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Physiological and proteomic analysis of the response to drought stress in an inbred Korean maize line

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Abstract

Understanding the response of a crop to water deficiency is the first step towards breeding drought-tolerant varieties. In this study, inbred maize (*Zea mays* L.) line KS140 was subjected to drought stress by withholding water for 10 days at the V5 or V6 leaf stage. Water-deficient plants experienced a decrease in relative leaf water content, stomatal conductance, net CO_2 assimilation rate, and water use efficiency compared to well-watered plants. This was accompanied by a decrease in the relative water content that resulted in severe growth retardation (75% decrease in leaf area, and 64% and 56% decrease in aerial tissue and root dry matter, respectively). Leaf chlorophyll content was unchanged. Two-dimensional electrophoresis protein expression profiles were compared between well-watered and water-deficient plants. Differential expression was observed for 29 protein spots, and these were identified using MALDI-TOF mass spectrometry. Of these proteins, 34% were involved in metabolism, 24% in response to stress, 14% in photosynthesis, 7% in protein modification, and 14% were proteins of unknown function. Of the 29 differentially expressed proteins, 24 and 5 protein spots were up- and down-regulated in water-deficient plants, respectively. Two pathogenesis-related proteins, an abscisic stress-ripening protein and heat shock protein 1, were expressed only under drought conditions. This study provides a protein profile of a Korean maize inbred line during drought stress, which will be valuable for future studies of the molecular mechanisms underlying drought resistance and for development of selective breeding markers for drought tolerance in maize.

Keywords: Drought, Maize, MALDI-TOF, Proteome, 2-DE.
Abbreviations: ACN_acetonitrile; CBB_coomassie brilliant blue; DAW_days after withholding water; MALDI-TOF_ matrix-assisted laser desorption/ionization time-of-flight; IPG_immobilized pH gradient; PPFD_photosynthetic photon flux density; SPAD_portable chlorophyll meter; TFA_trifluoroacetic acid; 2-DE_two-dimensional gel electrophoresis.

Introduction

Drought is a severe environmental factor that has substantial effects on plant growth and development. Previous research on plant responses to drought stress revealed that insufficient water supply leads to physiological, biochemical, and molecular changes (Pinheiro and Chaves, 2011). Limited water availability leads to reduced growth of aerial tissues and, to a lesser extent, of the root system. Physiological studies showed that sugars, sugar alcohols, amino acids, and amines accumulate under drought stress conditions in various plant species (Seki et al., 2007). Plant responses to drought include reduction in vegetative growth, stomatal closure, and a decrease in the rate of photosynthesis (Chaves et al., 2003; Mahajan and Tuteja, 2005). Maize (*Zea mays* L.) is a major worldwide crop cultivated both for human consumption and for animal feed. Maize is also a key raw material for industrial applications and bio-energy production. Maize is highly productive under optimal environmental and crop management conditions. However, maize plants are highly susceptible to various stresses, including drought (Lobell et al., 2011). Drought stress reduces plant growth and inhibits maize development in the early growth stages (Shaw, 1983). In particular, drought stresses during the V8–V17 growth period have a substantial effect on maize growth, architecture, ear size, and kernel number (Farré and Faci, 2006). Drought occurring at the silking stage can cause significant declines in kernel set and kernel weight (Bassetti, 1990), resulting in approximate yield losses of 20–50% (Nielsen, 2007). Plant drought also induces the expression of proteins that are not specifically related to water deficit, but which are induced by cellular damage. These include different classes of heat shock protein genes or cognates (Kiyosue et al., 1994), thiol proteases (Williams et al., 1994), proteinase inhibitors (Reviron et al., 1992), and osmotin (Kononowicz et al., 1993) in plants. In maize, a ferritin gene induced by iron stress was also induced by drought and abscisic acid (ABA) (Fobis-Loisy et al., 1995). Recent rapid advances in high-throughput analysis methods, such as transcriptomics and proteomics, have enabled researchers to investigate the molecular events underlying plant responses to drought on a genomic scale. Transcriptomic analyses were used to discover several genes that were induced or repressed in response to dehydration (Hayano-Kanashiro et al., 2009; Shinozaki and Yamaguchi-Shinozaki, 2007; Zheng et al., 2004). However,

transcriptional studies alone are not sufficient to fully understand plant responses to drought. Physiological and molecular changes occurring in response to water deficiency ultimately depend on the interactions between proteins in various metabolic, signaling, biosynthetic, and degradation pathways. Plant proteomic analysis allows for the large-scale study of molecular changes occurring at the protein level. Proteomics has already been used to evaluate drought-responsive proteins in important crop species such as rice (Shu et al., 2010), maize (Benešová et al., 2012; Riccardi et al., 2004), wheat (Peng et al., 2009), and sugar beet (Hajheidari et al., 2005). Preliminary studies focused primarily on qualitative and quantitative changes to dehydration-induced or repressed proteins. More complex studies compared the proteomic responses of drought-tolerant and drought-sensitive maize genotypes to water deficit (de Vienne et al., 1999; Riccardi et al., 2004). Such analyses allow the discovery of proteins that are directly involved in the mechanisms underlying drought tolerance. These proteins can then serve as molecular markers in marker-assisted selection and breeding programs or in transgenic approaches to improving plant drought tolerance.

Transcriptomic and proteomic changes in maize in response to drought have been examined for a number of cultivars and conditions, including water deprivation, osmotic stress, and ABA treatment. However, maize cultivars developed by the Rural Development Administration (RDA) in Korea have not been investigated to date. The aim of this study was to analyze the physiological and protein expression responses to drought stress of KS140 inbred line, which has been majorly used for normal corn breeding in Korea. Successive breeding over numerous generations has adapted this cultivar to Korean climatic conditions, and drought responses in this line might therefore differ from responses in previously examined maize varieties. In this study, the KS140 inbred line was subjected to drought stress treatments (10 days without water) and global protein expression was evaluated using two-dimensional gel electrophoresis (2-DE) analysis combined with MALDI-TOF mass spectrometry and RT-PCR.

Results and discussion

Responses of maize plants to drought stress

The physiological response of a Korean inbred line, KS140, to drought stress was evaluated in 25-day-old plants that had been subjected to water deficiency for 10 days under greenhouse conditions. Leaf rolling, a visible sign of drought, was observed in the water-deficient plants after 3 days of drought treatment (data not shown). The degree of leaf rolling became more pronounced as the duration of the drought progressed. Leaves on well-watered control plants remained unrolled. Rolling rapidly reduces effective leaf area and transpiration, and is therefore a useful drought-avoidance mechanism in arid areas (Clarke, 1986). Relative water content (RWC) of the youngest fully expanded KS140 leaf was significantly lower in water-deficient plants than in well-watered plants (~15% lower 3 days after withholding water (DAW) and ~45% lower 10 DAW, which indicated that the maize plants suffered from drought stress under water-withholding conditions (Fig. 1A). After 3 days without water, leaf area and dry matter of aerial tissues and roots were reduced by approximately 50%, 25%, and 33%, respectively, when compared to well-watered plants (Figs. 1B and C), suggesting that even a short 3-day period of water deficiency had a substantial impact on growth and development of the KS140 plants. After 10 days without water, dry matter accumulation in drought-stressed plants was severely inhibited, by ~64% in aerial tissues and ~56% in roots, compared to well-watered plants (Fig. 1D). Leaf area was most severely affected by drought stress (Fig. 1B). These results are consistent with previous research showing the effects of drought stress on maize, albeit at a different developmental stage (Zheng et al., 2010). To determine the effect of drought stress on photosynthesis, stomatal conductance, net CO_2 assimilation rate, water use efficiency, and leaf chlorophyll were measured on the youngest fully expanded leaf (Fig. 2). At 3 DAW, stomatal conductance, net CO_2 assimilation rate, and water use efficiency were respectively 90%, 78%, and 69% lower in drought-stressed leaves than in leaves from well-watered plants (Figs. 2A–C). At 10 DAW, there was little net CO_2 assimilation rate in the drought-stressed leaves, implying that no further dry matter accumulated. However, even after 10 days without water, drought had no impact on leaf chlorophyll content or the quantum efficiency (Fv/Fm) of photosystem II (data not shown) (Fig. 2D). Recently, Lua et al. (2011) reported that plant height and grain yield significantly decreased and anthesis-silking interval, chlorophyll content, root capacitance, and leaf senescence significantly increased in water-stressed plants compared to well-watered plants (Lua et al., 2011). These data suggest that maize responds quickly to drought stress through closure of stomata to reduce water loss and through protection of photosynthetic components such as chlorophyll and photosystem II.

Proteins differentially expressed between well-watered and water-deficient plants identified by 2-DE comparative proteomics

Proteomic identification was used previously to identify proteins differentially expressed between well-watered and drought-stressed plants (Kim et al., 2013). Separation of proteins by two-dimensional electrophoresis is advantageous as it provides an overview of the proteome through the separation of proteins by isoelectric point (p*I*) and molecular mass. In this study, proteins were extracted from pairs of KS140 leaves (well-watered vs. 10 days drought stress) using phenol extraction. Neutral IPG strips (pH 4–7) were used for isoelectric separation to achieve optimal two-dimensional protein gel resolution. 2-DE experiments were performed in triplicate, and gels were CBB-stained. Twenty-nine differentially expressed protein spots were identified through comparison of gels from the well-watered and drought-stressed samples using ImageMaster software (Figs. 3 and 4). Of these, 25 protein spots were markedly larger or were newly-induced (spots 4, 5, 7, 11, and 29) under drought conditions, and four spots were smaller (2, 12, 19, and 24) (Figs. 4 and 5). These results indicate that drought stress affected the abundance of several proteins in KS140 leaves, and that most of the affected proteins were up-regulated rather than down-regulated when water was withheld.

Identification of proteins involved in response to drought stress in KS140 leaves

To further understand the mechanisms underlying the response to drought stress in maize, we analyzed the differentially expressed proteins by MALDI–TOF and used Protein Prospector and Mascot database searches for identification. Proteins were classified based on functional categories established by Schnable et al. (2009). The differentially expressed proteins were related to diverse biological processes and comprised ten metabolism-related proteins, seven defense/stress-related protein, four photosynthesis related

Table 1. Proteins identified by MALDI-TOF MS.

Spot No.	Accession No.	Putative Function	Score	Expect	MP	SC (%)	Mr(kD) /pI	Biological process	Organism
1	B6TA31	Fruit protein PKIWI502	325	1.1e-025	24	54	31.7/6.62	Development	Zea mays
2	B6U5I1	Peptidyl-prolyl cis-trans isomerase	146	8.5e-008	22	30	46.8/4.91	Protein folding	Zea mays
3	gi|414883697	TPA: hypothetical protein ZEAMMB73_937583	128	5.4e-006	14	86	14.1/4.83		Zea mays
4	B6SXF5	Pathogenesis-related protein 1	103	0.0017	11	67	17.1/5.39	Plant defense	Zea mays
5	D4HR93	TPA: pathogeneis protein 10	110	0.00034	10	51	17.1/5.36	Plant defense	Zea mays
6	B6STK3	Cytochrome b6-f complex iron-sulfur subunit	138	5.4e-007	14	40	24.3/8.52	Photosynthesis	Zea mays
7	gi|514787580	Abscisic stress-ripening protein 2-like	63	17	7	21	11.5/9.80	Plant stress	Setaria italica
8	B6UB73	APx1 - Cytosolic Ascorbate Peroxidase	254	1.3e-018	19	63	27.5/5.65	Plant stress	Zea mays
9	P12653	Glutathione S-transferase	130	3.4e-006	11	27	23.5/5.28	Plant stress	Zea mays
10	B6TPH0	Lactoylglutathione lyase	177	6.7e-011	21	53	35.3/6.62	Metabolism	Zea mays
11	gi|226533140	Hypothetical protein	166	8.5e-010	16	40	33.6/5.96		Zea mays
12	B6T171	Serine-glyoxylate aminotransferase (LOC100281949)	274	1.3e-020	24	51	44.4/6.72	Metabolism	Zea mays
13	B6SSU6	Fructose-bisphosphate aldolase, cytoplasmic isozyme 1	268	5.4e-020	20	48	38.5/6.26	Metabolism	Zea mays
14	K7V067	Isocitrate dehydrogenase (ZEAMMB73_038317)	389	4.3e-032	37	59	46.5/6.11	Metabolism	Zea mays
15	B6T9J4	Aspartate aminotransferase	356	8.5e-029	28	48	50.5/8.15	Metabolism	Zea mays
16	B6TUD4	ATP synthase subunit gamma, chloroplastic precursor	288	5.4e-022	19	32	40.1/8.44	Photosynthesis	Zea mays
17	C0PD30	Fructose-1,6-bisphosphate aldolase	187	6.7e-012	18	52	38.4/6.37	Metabolism	Zea mays
18	gi|226509797	Uncharacterized protein LOC100274579	586	8.5e-052	31	70	42.5/5.65		Zea mays
19	P25462	Glutamine synthetase	228	5.4e-016	15	28	46.3/6.42	Metabolism	Zea mays
20	gi|242083462	Hypothetical protein	66	9.5	6	19	72.02/8.44		Sorghum bicolor
21	B6TG70	Mitochondrial-processing peptidase beta subunit	332	2.1e-026	33	51	58.5/5.87	Proteolysis	Zea mays
22	Q6L3A1	ATP synthase subunit alpha	381	2.7e-031	35	50	55.8/5.87	Photosynthesis	Saccharum hybrid
23	P93804	Phosphoglucomutase	301	2.7e-023	28	41	63.3/5.46	Carbohydrate metabolism	Zea mays
24	C0P4M0	Pyridine nucleotide-disulphide oxidoreductase	95	0.01	9	29	46.6/5.60	Plant stress	Zea mays
25	B6T416	Ribulose bisphosphate carboxylase/oxygenase activase	107	0.00067	9	24	48.1/6.29	Photosynthesis	Zea mays
26	K7VII1	Putative actin family protein isoform 1	324	1.3e-025	21	52	41.9/5.24	Structure	Zea mays
27	P15719	Malate dehydrogenase (NADP)	207	6.7e-014	23	40	47.3/6.49	Carbohydrate metabolism	Zea mays
28	B4G072	UDP-glucosyltransferase BX9	376	8.5e-031	29	53	50.6/5.22	Metabolism	Zea mays
29	C4J410	Heat shock protein 1 (LOC100501536)	319	4.3e-025	33	46	71.2/5.08	Plant stress	Zea mays

SC, sequence coverage. Mr/pI, Theoretical molecular weight/isoelectric point.

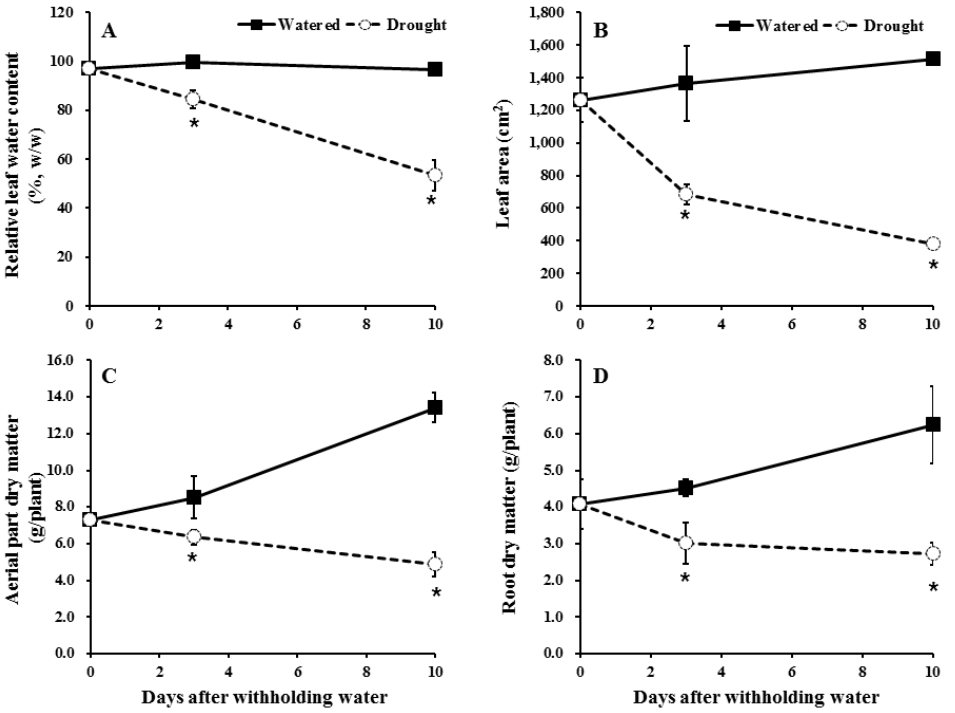

Fig 1. (A) Relative leaf water content, (B) leaf area, dry matter of aerial tissue (C) and root (D) of well-watered and drought-stressed plants at 0, 3, and 10 days after withholding water. Values are means ± standard error (n=3 or 4). An asterisk indicates that means are significantly different between the well-watered and the drought-stressed plants as determined by LSD test (α=0.05).

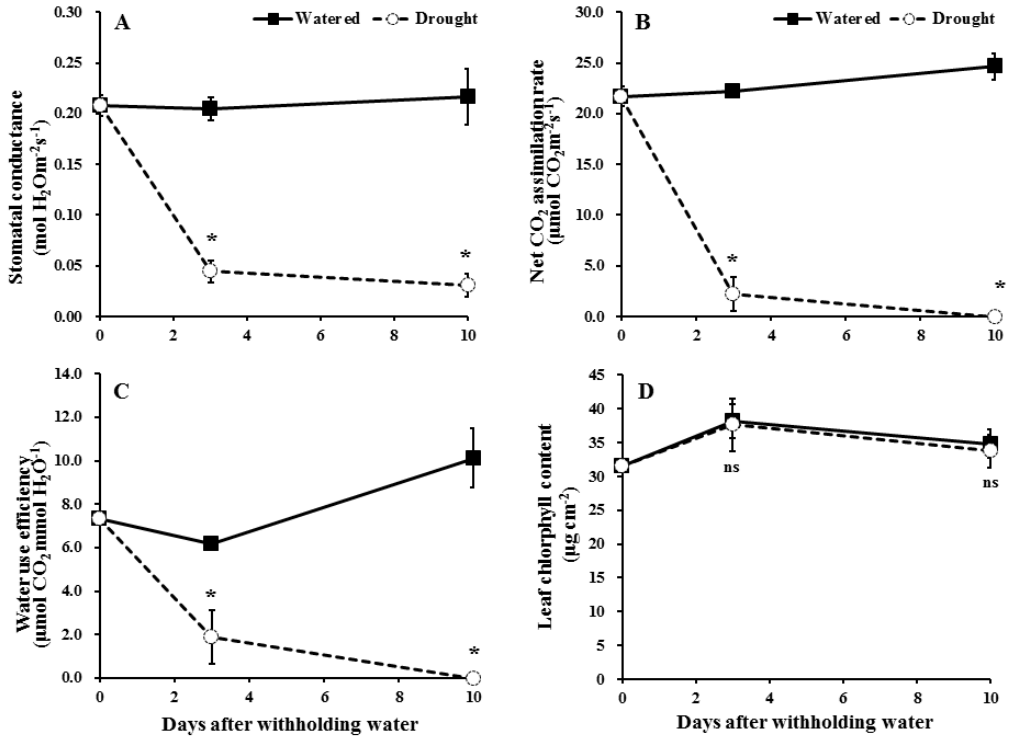

Fig 2. (A) Stomatal conductance, (B) net CO_2 assimilation rate, (C) water use efficiency, and (D) leaf chlorophyll content of well-watered and drought-stressed maize plants at 0, 3, and 10 days after withholding water. Values are means ± standard error (n=3 or 4). An asterisk indicates that means are significantly different between the well-watered and the drought-stressed plants as determined by LSD test (α=0.05).

proteins, two protein-folding/proteolysis-related proteins, two development/structure related proteins, and four proteins of unknown function (Table 1).

Metabolism-related proteins

A number of enzymes related to energy metabolism responded to drought pressure in the leaves of the KS140 inbred maize line. Two isoforms of fructose-bisphosphate aldolase (FBA, spots 13 & 17), which are important metabolic enzymes in the glycolysis/gluconeogenesis pathway, and phosphoglucomutase (PGM, spot 23) and malate dehydrogenase (MDH, spot 27), which are involved in carbohydrate synthesis, were identified as drought-induced proteins (Table 1). Abundance of other metabolism-related proteins such as isocitrate dehydrogenase (IDH, spot 14), lactoylglutathione lyase (LGL, spot 10), aspartate aminotransferase (AST, spot 15), and UDP-glucosyltransferase (UGT, spot 28) also increased under drought conditions, whereas glutamine synthetase (GS, spot 19) and serine-glyoxylate aminotransferase (SGAT, spot 12) levels decreased (Figs. 4 and 5; Table 1). Previous research found that FBA mRNA levels increased in response to high salinity, drought, and/or ABA in maize (Hu et al., 2012), and *Arabidopsis thaliana* (Lu et al., 2012). González et al. (2005) reported that IDH activity showed a gradual increase during moderate drought stress, and the isoenzyme pattern further supported the observation of a higher IDH activity in drought-stressed pea nodules (Gálvez et al., 2005). Activity of AST declined by 40% in *Pisum sativum* nodules in water-deficient plants (Gálvez et al., 2005). MDH was up-regulated by drought stress in wild watermelon root and grapevine (Cramer et al., 2013). To our knowledge, no prior studies found a drought effect upon PGM, LGL, UGT, GS, or SGAT abundance. Further research is required to determine the roles of these metabolism-related proteins in the drought response in maize. In summary, several metabolism-related proteins were found to be up- or down-regulated 10 days after the cessation of watering, suggesting that a global metabolic change occurred in maize leaves under drought stress.

Stress-related proteins

The second major group of proteins to be affected by water deficiency was that of stress-related proteins. Four of the seven identified stress-related proteins were newly induced by drought stress: two pathogen related proteins (PR) (PR-1 and PR-10, spots 4 and 5), abscisic stress-ripening protein 2-like protein (ASR2, spot 7), and heat shock protein 1 (HSP1, spot 29). Levels of ascorbate peroxidase (APx1, spot 8) and glutathione S-transferase (GST, spot 9) were higher in drought-stressed plants than in well-watered plants, whereas levels of pyridine nucleotide-disulfide oxidoreductase (PNDO, spot 24), which is involved in detoxification, were lower in drought-stressed plants (Figs. 4 and 5). Previous study, drought caused the up-regulation of protective and stress-related proteins such as chaperones and dehydrins (Benešová et al., 2012). In addition, PR protein activities increased in response to drought stress in white clover leaves (Lee et al., 2008). Maize PR-10 showed significant sequence identity to proteins from the PR-10 family, including one from white lupin that was shown to possess RNase activity (Bantignies et al., 2000). Expression levels of a number of ASR genes rapidly increased in response to water deficit, cold, salt, and limited light (Kalifa et al., 2004), and cloning of maize ASR revealed its role in drought resistance (de Vienne et al., 1999; Riccardi et al., 2004). Generally, plants reacted rapidly to low water availability via induction of ABA synthesis, which corresponded with the possible induction of genes encoding various PR proteins by ABA (Pechanova et al., 2013). Plants have developed several strategies to minimize the damage caused by reactive oxygen species (ROS) (Mittler et al., 2004). *APX* transcription was up-regulated in two inbred maize inbred lines (Han21 and Ye478) under moderate or severe drought stress conditions (Zheng et al., 2010). GST expression can be induced by a range of abiotic stressors such as drought, salt, and cold (Gallé et al., 2009). HSPs are usually induced under conditions of stress and mediate tolerance to stressors, including drought, salinity, ROS, and low temperatures (Pechanova et al., 2013). In this study, we found that the affected stress-related proteins were mostly up-regulated in response to drought stress. This suggests that these stress-related proteins may play important roles in plant tolerance to drought stress.

Photosynthesis related proteins

A third group of proteins whose abundance changed under drought conditions was associated with photosynthesis. Levels of cytochrome b6-f complex (cyto b6/f, spot 6), ATP synthase subunit gamma (spot 16), ATP synthase subunit alpha (spot 22), and ribulose bisphosphate carboxylase/oxygenase activase (RCA, spot 25) were higher under drought stress than in well-watered conditions. Cytochrome b6-f complex protein mediates electron transfer between photosystems I and II, governs cyclic electron flow around PSI, and controls state transitions in the thylakoid membrane (Hurt and Hauska, 1981). These results corresponded with previous studies. The cyto b6/f complex was upregulated in drought-stressed rice (Ali and Komatsu, 2006) and chloroplast ATP synthase was up-regulated by drought stress in *Phalaenopsis* (Ali et al., 2005). RCA is a molecular chaperone that is involved in switching Rubisco from an inactive to an active conformation (Spreitzer and Salvucci, 2002), and the up-regulation of these photosynthesis related proteins might act to alleviate the damage to Rubisco caused by drought stress (Ji et al., 2012).

Protein related modification, development, and structure

Proteolysis-related proteins, which are necessary for maintaining cellular protein homeostasis, were also more abundant in water-stressed samples than in samples from control plants. This group included mitochondrial-processing peptidase beta subunit (spot 21), which was induced by drought stress in KS140. Proteins damaged by cell stress are degraded by proteasomes and proteolytic enzymes (Kurepa et al., 2009). A protein-folding protein, peptidyl–prolyl cis-trans isomerase (PPIase, spot 2), was down-regulated in response to drought stress in KS140. This differed from the PPIase abundance profile observed in sorghum under drought, and the authors suggested that PPIase might be connected to different regulatory pathways in different species (Sharma and Singh, 2003). Finally, we found several proteins of unknown function, such as fruit protein PKIWI1502 (spot 1) and putative actin family protein isoform 1 (spot 26), that were upregulated in water-deficient plants compared to well-watered plants. These proteins were observed by 2-DE to be highly abundant, which suggested roles in central cellular functions. The roles of these proteins in maize await investigation.

Transcriptional expression profiling of genes corresponding to proteins regulated under drought stress

To determine whether the 2-DE derived protein expression

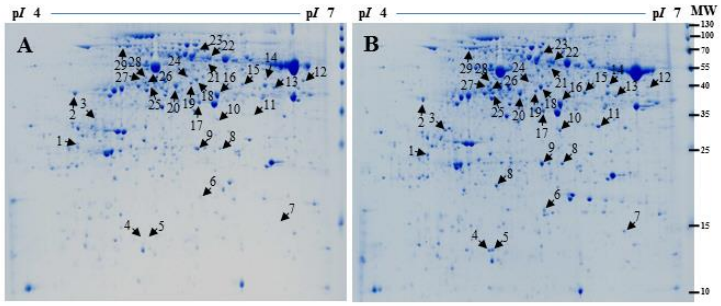

Fig 3. Representative 2-DE gel of Korean maize inbred line (KS140). (A) Well-watered, (B) Ten days drought stress. Diffentially expressed protein spots detected on the 2-DE gel are indicated by arrows. A total of 500 µg protein was used for each 2-DE gel.

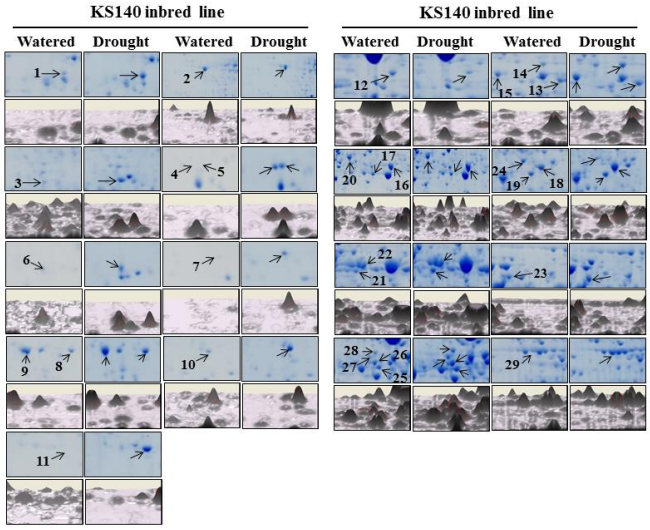

Fig 4. Expression profiles of protein spots in well-watered and drought-stressed KS140 inbred lines. A close-up view of differentially expressed protein spots is shown.

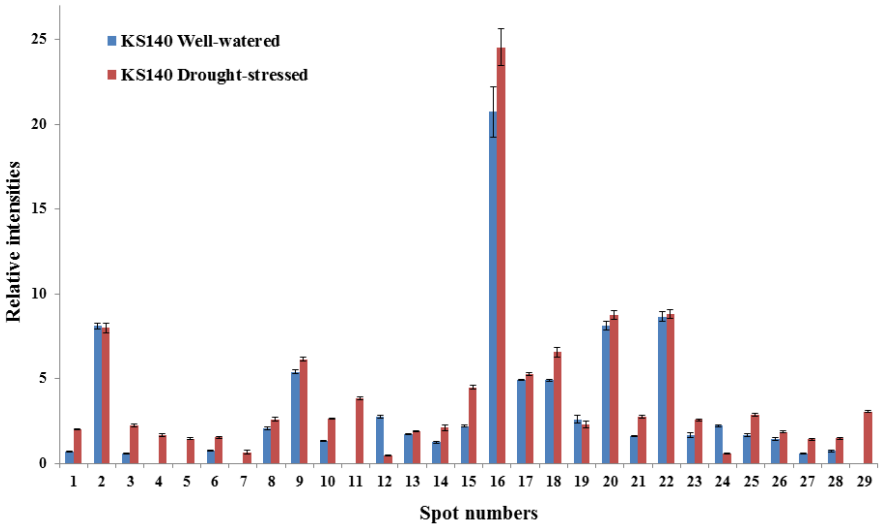

Fig 5. Bar graph showing relative intensities of each 2-DE protein spot as determined using ImageMaster software analysis of three replicate gels.

profiles of identified proteins corresponded with their transcript levels, a semi-quantitative RT-PCR analysis was performed using gene-specific primer pairs for 13 randomly chosen genes encoding identified proteins. Transcript levels were higher in drought-stressed plants than in well-watered plants for *PR-1* (spot 4), *PR-10* (spot 5), *Cyto b6/f* (spot 6), *APx1* (spot 8), *SGAT* (spot 12), *FBA* (spot 13, 17), *IDH* (spot 14), and *PBM* (spot 23) (Fig. 6). Conversely, *PPIase* (spot 2), *AST* (spot 15), GS (spot 19), and *RCA* (spot 25) transcript levels were lower in drought-stressed plants than in well-watered plants (Fig. 6). RT-PCR analysis was reproducible for at least two replicates. These data showed that protein and mRNA were similar with the exception of *AST* (spot 15) and *RCA* (spot 25) (Fig. 6). These two proteins might therefore be regulated at the post-transcriptional level (Cramer et al., 2013).

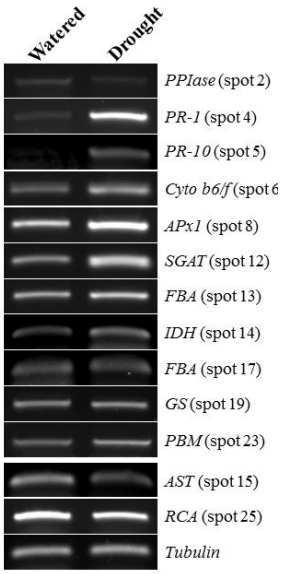

Fig 6. RT-PCR analysis of genes corresponding to 13 differentially expressed proteins.

Materials and Methods

Plant materials and growth conditions

Maize (*Zea mays* L.) KS140 plants were used. KS140 is a parent of cv. Gangdaok, an elite normal corn F_1 hybrid released by the Rural Development Administration (RDA) in South Korea. KS140 seeds were planted into 1/5000a Wagner's pots filled with sandy loam soil on August 1, 2013. Plants were cultivated in a greenhouse (Suwon, South Korea) and were thinned to a single plant per pot at the V2 leaf stage. Volumetric soil moisture content was monitored at a 10 cm soil depth with a capacitance-type moisture sensor (WaterScout SM 100 Soil Moisture Sensor, Spectrum Technologies, Inc., IL, USA). Soil moisture content was maintained at >10% volumetric soil moisture content using an automatic irrigation system (Aqua Pro, Netafim Ltd., Israel), with 2 L of tap water applied per irrigation (Supplementary Fig. 1). Water was withheld for 10 days commencing at the V6 leaf stage. The leaves of plants in water-withheld pots began to roll after three days without irrigation, corresponding with soil moisture content <5%. Leaf area and dry matter of aerial and root tissues were determined after 3 and 10 days without water. Samples were examined from 3–4 plants each from the drought-stressed and well-watered groups.

Determination of leaf chlorophyll

Fifteen fresh maize leaves with portable chlorophyll meter (SPAD) values in the 8–59 range were harvested and their leaf areas measured. To extract chlorophyll, leaves were diced and placed into 15 ml conical tubes filled with 5 ml of 95% (v/v) ethanol. Tubes were incubated at 4°C for 48 hours. Extract absorbance was measured at 648 nm and 664 nm using a spectrophotometer (U-2900, Hitachi High-Technologies Corp., Japan). Concentration (μg ml^{-1}) of chlorophyll a and b in the extracts was determined using the following equations (Miazek and Ledakowicz, 2013):

$$Chl_a = 13.36 \times A_{664} - 5.19 \times A_{648}$$
$$Chl_b = 27.43 \times A_{648} - 8.12 \times A_{664}$$

Leaf chlorophyll content based on leaf area correlated positively with the SPAD value (r = 0.97). The standard curve for leaf chlorophyll content determination was obtained using the SPAD values as follows:

$$Chlorophyll_{a+b}\ (\mu g\ cm^{-2}) = 0.9155 \times \text{SPAD value} - 8.5575\ (R^2 = 0.95)$$

The SPAD value of the youngest fully expanded leaf was measured with a chlorophyll meter at 3 and 10 days after withholding water and its chlorophyll content was calculated using the standard curve.

Measurements of leaf photosynthesis and stomatal conductance

Net CO_2 assimilation rate (A), stomatal conductance, and leaf transpiration rate (Tr) were measured on the youngest fully expanded leaf using a portable gas-exchange system (Li-6400, LI-COR, Lincoln, NB, USA) at 3 and 10 days after withholding water. A and Tr values were used to calculate the water use efficiency (A/Tr). All other variables within the leaf chamber of the Li-6400 were standardized during measurements; leaf temperature was maintained at 25°C and photosynthetic photon flux density (PPFD) at 1,500 μmol quanta m^{-2} s^{-1}. All measurements were replicated 3 times for each three plants of well-watered and drought stressed, respectively.

Relative leaf water content

A leaf cut was taken from the middle of the youngest fully expanded leaf at 3 and 10 days after withholding water. Fresh weight was determined and the leaf cut was then floated on water for up to 48 hours. The turgid weight was then noted, and the leaf cut was subsequently oven-dried at ~70°C for 5 days for determination of dry weight. Relative water content (RWC) of the leaf was calculated as follows (Smart and Bingham, 1974):

$$\text{RWC} = \frac{(\text{fresh weight} - \text{dry weight})}{(\text{turgid weight} - \text{dry weight})} \times 100$$

Protein extraction, 2-DE, and image analysis

Maize leaf proteins were extracted by phenol extraction method combined with Mg/NP-40 buffer containing 0.5 M Tris-HCl (pH 8.3) and 2% β-mercaptoethanol. Extraction procedure of proteins was carried out as described by Kim et al. (2008). Protein content was measured using a 2-D quant kit (GE healthcare, Waukesha, WI, USA). Two-dimensional gel

electrophoresis (2-DE) analysis was performed according to Kim et al. (2008). Immobilized pH 4-7 gradient (IPG) strips (24 cm) were rehydrated in rehydration solution containing equivalent samples (500 µg). IPG focusing was then performed at 50 V for 4 hr, 100 V for 1 hr, 500 V for 1 hr, 1000 V for 1 hr, 2000 V for 1 hr, 4000 V for 2 hr, 8000 V for 5 hr, 8000 V for 9 hr, and 60 V for 6 hr using the IPGphore II platform (GE Healthcare, Waukesha, WI, USA). Each focused IPG strip was then placed into a 20 ml screw-cap tube with 5 ml of equilibration buffer [50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT, and 0.1 mg/ml bromophenol blue] and agitated gently at room temperature for 20 min. A second equilibration was performed in equilibration buffer with 55 mM iodacetamide solution and without DTT under dark conditions for 20 min with gentle agitation. Electrophoresis was performed using 13% SDS-polyacrylamide gels, after which the 2-DE gels were stained with colloidal Coomassie Brilliant Blue (CBB) (Kim et al., 2013). Images were acquired using a transmissive scanner (PowerLook 1120, UMAX) with a 32 bit pixel depth, 300 dpi resolution, and brightness and contrast set to default. Gel spots were detected automatically using Image Master 2D Platinum software 6.0 (GE Healthcare, Waukesha, WI, USA). The volume of each spot was then normalized to an average of the volume of spots on the gels.

In-gel digestion

Differentially expressed protein spots were subjected to in-gel trypsin digestion according to the method described by Kim et al. (2008). CBB-stained target spots were excised using a razor blade, washed with 50% (v/v) acetonitrile (ACN) in 0.1 M NH_4HCO_3, and vacuum-dried. Dried gel fragments were then treated with 10 mM DTT in 0.1 M NH_4HCO_3 for 45 min at 55°C. The DTT solution was then immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and samples were incubated for 30 min at room temperature in the dark. Gel pieces were then washed with 50% ACN in 0.1 M NH_4HCO_3 and then digested at 37°C overnight in 10 µl of digesting solution with 12.4 ng/µl trypsin and 25 mM NH_4HCO_3. Samples were air-dried after digestion.

MALDI-TOF MS analysis

Nitrocellulose (20 mg/ml) and a-cyano-4-hydroxycinnamic acid (40 mg/ml) (Sigma-Aldrich) solutions were prepared in acetone (Kim et al., 2013). The a-cyano-4-hydroxycinnamic acid solution, the nitrocellulose solution, and isopropanol were then mixed at a 100:50:50 ratio, and 2 µl of the mixture was added to 2 µl of peptide sample solution. A 1 µl sample of the final solution was spotted immediately onto a matrix-assisted laser desorption/ionization (MALDI) plate and left for 5 min. The MALDI plate was then washed with 0.1% (v/v) trifluoroacetic acid (TFA). The gel spots were analyzed using a Voyager-DE STR MALDI time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA). Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, grid voltage of 76.000%, guide wire voltage of 0.010%, and a delay time of 150 ns. Des-Arg1-bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1296.6853) were used as a two-point internal standard for calibration. Peptides were selected in the mass range of 500–3000 Da. The software package PerSeptive-Grams was used for data processing. Database searches were performed using the Protein Prospector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) websites.

Semi-quantitative RT-PCR analysis

Total RNA was extracted using the SDS-phenol method from leaves of 25-day-old maize seedlings that were well-watered or exposed to drought stress for 10 days (Kim et al., 2013). Total RNA samples (5 µg per reaction) were used for cDNA synthesis according to the manufacturer's instructions (Invitrogen, Madison, WI). RT-PCR was performed with gene-specific primers corresponding to the genes encoding the identified proteins. The primers are summarized in Supplementary Table 1. Primers were designed to generate PCR products of 250–450 bp. Tubulin transcription was used as an internal control for normalization of cDNA input.

Statistical analysis

The analysis of variance (ANOVA) for physiological and protein spot volumes values were performed to determine statistically different values at a significance of $p \leq 0.05$ using SAS software (ver. 9.2).

Conclusion

In this study, proteomic analysis was used to assess responses to drought in a maize inbred line (KS140). Leaves were analyzed for physiological and proteomic changes occurring in response to water deficiency. Drought affected relative leaf water content, leaf area, aerial and root tissue dry matter, stomatal conductance, net CO_2 assimilation rate, and water use efficiency. Proteins modulated by drought stress were involved in glycolysis and carbohydrate metabolism, stress and defense, photosynthesis, and protein modification. Protein responsiveness to drought stress corresponded with transcript level for most of the randomly selected proteins. These integrated data provide a perspective on cellular events regulated by drought stress in maize inbred line leaves. These findings provide new insights into stress responses in maize that will be valuable in the development of novel drought-tolerant maize varieties.

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