level also causes a chain of metabolic disturbance. For instance, an appropriate increase in exogenous trehalose can improve rice salt tolerance by reducing the Na^+/K^+ ratio but strongly decreased endogenous proline (Nounjan et al., 2012). Another highlight was that some metabolites were obviously of tissue-specific response or tissue-common response under WD and/or RW. Some of the stress treatment-responsive metabolites in both leaves and roots showed diametrically opposite expression pattern in these two tissues (Fig 3; Suppl. Tables 2, 3 and 4). Therefore, individual metabolites in a certain metabolic network should act in a self-stabilizing and change-minimized manner (Stitt et al., 2010).

Amino acids and organic acids are groups of metabolites essential to maize WD tolerance

Amino acids usually serve as precursors of organic acids (Obata and Fernie, 2012). Regardless of the number or the metabolite level, the amino acids were higher than organic acids in both leaves and roots of WD-stressed maize seedlings (Suppl. Tables 2 and 3), agreeing with the findings in salt-stressed *Lotus japonicus* (Sanchez et al., 2008; Obata and Fernie, 2012, and references therein). Increased amino acid levels usually result from stress-induced protein degradation (Ingram and Bartels, 1996; Obata and Fernie, 2012). However, the overall level of metabolism of the organic acids did not increase with increase in amino acid levels (Suppl. Tables 2 and 3) likely because a large amount of the amino acids could run off from plants through exudation from the roots into the rhizosphere under drought (Henry et al., 2007). The metabolism of organic acids has not been well-defined (Thimann and Bonner, 1950), but they have been demonstrated to confer plant tolerance to some special stresses (Mariano et al., 2005) because they are sources for drought-induced proline synthesis (Venekamp et al., 1989). More importantly, they are also intermediates in tricarboxylic acid cycle (Mariano et al., 2005). The latest speculation is that organic acids maybe act as signaling messengers sensing extreme stress because their increase can lead to increase in cytosolic pH (Bowne et al., 2012). In these organic acids, the benzoic acid is a critical regulator in the plant-environment interaction because it is a key moiety for biosynthesis of salicylic acid which is an important signaling molecule (Wildermuth, 2006) and can therefore confer plant drought tolerance (Hayat et al., 2010).

Maltose: a unique metabolite in maize WD tolerance

Maltose has the protective effects on proteins, membranes and the photosynthetic electron transport chain at physiologically relevant concentrations during temperature shock, giving plants additional time to produce their complement of long-term stress related proteins and metabolites (Kaplan and Guy, 2004).

Maltose was of responses to both WD (Suppl. Table 2) and RW (Suppl. Table 4). It presented a unique metabolite profile different from those of other metabolites such as glucose, fructose and sucrose (Fig 4). This is likely that although maltose and glucose are all major products of amylolytic pathway in plants these two sugars are produced by different paths (Fig 5). For example, maltose is produced via plastidial β-amylases and glucose is formed through disproportionating isozyme 1 during hydrolytic degradation of starch (Fettke et al., 2009). However, amylolytic enzymes like β-amylase is catalytically inactive on native starch grains without ample prior digestion (Kaplan and Guy, 2004; and references therein). The maltose-generating hydrolytic (amylolytic) pathway is the primary source of sugars (Kaplan and Guy, 2004; Weise et al., 2011). However, starch degradation in plastid is usually stress-induced (Geigenberger et al., 1997; Sparla et al., 2006; Harb et al., 2010). Taken all together, maltose follows a metabolic route distinctive from other sugars to affect maize WD tolerance.

Metabolism of maize growth recovery from WD stress

In the fields, maize has to cope with changing environments from WD to RW in order to survive. Drought can exert various damages on plants such as membrane integrity (Mahajan and Tuteja, 2005) and peroxidation by enhanced production of reactive oxygen species (Mittler, 2006). Among known metabolites identified in this study, alanine, fructose, glutamine, isoleucine, spermine and threonine appear to be more important to whole-maize growth recovery under RW because they had significantly increased metabolic levels (Suppl. Tables 2, 3 and 4). Additionally, they are also likely key linking-up metabolites between the metabolic networks related to both WD-tolerance and growth recovery under RW. On the other hand, the great majority (41%) of known RW-responsive metabolites were amino acids, at least indicating that maize growth recovery has the large demand for building block metabolites. Some of the WD- and RW-affected metabolites such as amino acids can sustain the ordered vicinal water around proteins by decreasing

protein-solvent interactions at low water activities (Obata and Fernie, 2012, and references therein). Plant growth recovery from the stresses such as drought is associated with a repair process of drought-injured photochemical systems (Miyashita et al., 2005), which could be evidenced by recovery of maize photosynthesis under RW (Fig 2). It can be reasonably speculated that maize growth recovery likely adopts an emergency repair mechanism to prevent against damage to molecular functions under RW by means of production of abiotic-stress tolerance-related metabolites such as spermine (Capell et al., 2004; Gill and Tuteja, 2010). There were 21 out of the identified metabolites whose change in metabolic level could be directly or indirectly coupled with differential expression of the related genes of our previous study on a large-scale gene expression profile of WD-stressed maize (Li et al., 2009). Taken our results with the above-mentioned literature, a preliminary metabolic network to respond to WD and RW can be clearly schemed out (Fig 5). The mode emphasizes the pivotal roles of pathways of glycolysis, starch and protein degradation in maize WD tolerance and growth recovery from the stress (Fig 5). However, numerous unknown metabolites suggested that there are still some unknown metabolic pathways that are responsive to WD and RW and needed to be further developed.

Materials and Methods

Planting and management of maize

Seeds of maize inbred line HZ4 were surface-sterilized for 10 min in 75% ethanol, washed five times (2 min each) in sterile water, and then germinated in sterilized wet sand. After emergence, the seedlings were randomly distributed by randomly swapping their positions every 4 h to eliminate potential effects of differential light intensity in different positions in the chamber. The seedlings with fully expanded euphylla were transferred into vigorously aerated hydroponic tank containing 0.5× Hoagland's nutrient solution (Hoagland and Arnon, 1938) and grown in a greenhouse with a 12 h photoperiod of 120 mol m^{-2} s and under a 60-80% relative humidity at 25-31 ºC.

Treatments of maize seedlings

When growing up to the three-leaf stage, the seedlings with the same size were selected to be exposed to progressive WD by sequential transfer at a 24 hour's interval to the nutrient solutions with 10%, 15%, and 20% PEG 6000, respectively. The WP in the solution was -0.15 megapascals (MPa) at 10% PEG 6000, -0.30 MPa at 10% PEG 6000, and -0.50 MPa at 20% PEG. After 24 h of treatment in the nutrient solutions with 20% PEG, seedlings were grown for 72 h for RW treatment in nutrient solution without PEG. Control seedlings were those which were synchronously treated in solutions without PEG. During the growth of the seedlings, the nutrient solution was renewed every 2 d and vigorously aerated for 15 min every 1 h, and the pH of the solution was adjusted to 7 ± 0.2 every day.

Measurement of physiological parameters

Photosynthetic parameters and WP of the second leaves of maize seedlings were measured. Photosynthetic parameters were analyzed at 10:00 a.m. daily to reduce the impact of circadian variation by using a portable photosynthesis system LI-6400 (Li-Cor, Lincoln, NE, U.S.). The photosynthesis system LI-6400 was operated under the conditions of 28 ºC in the chamber, carbon dioxide concentration of 385 ± 5 μL/L, and photosynthetic photon flux density of 1000 $\mu M/m^2/s$. WP of the leaves was determined by using a WP4 water potentiometer according to the operating instructions given in the manual (Version 2.2, Decagon Devices Inc.).

Extraction of polar metabolites

The second fully-expanded leave and all the roots of each seedling were separately collected at 10:00 a.m. Collected leaves were immediately frozen in liquid nitrogen, but the roots were quickly and fully washed with sterile distilled water before freezing. Maize tissues were ground into powder in liquid nitrogen. Extraction of metabolites was conducted with powdered tissues according to the methods described by Roessner et al. (2000) but with minor modifications. Briefly, a 100-mg aliquot of the powdered tissues was treated for 15 min at 70 ºC in the solution composed of 1400 μL pre-cooling methanol and 50 μL pre-cooling quantitative internal standard solution containing 2 mg ribitol/mL. The resultant mixture was then vigorously oscillated for 5 min in the solution containing equivalent volume of deionized water as well as 750 μL chloroform followed by centrifugation for 10 min at 4000 g at 4 ºC. After centrifugation, the upper methanol-water phase was transferred to a new glass tube and then immediately dried via vacuum at room temperature.

Derivatisation of polar metabolites

For derivatisation, the residues resulting from vacuum dry were re-dissolved at 37 ºC in 90 μL of methoxyamine hydrochloride (20 mg/mL)-pyridine solution. A 30-μL aliquot of the mixture of the standard substances was then added, which was composed of n-heptane solution containing C_{10}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, C_{30}, C_{32}, C_{34}, C_{36}, C_{38}, and C_{40} alkanes (Sigma), 50 mg/L each. Following adding retention time standard mixtures, a 90-μL aliquot of N-methyl-N-[trimethylsilyl] trifluoroacetamide was added. The resulting mixture was fully mixed by gently shaking and then allowed to react for 30 min at 37 ºC, resulting in the reaction solution. A 1-μL aliquot of the reaction solution was injected into the gas chromatograph (GC) column as the general hot needle technique.

Manipulation of GC-MS

GC-MS was used to identify polar metabolites from maize seedlings. GC-MS analysis was conducted using the Trace DSQ GC-MS system (Thermo Electron, U.S.) comprising an AS 2000 autosampler (Thermo Electron, Dreieich, Germany), a GC 8000 gas chromatograph, and a DSQ quadrupole mass spectrometer (ThermoFinnigan, Manchester, UK) , the temperature for injection of the sample was at 230 ºC, the interface temperature was at 250 ºC, and the temperature for the ion source was adjusted to 200 ºC. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC was performed following the previous approach (Roessner et al., 2000) but with some modifications. In brief, isothermal heating took place for 5 min at 70 ºC. The temperature was then increased at a rate of 5 ºC/min up to 350 ºC. This temperature was held for 1 min, and then slowly decreased to 330 ºC and maintained for 5 min at this temperature. The temperature in the GC system was equilibrated for 1 min at 70 ºC prior to injection of the next sample. Following GC analysis, MS analysis was conducted as ionization mode. The ionization mode was operated in an electronic impact manner, where electronic volt was at 70 eV, temperature of ion source was at 200 ºC and temperature of ion transmission line was at.250 ºC. Mass spectra were analyzed within a scanning range from 50 to 600 m z^{-1} at 2 scans s^{-1}.

Identification of metabolites

GC-MS-based mass spectra were used to detect and quantify specific mass spectral fragments. The retention index (RI) of each component was calculated through comparison with retention time of the mixture of the standard substances. Analysis of the mass spectra was based not only on a mass spectrum library of authentic standards that was constructed in-house but also on matching against reference compounds of the NIST Mass Spectral Library 2002. If a target component showed a match of ≥750 with a specific standard substance on a scale of 0 to 1,000 and its RI deviation value fell into a range of -3.0 to 3.0, it was identified as the known metabolite. If the RI deviation values of metabolites from different samples fell into a range from -0.1 to 0.1, these metabolites were considered to be the same metabolite. Metabolites that did not meet the above criteria were defined as "unknown". Unknown metabolites were named as a capital P (polar) plus RI value such as P1190.6.

Data analysis of the GC-MS

After obtaining original metabolic maps by GC-MS analysis, the manual procedure was carried out for inspection of three repetitive data from three individual seedlings from each treatment to exclude biological deviation resulting from growth differences of individual seedlings. In brief, if an unusual map with a very high or extremely low value was found in a certain treatment, tissue samples from another batch of three individual seedlings under that treatment condition would be analyzed again by GC-MS for verification. The data verified were imported into Microsoft Excel 2003 followed by manual peak alignment, and then normalized. For normalization within each chromatogram, the peak area of each metabolite was divided by the peak area of the internal standard (i.e. ribitol) present in the same chromatogram, giving the response ratio of the metabolite. Then, the response ratio was subsequently converted to a relative value. Conversion of the response ratio was performed by dividing it by the fresh weight of each sample (Roessner et al., 2000). After log10 transformation of relative response ratios in metabolite response matrix data, HC and PC analyses were conducted with SPSS 16.0 software as default parameters (SPSS Inc, U.S.). Missing values were replaced with 0. Student's *t*-tests were conducted using the algorithm in Microsoft Excel 2003. If the level of a given metabolite differed significantly ($P < 0.05$) and the fold change of its level was ≥ 2 and ≤ 0.5 according to a comparison of WD-stressed tissues vs. control tissues at the same time point, the metabolic level of the metabolite was considered to be increased and decreased, respectively. Dynamic patterns of metabolism of the metabolites were drawn through SOM analysis as described by Kim et al. (2007).

Statistical analysis

Statistical analysis was conducted using the SPSS 16.0 software according to the instructions.

Conclusions

The survival of maize under WD is associated not only with WD tolerance during WD stress but also with growth recovery ability under RW. Maize WD tolerance is involved in a very complex interplay among multidimensional factors including stress time course, coordination of metabolic pathways between tissues, stress strength, and dynamic changes of metabolic levels in the metabolic networks. Changes of the metabolites in metabolic level in the roots are to maximize water uptake whereas the changes in the leaves are to prevent water loss and increase water-use efficiency. WD tolerance depends on balance between related metabolic networks. Metaolic balance of the metabolic networks must be based on controlled metabolic level of the metabolites. There is a significant crosstalk between metabolisms of WD tolerance and growth recovery, where glycolysis, starch and protein degradation are pivotal pathways. Growth recovery of maize from WD stress depends on an emergency repair mechanism.

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