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ORIGINAL PAPER

The panorama of physiological responses and gene expression of whole plant of maize inbred line YQ7-96 at the three-leaf stage under water deficit and re-watering

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Abstract Changes in water potential, growth elongation, photosynthesis of three-leaf-old seedlings of maize inbred line YQ7-96 under water deficit (WD) for 0.5, 1 and 2 h and re-watering (RW) for 24 h were characterized. Gene expression was analyzed using cDNA microarray covering 11,855 maize unigenes. As for whole maize plant, the expression of WD-regulated genes was characterized by up-regulation. The expression of WD-regulated genes was categorized into eight different patterns, respectively, in leaves and roots. Newly found and WD-affected cellular processes were metabolic process, amino acid and derivative metabolic process and cell death. A great number of the analyzed genes were found to be regulated specifically by RW and commonly by both WD and RW, respectively, in leaves. It is therefore concluded that (1) whole maize plant tolerance to WD, as well as growth recovery from WD, depends at least in part on transcriptional coordination between leaves and roots; (2) WD exerts effects on the maize, especially on basal metabolism; (3) WD could probably affect CO_2 uptake and partitioning, and transport of fixed carbons; (4) WD could likely influence nuclear activity and genome stability; and (5) maize growth recovery from WD is likely involved in some specific signaling pathways related to RW-specific responsive genes.

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Abbreviations

ASI	Anthesis-silking interval
CC	CO_2 concentration
Down	Down-regulated
ER	Early response
EST	Expressed sequence tag
GO	Gene ontology
H	Hour
LR	Late response
nARVOL	Normalized artifact removed volume
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PR	Photosynthetic rate
qRT-PCR	Quantitative real-time PCR
RW	Re-watering
SC	Stomatal conductance
TR	Transpiration rate
Up	Up-regulated
WD	Water deficit
WP	Water potential

Introduction

Drought is one of the extreme environmental conditions that curtail agricultural crop productivity (Bray 1993; Bruce et al. 2002). Improved productivity under periodic drought stress is still a major challenge for world agriculture in part because of gaps in our understanding of drought biology (Nelson et al. 2007). Drought tolerance, which involves surviving moderate water loss (e.g., 90% relative water content), is the ability to survive absolute water contents of 0.1 g H_2O g^{-1} (Moore et al. 2009). Plant drought adaptation can be divided into drought resistance and drought escape (flowering to complete life cycle before drought), where drought resistance includes drought (stress) avoidance (maintenance of tissue water potential) and drought (stress) tolerance. Drought tolerance is further divided into dehydration avoidance and dehydration tolerance (Levitt 1980; Price et al. 2002).

Maize (*Zea mays* L.) is a major crop, which is now used as an important model organism for addressing fundamental issues such as stress tolerance in monocotyledonous plants (Dong et al. 2003). Maize crops are impacted by drought throughout their life cycle (Nelson et al. 2007). The mechanism of maize drought tolerance is very complex and includes: changes in many morpho-physiological characteristics such as leaf water status, osmotic adjustment, stomatal behavior, chloroplast activity and dry matter production (Camacho and Caraballo 1994); membrane permeability and enzymatic antioxidant system (Bai et al. 2006); photosynthesis and accumulation of some compounds such as phenolics, glutamine and free proline; activity of some enzymes such as caffeic acid/5-hydroxyferulic 3-*O*-methyltransferase and levels of plant hormones such as abscisic acid (Wu et al. 2001; Bahrun et al. 2002; Vincent et al. 2005; Fan et al. 2006; Harrigan et al. 2007); root volume, root weight and leaf area (Camacho and Caraballo 1994). Unfortunately, all these water deficit (WD)-responsive characteristics are hardly applied as traits to practical breeding and selection programs of maize with WD tolerance, because they are unstable or non-inheritable and, more importantly, do not always correlate with the ability of drought tolerance (Harrigan et al. 2007).

It has been demonstrated that regulation of gene expression is a central process both in plant growth and development and in plant response to external factor induction (Eveland et al. 2008). Gene expression regulation is also a basis for the bottom–up (gene-to-phenotype) discovery approach to integrate information across the levels of organization involved in studying and understanding gene-to-phenotype relationships (Campos et al. 2004). Transcriptomics, as one of 'omics' technologies, is a most powerful and direct tool to discern differently regulated genes and responsive pathways (Moore et al. 2009). A suggested strategy to significantly impact crop performance under field conditions is to integrate the above-mentioned stress-responsive pathways during critical vegetative and reproductive windows through transgenic approaches (Nelson et al. 2007; Bressan et al. 2009).

The cDNA microarray technique was first used in 1999 to analyze gene expression of maize ear under WD (Sun et al. 2001). It was then applied by other researchers to unravel the gene expression of female reproductive tissues (Zinselmeier et al. 2002), placenta and endosperm in developing kernels (Yu and Setter 2003), leaves and roots of three-leaf-stage maize (Zheng et al. 2004; Jia et al. 2006; Li et al. 2009), developing immature ear and tassel (Zhuang et al. 2007) and primary roots (Bassani et al. 2004; Poroyko et al. 2005; Spollen et al. 2008). These results have revealed that maize response to WD is involved in complex networks of gene expression regulation, with a considerable difference between the stressed maize lines. However, species-specific mechanisms are overlooked (Moore et al. 2009).

A short anthesis-silking interval (ASI) has been used as a secondary trait for breeding and selection of WD-tolerant maize lines (Bolaños and Edmeades 1996), because the short ASI could increase yield-associated pollination frequency under drought, therefore endowing maize floral organs with adaptation to drought during the flowering stage (Bruce et al. 2002).

Maize drought tolerance depends on whole plant rather than a few specific tissues or organs throughout the life cycle. The three-leaf stage is both a hinge point of maize growth from autotrophy to heterotrophy and a crucial growth stage for root formation and differentiation of leaves and stems (Guo et al. 2004). Occurrence of drought at this stage will affect the subsequent growth and development of maize. In the fields, maize frequently undergoes a process from growth retardation by WD to growth recovery by re-watering (RW). However, the existing studies lack information about the bird's-eye view of gene expression of the whole plant of maize, especially maize lines with a short ASI and under WD and RW.

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 (Hohl and Peter 1991; Lu and Neumann 1998; Verslues et al. 1998) and is therefore used to induce WD in the laboratories, because it mimics dry soil more closely than solutions of low M_r osmotica, which tend to infiltrate the cell wall with solutes (van den Berg and Zeng 2006).

We bred a maize inbred line YQ7-96, which is characterized by a short ASI of 0–1 day. The plants of this line

did not wither at the three-leaf stage and, on the other hand, these could normally produce some seeds under drought in the field trials (data not shown). In the present study, we focus on gene expression of this maize line under PEG-imposed WD and after RW to provide a complement for maize WD- and RW- responsive genes and to lay a foundation for in-depth insight into the molecular mechanisms of WD response and growth recovery of whole plant of maize lines with a short ASI at the three-leaf stage.

Materials and methods

Plant growth and WD treatment

Seedlings of maize inbred line YQ7-96 were grown as described by Qing et al. (2009) in vigorously aerated hydroponic tanks containing 0.25× Hoagland's nutrient solution (Hoagland and Arnon 1938) in a greenhouse of Zhejiang University. The greenhouse had a photosynthetic photon irradiance of 350 μmol m^{-2} s^{-1}, 25 (night) −30°C (day), a 12-h photoperiod and a relative humidity of 60–80%. On reaching the three-leaf stage, the seedlings suffered from WD stress treatment for 0.5, 1 and 2 h in 1× nutrient solution with −0.5 MPa WP imposed by PEG8000 (Sigma). WD stress treatment was conducted at 10:00 a.m. The seedlings that were stressed for 2 h by WD were transferred into 1× nutrient solution without PEG8000 and grown for another 24 h for growth recovery treatment. Control treatment of the seedlings was conducted in parallel with procedures of WD treatment in the nutrient solution without PEG8000. The nutrient solutions used were renewed once every 3 days. The leaves and roots were separately harvested at 10:00 a.m. The harvested tissues were immediately rinsed with sterile distilled water and then immediately frozen in liquid nitrogen.

Growth elongation assay of roots and leaves

Growth of primary roots and the third leaves was assayed. The roots chosen for assay before stress treatment were 100-mm long, and the leaves chosen were 600-mm long from leaf base to leaf apex. The leaves were scored at the middle part of the primary vein before treatment, and the length from the scored point to the leaf apex was measured after treatment. The measure was conducted at 10:00 a.m. The resulting data were statistically analyzed using the software SPSS 13.0 (http://www.spss.com/).

WP measurement

WP of three-leaf-old maize seedlings was analyzed with a WP4 water potentiometer (Version 2.2, Decagon Devices Inc.) following the instrument specification. The resulting data were statistically analyzed using the software SPSS 13.0.

Assay of photosynthesis-related parameters

Photosynthetic parameters were assayed on the middle part of the second leaves of three-leaf-old maize seedlings using a portable photosynthesis system LI-6400 (Li-Cor, Lincoln, Neb.). The chamber of LI-6400 was set up at 28°C and had 385 ± 5 μl^{-1} intercellular carbon dioxide and 1,000 μmol m^{-2} s^{-1} photosynthetic photon flux. The statistic analysis of the data was conducted using the software SPSS 13.0.

RNA isolation

Total RNA was isolated using TRIzol kit (Gibco-BRL, Gaithersburg, MD, USA) and then treated with RNasin (Promega, Madison, WI, USA) and RNase-free DNase (Promega, Madison, WI, USA) to remove genomic DNA according to the manufacturer's specification. The quality of total RNA was assessed by separating RNAs in 1% agarose gel containing formaldehyde. The mRNA was isolated from total RNA with the PolyATtract mRNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's specification.

Preparation of cDNAs

cDNAs were obtained through a large-scale polymerase chain reaction (PCR) amplification of cDNA clones from the cDNA library developed by us. Bacteria with cDNA clones were cultured overnight at 37°C on an LB agar plate containing 100 μg/ml ampicillin. The individual colonies were transferred into the wells of the standard 96-well plate, where each well contained 1 ml of 2× (double-strength) YT liquid medium and 50 μg/ml of ampicillin, and then cultured overnight at 37°C with shaking at 200 rpm in an orbital shaker. The overnight bacteria culture was centrifuged at 5,000 rpm for 5 min. After centrifugation, the supernatant in each well was discarded and the precipitated bacterial pellet was left. After that, the following solutions were added to each well: 200 μl of solution I, composed of 50 mM glucose, 25 mM Tris–HCl with pH 8 and 10 mM EDTA with pH 8; 200 μl of solution II comprising 200 mM NaOH and 1% SDS; 200 μl of solution III with pH 5.5 and containing 3 M KAc. The plate was sealed with adhesive foil and then turned upside down several times. The foil corresponding to each well was punched with a toothpick. The plate was then heated for 5 min for lysis of cells in boiling water and then immediately cooled on ice water. The 300-μl bacterial cell lysate from each well was transferred to a well of the

96-well MultiScreen®-DV Clear Plate (Millpore SA, France). The Clear Plate was sealed with adhesive foil and then placed onto the new standard 96-well plate, and two plates were fixed with a rubber band. The fixed plates were centrifuged at 5,000 rpm for 5 min. The Clear Plate was then moved, and 300 µl isopropanol was added to each well of the standard 96-well plate at the bottom of the fixed plates. The 96-well plate of added isopropanol was sealed, turned upside down several times, and then placed at −20°C for 2 h followed by centrifugation at 10,000 rpm for 25 min to precipitate plasmid DNA. The precipitated plasmid DNA was further dissolved in 50 µl Milli-Q water, and 10 ng of the precipitated plasmid DNA was used as template for PCR amplification of cDNAs. The PCR was performed in a standard 96-well PCR plate, where each well contained 100 µl of reaction mix composed of 10 ng plasmid templates, 50 mM KCl, 10 mM Tris–Cl with pH 8.3, 1.75 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP and dTTP each, 5 units of DNA Taq polymerase and 1 µM of each of universal M13 primers (forward 5′-CCCAGTC ACGACGTTGTAAAACG-3′ and reverse 5′-AGCGGA TAATTTCACACAGG-3′). The PCR reactions were run for 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 60 s at 58°C, and 3 min at 72°C, and then kept for 10 min at 72°C. All PCR products were examined on 1% agarose gel before further use.

cDNA microarray design and construction

The cDNA microarray was made with PCR-amplified cDNAs of 11,855 unigenes from the maize cDNA library developed by our laboratory according to the above-mentioned procedures. The cDNA library was constructed with leaf, stem and floral bud tissues of normally grown maize plants and maize plants treated for 48 h by a combined stress of WD, salt and alkalinization with the solution comprising 25 mM NaCl, 25 mM Na_2SO_4, 25 mM $NaHCO_3$, 25 mM Na_2CO_3 and with −0.5 MPa WP imposed by 20% PEG8000 (Sigma). The plant tissues used for construction of the library were ones from the 12th fully expanded leaf, the third internode below the 12th leaf and floral bud. Equal amounts of total RNA from each of the tissues of control and stressed plants were pooled. About 5 µg of the pooled RNA was used for each reaction for isolation of mRNA. About 500 ng of pooled mRNA was used to synthesize the first-strand cDNA using the SuperScriptTM III RNase H^- (Invitrogen, Carlsbad, CA, USA). The double-strand cDNA was synthesized by PCR in a 100 µl of reaction mix containing 2 µl of the first-strand cDNA, 1 µM of each of specific primers (forward 5′-AACATATGCGGCCGCATTATGG-3′ and reverse 5′-GATCTTCCACGCGTCGACT-3′), 1× PCR buffer (20 mM Tris–HCLl with pH 8.4, 50 mM KCl), 1.75 mM $MgCl_2$, 0.5 mM dCTP, dATP, dGTP, and dTTP each, and 5 units of DNA Taq polymerase (Promega, Madison, WI, USA). The PCR reactions were run for 1 min at 95°C followed by 16 cycles of 10 s at 95°C, 45 s at 60°C and 3 min at 72°C, and then kept for 10 min at 72°C. The double-strand cDNAs were subject to size fractionation using Sephacryl S-400 Matrix (Promega, Madison, WI, USA) and further separated by running 1% agarose gel. cDNAs with a size of 1 kb or longer were then recovered from the gel using SanPrep Column Kit (Sangon Biotech, Shanghai), directionally ligated into the *Not*I and *Sal*I sites of pBluescript SK(+) and transformed by the electroporation with Gene Pulser XcellTM Electroporation System (Bio-Rad) into *Escherichia coli* strain DH5α cells to generate the cDNA library. After sequencing of clones randomly selected form the library as well as sequence assembling, the expressed sequence tags (ESTs) representing 11,855 unigenes were obtained and have been released to the GenBank EST database annotated under accession numbers from EC855394 to EC872155. The ESTs of arrayed genes were analyzed using the BLASTn and BLASTx programs with ≤1e−5 as a cutoff score against an integrative PlantGDB database (http://www.plantgdb.org/, as of 20 January 2011), and maize genome database (http://www.maizesequence.org/index.html, as of 20 January 2011) (Supplementary Table S1).

To make the cDNA microarray, the PCR-amplified cDNAs were re-arranged and denatured in the standard 384-well plates, where each well contained denaturing solution composed of 0.4 M NaOH and 10 mM EDTA with pH 8.0, heated at 95°C for 5 min and then immediately cooled on ice. Afterward, cDNA samples were spotted from 384-well plates onto 8 × 2 cm Immobilon-TM-Ny^+ membranes (Millipore, Bedford, MA, USA) using a GeneTACTM G3 arrayer (Genomic Solutions, Ann Arbor, MI, USA) equipped with 384 printing pins of 0.4-mm diameter. The microarray on each piece of membranes included 384 subgrids, and each subgrid had 22 cDNA spots arranged within a 5 × 5 array. In each subgrid, the cDNA sample representing one gene was spotted twice. The membranes spotted with cDNAs were exposed for fixing cDNA to ultraviolet of 60 mJ/cm^2. The PCR-amplified maize 18S rDNA were spotted as the positive control. For each array membrane, a total of 12 positive control spots were designed, which were distributed in three subgrids (4 spots each) located at three corners of the array membrane.

Target cDNA labeling and cDNA microarray hybridization

Total RNAs prepared with different tissue materials were used for synthesis of the first-strand cDNAs in the presence of [^{33}P]-dCTP to generate labeled target cDNA available for cDNA microarray hybridization. Target first-strand

cDNA labeling was conducted in 50 μl reaction mix containing 10 μg total RNA, 0.1 μg random hexamer primer, 0.3 μg oligo(dT)17, 10 μl 5× first-strand buffer, 3.5 μl dNTP mix (dATP, dGTP and dTTP, 10 mM each), 60 μCi [^{33}P]dCTP (Amersham Biosciences, Buckinghamshire, UK) and 400 units of SuperScript™ III RNase H (Invitrogen, Carlsbad, CA, USA). The reaction mix was heated for 5 min at 70°C and immediately cooled for 5 min on ice; [^{33}P]-dCTP and reverse transcriptase were then added. The reaction mix was then incubated at 46°C for 3 h, followed by adding 50 μl TEN buffer comprising 10 mM Tris–HCl with pH 8.0, 1 mM EDTA with pH 8.0, and 100 mM NaCl to stop the reaction. The labeled target cDNAs were purified through a Sephadex G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA), heated at 95°C for 5 min, and then immediately cooled on ice before hybridization.

The microarray membrane was rehydrated in 0.5 mM sodium phosphate buffer containing 179 g $Na_2HPO_4 \cdot 12H_2O$, 4 ml of 85% H_3PO_4 and 1,000 ml Milli-Q water of pH 7.2 before pre-hybridization. Pre-hybridization was conducted at 60°C for 2 h in the hybridization solution containing 1% (w/v) BSA, 1 mM EDTA with pH 8.0, 50 mM sodium phosphate buffer and 7% (w/v) SDS. Following pre-hybridization, hybridization was conducted overnight at 60°C in the hybridization solution containing the labeled target cDNAs. The hybridized membranes were washed twice (10 min each) at 46°C with a solution containing 2× SSC and 0.1% (w/v) SDS in an orbital shaker, and further washed twice (10 min each) with a solution containing 0.1× SSC and 0.1% (w/v) SDS.

Microarray data analysis

The hybridized membranes were sealed with thin plastic films, exposed for 48 h in a storage phosphor screen (Kodak, Rochester, New York, USA), and then scanned with a Typhoon 9200 scanner (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to obtain the image data. The image data were analyzed using the ArrayVision 6.0 program (Imaging Research, Ontario, Canada) under default settings to generate a normalized artifact removed volume (nARVOL) value of each spot following the previous method (Wolfinger et al. 2001). Microarray hybridization of each sample of control or stressed plants at each treatment time point was biologically repeated twice, generating four nARVOL values for each gene. The signal intensity for each gene in the same tissue of control or stressed plants at the specific time point was expressed with the value of ln (natural logarithm) transform of the mean of four nARVOL values. The reproducibility of the experiment was evaluated with the Pearson's correlation coefficient (r^2) calculated by Microsoft Excel and Access program.

Gene expression was based on a comparison of ln (natural logarithm) transform of four nARVOL values between hybridization signal intensities of the same gene element of the same tissues of both control and stressed plants at the specific treatment time point. The differential expression of genes was judged through false discovery rate (FDR) with a Q value of <0.01 (Pounds and Cheng 2006; http://www.stjuderesearch.org/site/depts/biostats/robustfdr). If the fold of ln nARVOL, i.e., WD:control, was >1 and had a Q value of <0.01, the gene would be identified as up-regulated. If the fold of ln nARVOL was less than −1 and had a Q value of <0.01, the gene would be identified as down-regulated.

Biological annotation and expression clustering of genes

The genes were biologically annotated according to the method of Gene Ontology annotation, GO slims (Camon et al. 2004; Harris et al. 2004; http://www.geneontology.org). GO consists of over 16,000 terms, distributed over three ontologies, which describe what a protein does (molecular function), how it does it (biological process) and where it performs this task in a generic cell (cellular component) (Camon et al. 2004).

The gene expression pattern was clustered according to hierarchical clustering and K-means clustering methods, which are based on heuristic clustering algorithm and commonly used to interpret patterns of gene expression (Yeung et al. 2001; Yeung and Ruzzo 2001).

Quantitative real-time PCR (qRT-PCR)

The template for qRT-PCR was the first-strand cDNA synthesized with the total RNA samples used in the cDNA microarray hybridization using SuperScriptTM III RNase H$^-$ (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. qRT-PCR was conducted using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Inc. Hercules, CA) on the iCycler system (Bio-Rad Laboratories, Inc. Hercules, CA). The maize 18S rDNA was used as an internal control. Primers were designed using Primer Express software (Applied Biosystems) to allow for amplification of about 100-bp products. qRT-PCR reactions were run for 1 min at 93°C followed by 50 cycles of 10 s at 93°C and 20 s at 60°C, and 10 s at 72°C. Three repeat qRT-PCR reactions were conducted for each total RNA sample. Standard curves were established for all investigated genes using a series of amplicon dilutions. The relative mRNA level was calculated as $2^{-(\Delta CT\ \text{treatment} - \Delta CT\text{control})}$. Primers used are listed in Supplementary Table S2.

Results

Characterization of phenotypic and physiological responses of maize inbred line YQ7-96 under WD

To provide a base for analysis of gene expression, some physiological responses of the three-leaf-old maize plants were investigated under WD and RW. When compared to the control group, the WP in stressed leaves significantly declined with stress time course, but returned to the control level 24 h after RW treatment (Fig. 1a). Leaf elongation of WD-stressed seedlings was not significantly affected within 0.5 h after WD, started to significantly ($p < 0.05$) decrease 1 h after WD, and recovered obviously but not completely ($p > 0.05$) to the control level 24 h after RW treatment (Fig. 1b). Root elongation of the stressed seedlings showed no significant difference from that of the control seedlings throughout WD, but was 2.4 times that of control roots 24 h after RW treatment (Fig. 1b).

When compared with the control seedlings, WD-stressed seedlings showed significant ($p < 0.01$) decrease in photosynthetic rate (PR) (Fig. 2a), transpiration rate (TR) (Fig. 2b) and stomatal conductance (SC) (Fig. 2c), agreeing with the previous viewpoint that when maize encounters water deficits, there is a decline in photosynthesis per plant (Bruce et al. 2002). However, intracellular CO_2 concentration (CC) (Fig. 2d) of the WD-treated seedlings significantly ($p < 0.01$) decreased 0.5 h after stress treatment, but increased after 1 h, consistent with a previous study (Lawlor 2002). After RW treatment, PR, TR and SC of the stressed seedlings recovered to the control levels, but CC was still higher than that of the control level. All these results indicate that the PEG treatment indeed led to WD effects on the maize seedlings.

The outline of genes arrayed on the cDNA microarray

Unique cDNAs representing 11,855 maize unigenes were PCR amplified and then arrayed on the microarray membranes, which accounted for approximately 20% of the estimated 59,000 maize genes (Messing et al. 2004). The results of functional annotation and categorization of the genes can be found in Supplementary Table S1. Of these arrayed genes, 10,817 (91.2%) were homologous to known maize EST sequences, whereas 1,038 (8.8%) were identified as novel maize genes because they had no hits, an *E* value of >1e−5, or homology with the EST sequences from non-maize organism. In these genes, 4,559 (38.5%) were annotated with a description of either definitive function or unknown protein, of which 897 (19.7%) belonged to protein-encoding genes from maize, 3,035 (66.6%) to protein-encoding genes from rice and 627 (13.7%) to protein-encoding genes from other organisms.

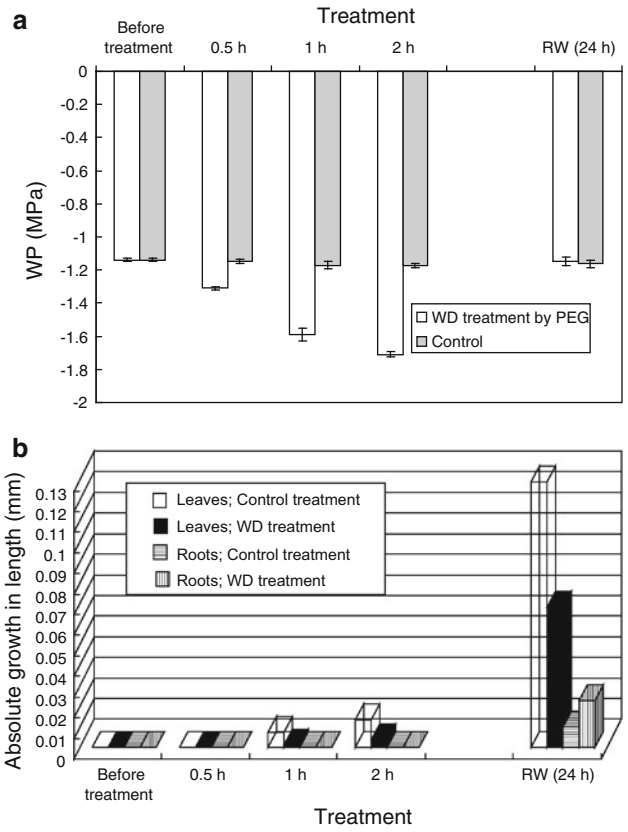

Fig. 1 Leaf WP and growth elongation of seedlings of maize inbred line YQ7-96 at the three-leaf stage under WD and RW. **a** Leaf WP. **b** Growth elongation of leaves and roots. WD treatment of the seedlings was conducted in 1× nutrient solution with −0.5 MPa WP imposed by PEG 8000. WP datum presented at each treatment time point was the mean ± standard error of data of the second leaves from ten individual seedlings in two independent experiments, with five plants for each experiment. The datum of growth elongation at each treatment time point was the mean ± standard error of data of the third leaves from five individual seedlings in two independent experiments, with two to three plants for each experiment. RW treatment was conducted after a 2-h WD treatment. Measurements were conducted at 10:00 h. *RW* re-watering, *WD* water deficit, *WP* water potential

The remaining 7,296 (61.5%) could not be annotated (Supplementary Table S1).

Of ESTs of arrayed genes, 8,082 (68%) could be well mapped onto maize chromosomes against maize genome database (http://www.maizesequence.org/index.html, as of 20 January 2011) by sequence homology analysis under the criteria of both *E* value ≤1e−5 and identity of at least continuous 100 nucleotides. Some ESTs could be mapped onto more than one site of a chromosome (Supplementary Table S3) and/or onto several chromosomes.

Global changes in the number of WD-regulated genes

As a result, the r^2 value of the microarray hybridization data ranged from 0.79 to 0.955 between repeats of the same

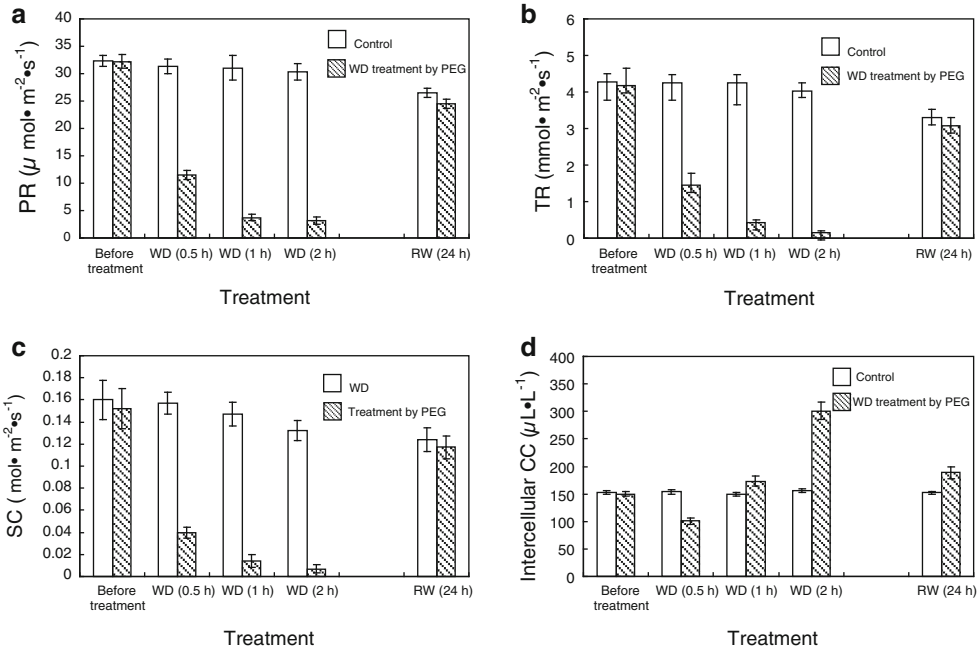

Fig. 2 Changes in photosynthetic parameters of maize inbred line YQ7-96 seedlings at the three-leaf stage under WD and RW. **a** PR; **b** TR; **c** SC; **d** Intercellular CC. WD and RW treatments of the seedlings were conducted as described in Fig. 1. The measurement was conducted at 10:00 a.m. The datum presented at each treatment time point was the mean ± standard error of data of second leaves from five individual seedlings in two independent experiments, with two to three plants for each experiment. *CC* CO_2 concentration, *PR* photosynthetic rate, *RW* re-watering, *SC* stomatal conductance, *TR* transpiration rate, *WD* water deficit

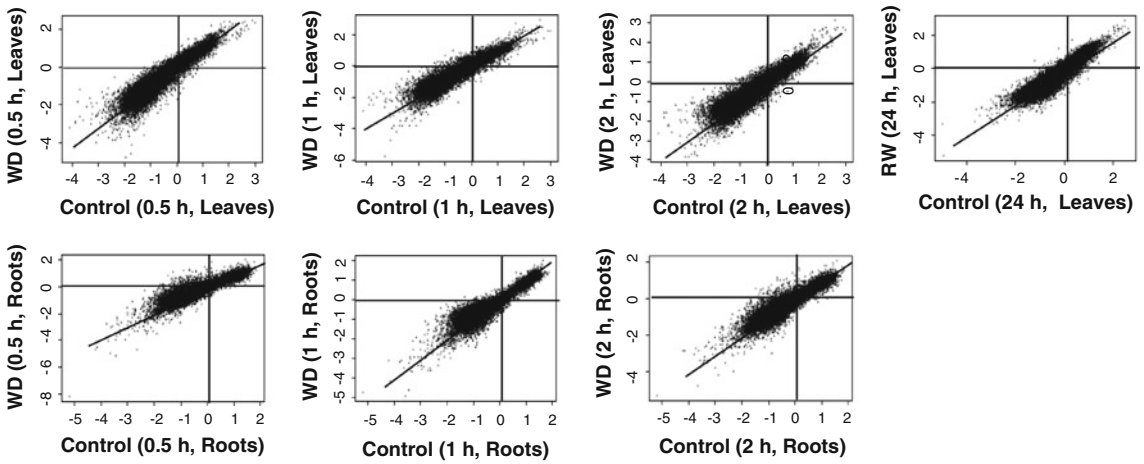

Fig. 3 The scatter plot of ln nARVOL values of signal intensity resulting from microarray hybridization. The datum corresponding to each spot was ln nARVOL of average of four data produced by two independent hybridizations with labeled cDNAs synthesized separately with total RNA from two individual plants. The nARVOL values were calculated using the ArrayVision 6.0 program (Imaging Research, Ontario, Canada) under default settings. RW treatment was conducted after a 2-h WD treatment. *In* natural logarithm, *RW* re-watering, *WD* water deficit

gene in the same membrane microarray and from 0.94 to 0.982 between two repeated membrane microarrays for the same biological sample (Supplementary Table S4). The qRT-PCR analysis of expression of total 126 time points of 18 genes substantiated that the microarray data were of high creditability (Supplementary Fig. S1). The signal intensity of the analyzed genes is presented in a scatter plot (Fig. 3). According to analysis by FDR with a Q value of <0.01 (Supplementary Table S5), a total of 3,400 (28.6%) of the arrayed genes showed differential expression under WD (Fig. 4a). Of these regulated genes, 1,481 (43.6%) and 1,471 (43.3%) were differentially regulated, specifically in leaves and roots, respectively; 448 (13.1%) were common to both leaves and roots (Supplementary Table S6).

Of WD-regulated genes in leaves, 1,278 (66.3%) and 651 (33.7%) were up- and down-regulated, respectively; the gene expression was characterized by up-regulation 1 h after WD treatment and by down-regulation 2 h after WD treatment (Fig. 4b; Supplementary Table S6). Of WD-regulated genes in roots, 1,889 (98.4%) and 30 (1.6%) were up- and down-regulated, respectively; the gene expression was characterized by significant up-regulation 0.5 h after WD treatment and by significant down-regulation 1 h after WD treatment (Fig. 4b; Supplementary Table S6).

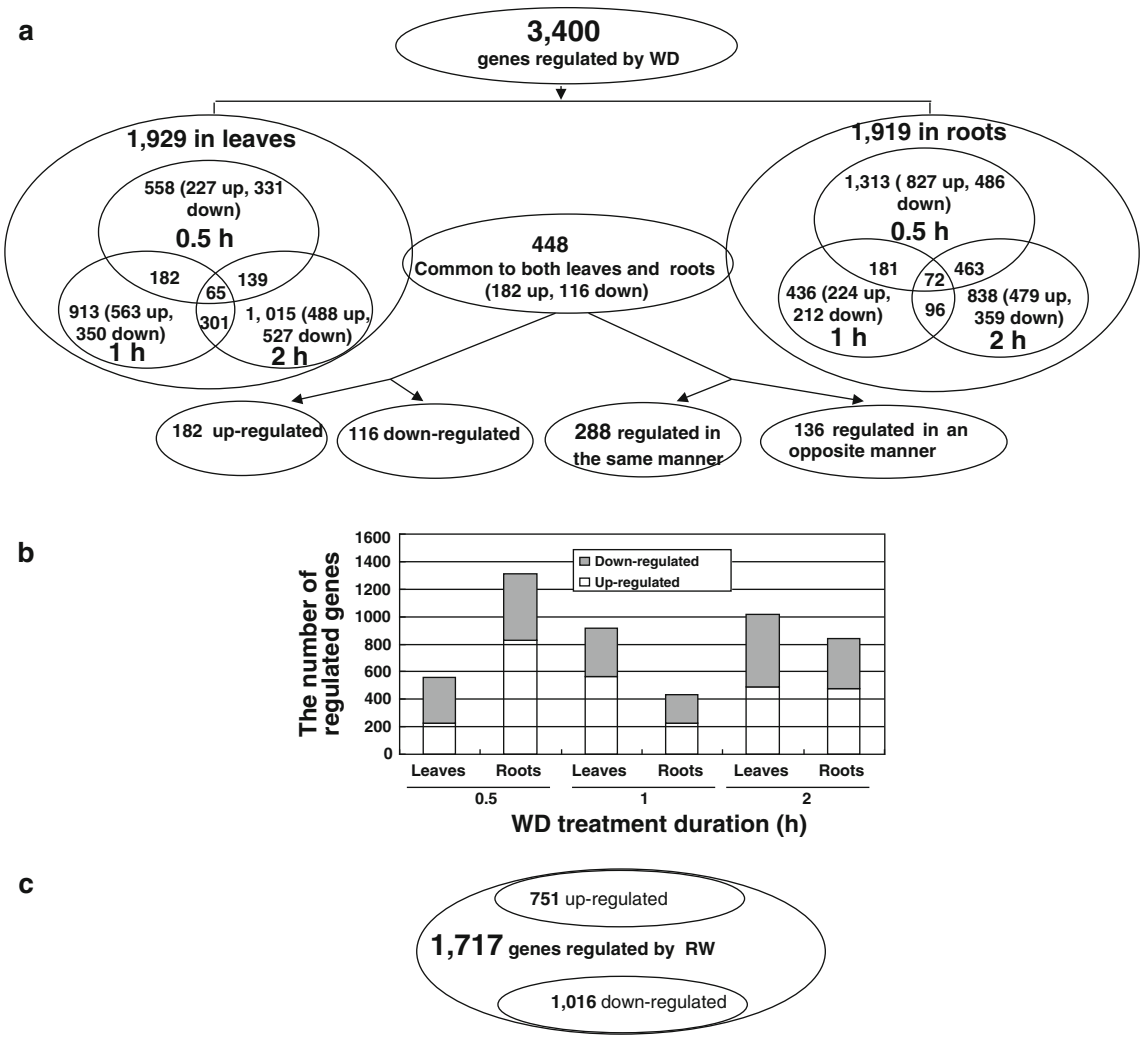

Fig. 4 The number of WD- and RW-regulated genes in maize inbred lineYQ7-96 at the three-leaf stage. **a** The total number of genes regulated specifically by WD. **b** Changes in WD-regulated genes with WD treatment course. **c** Genes regulated specifically by RW in leaves. RW treatment was conducted after a 2-h WD treatment. *Down* down-regulated, *RW* re-watering, *Up* up-regulated, *WD* water deficit

Evidently, for whole maize plant response to short-term WD, gene expression was characterized by up-regulation rather than down-regulation. However, such feature of gene expression depends greatly on WD conditions and the maize tissues (Yu and Setter 2003; Jia et al. 2006).

Of WD-regulated genes common to both leaves and roots (Fig. 4a), 182 (40.6%) and 116 (25.9%) were up- and down-regulated, respectively; 288 (64.3%) and 136 (30.4%) were regulated in the same manner and in an opposite manner, respectively.

Changes in the number of regulated genes with the time course of WD

In leaves, the total number of regulated genes obviously increased with WD (Fig. 4b). In roots, the change in the total number of regulated genes, however, coincided with that in the whole maize plant (Fig. 4b).

The number of regulated genes after a 0.5 h stress treatment was much higher in stressed roots than in stressed leaves, suggesting that gene expression response to WD was more rapid in roots than in leaves during this stress stage. In addition, according to the timing of expression, expression of WD-regulated genes could be classified into transient response at one time point, and recurrent response (RR) at two or three time points. Obviously, gene expression responses are fairly rapid between 0.5 and 2 h after WD (Fig. 4b), and this stage can be considered to be a "key" WD adaptation period of whole maize plants. Transient response can be further divided into early response (ER) 0.5 h after WD treatment and late response (LR) over 0.5 h after WD treatment.

Of WD-regulated genes in leaves, 332 (17.2%) were ER specific, 1,040 (53.9%) were LR specific, and 557 (28.9%) were of RR. More interestingly, of 18 regulated kinase genes, 4 (22.2%) were ER specific and down-regulated and 6 (33.3%) were LR specific and down-regulated. Of 9 transcription factor genes regulated, 5 (55.6%) were ER specific. Of 55 ribosome-related genes regulated, 31 (56.4%) were LR specific (Fig. 4a; Supplementary Table S6).

Of WD-regulated genes in roots, 741 (38.6%) were of ER, 510 (26.6%) were of LR and 668 (34.8%) were of RR (Fig. 4a; Supplementary Table S6). Of 60 ribosome-related genes regulated, 46 (76.7%) were ER specific. A total of 8 transcription factor genes were regulated, of which 5 (62.5%) were of ER and 6 (75%) were up-regulated (Fig. 4a; Supplementary Table S6).

Of the up-regulated genes common to both leaves and roots (Fig. 4a; Supplementary Table S5): 100 (20.5%) and 139 (28.5%) were up-regulated, respectively, 0.5 and 2 h after WD stress treatment in roots; 148 (30.3%) and 128 (28.6%) were up-regulated, respectively, 1 and 2 h after WD stress treatment in leaves.

Expression of WD-regulated gene in leaves following RW treatment

Compared to the expression under WD treatment, the WD-regulated genes presented the following expression tendencies (Supplementary Table S6) after RW treatment: (1) 1,549 (80.3%) returned to the control levels, (2) 43 (2.2%) continued to be down-regulated, (3) 20 (1%) continued to be up-regulated, and (4) 316 (16.4%) was regulated in a manner opposite to that under WD. Such expression profile was somewhat similar to results observed in the ear tissue of WD-treated maize (Bruce et al. 2002), indicating that the process of growth recovery of maize from WD was considerably complex. Expression of WD-regulated genes in roots under RW was not assayed because of constraints in root tissue materials.

Biological annotation of WD-regulated genes

Gene function category of WD-regulated genes was assigned using the GO method. One gene could, maybe, be assigned to more than one functional category because of its multiple functions.

A total of 877 WD-regulated genes in maize leaves could be functionally grouped. According to the number of the regulated genes in a specific category, the major effects of WD on cellular function were directed at catalytic activity (424 genes), binding (284 genes), structural molecule activity (107 genes) and transporter activity (39 genes). WD influence upon cellular location focused on the membrane (114 genes). As for cellular processing, WD produced great effects on the metabolic process (512 genes), electron transport (47 genes), transport (91 genes), cell communication (12 genes), response to stimulus (11 genes) and cell differentiation (5 genes) (Supplementary Table S6).

The major functional groups affected by WD in roots were similar to those in leaves, including catalytic activity (218 genes), binding (157 genes), structural molecule activity (76 genes) and transporter activity (17 genes) in terms of cellular function; membrane (56 genes), cytoplasm (131 genes), nucleus (37 genes) and extracellular region (13 genes) in terms of cellular location; metabolic process (300 genes), electron transport (17 genes), transport (43 genes) and response to stimulus (9 genes) in terms of cellular processing (Supplementary Table S6).

In comparison with similar studies (Bray 2004; Zhou et al. 2007), WD-affected functional categories that were newly found were catalytic activity, binding, oxidoreductase activity and motor activity in terms of cellular function; cytoplasm, extracellular region, chromosome and external encapsulating structure in terms of cellular location; metabolic process, amino acid and derivative metabolic process, and cell death in terms of cellular processing (Supplementary Table S1).

Expression of protein synthesis- and degradation-related genes under WD

A total of 55 ribosome-related genes were differentially regulated by WD in leaves, of which the majority (45 genes, 81.8%) were down-regulated; on the contrary, 51 (85%) of 60 ribosome-related genes were up-regulated by WD in roots (Supplementary Table S7). Another strong impression was that 31 (56.4%) of the regulated ribosome-related genes in leaves were LR specific, while 46 (76.7%) in roots were ER specific, maybe reflecting the timing of WD signal transduction from roots to leaves.

Of 12 WD-regulated proteosome and ubiquitin genes in leaves, 8 (66.7%) were down-regulated. Of 13 WD-regulated proteosome and ubiquitin genes in roots, 7 (53.9%) were up-regulated (Supplementary Table S7).

Expression of genes related to nuclear activity, transposons and retrotransposons under WD

A total of 15 tubulin and histone genes were regulated by WD in leaves, while 16 were regulated by WD in roots (Supplementary Table S7). These genes are thought be related to the cell cycle of plants (Yu and Setter 2003). The data indirectly agree with a previous finding that WD can affect cell division and nDNA endoreduplication (Setter and Flannigan 2001).

A total of 8 nuclear pore protein, nucleolar protein and nucleotide translocator genes were regulated by WD

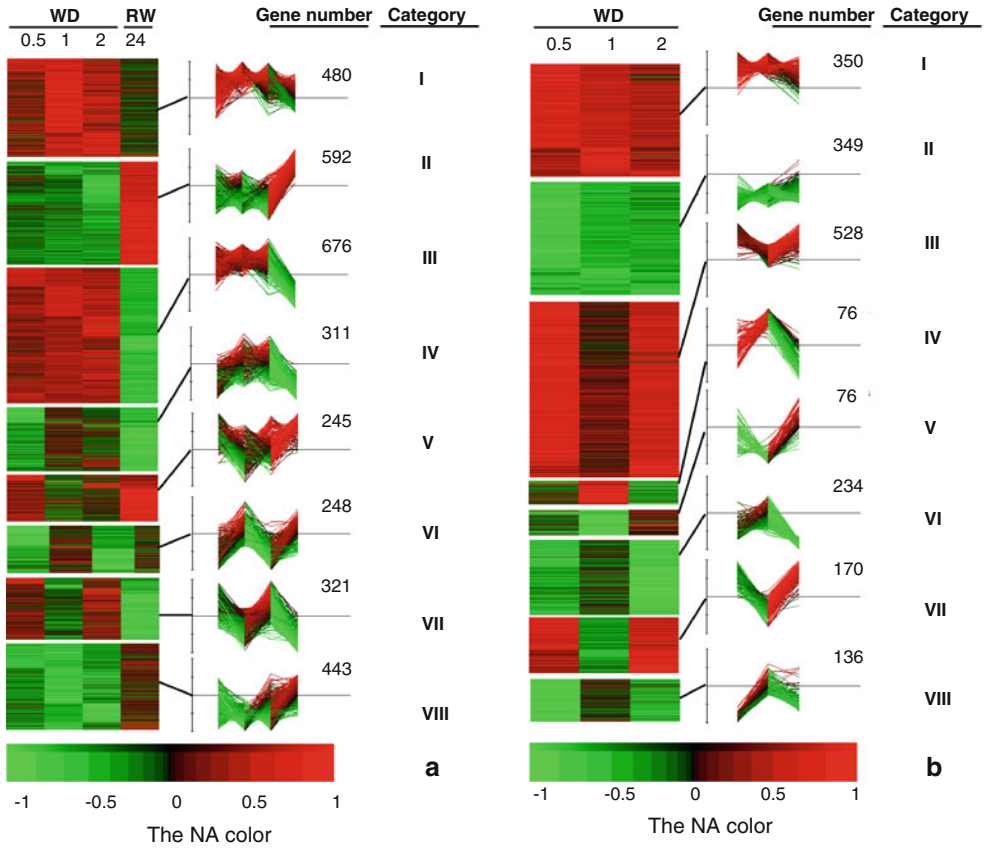

Fig. 5 Clustering of expression of the WD-regulated genes in leaves of maize inbred line YQ7-96 at the three-leaf stage. **a** Patterns of gene expression in leaves. **b** Patterns of gene expression in roots. Gene expression was clustered according to the hierarchical clustering and K-means clustering methods. RW treatment was conducted after a 2-h WD treatment. *RW* re-watering, *WD* water deficit

(Supplementary Table S7), suggesting that WD can probably affect functions of nuclear transport of maize cells. A total of 8 transposon and retrotransposon genes were regulated by WD in roots, of which 4 were up-regulated (Supplementary Table S7), indirectly suggesting that WD could affect the transposition of transposons and retrotransposons and therefore speculating that WD exerts an effect on genome stability of maize, because transposition of transposons and retrotransposons is one of the mechanisms that create genome instability (Baumel et al. 2002; Kazazian and Goodier 2002).

Expression patterns of WD-regulated genes

It has been believed that genes clustered into the same set tend to participate in common processes (Tavazoie et al. 1999). To give a clue to discern potential biological functions of functionally unknown genes spotted on the microarray, the expression patterns of WD-regulated genes were thereby clustered using hierarchical clustering K means methods (Fig. 5). Some genes could be assigned to more than one expression pattern. However, genes of functionally uncategorized genes still accounted for the overwhelming majority in each pattern (Fig. 5). For leaves, the expression of WD-regulated genes was categorized into eight distinct patterns (Fig. 5a). The largest expression pattern was category III with 676 genes. The expression of WD-regulated genes in roots was also categorized into eight patterns (Fig. 5b), with category III of 528 genes as the largest. Genes under each expression pattern can be obtained from Supplementary Table S8. The major subfunctional groups that were affected in each expression category are presented in Tables 1 and 2.

Genes regulated specifically by RW in leaves

Physiological responses of plant growth recovery from WD under RW seem somewhat different from those under WD conditions (Wong et al. 2006). To outline the gene expression of maize growth recovery from WD, we analyzed the expression of genes of leaves of maize seedlings that were stressed for 2 h under RW treatment. The criteria for defining

Table 1 Comparison between major WD-affected sub-functional groups of gene expression categories with an identical expression pattern in leaves and roots

Expression category matching between tissues	Cellular function	Gene percentage (% of genes in the expression category)		Cellular location	Gene percentage (% of genes in the expression category)		Cellular process	Gene percentage (% of genes in the expression category)	
		Leaves	Roots		Leaves	Roots		Leaves	Roots
I (480 genes) in leaves; I (350 genes) in roots	Catalytic activity	25.4	21.1	Intracellular	23.1	29.3	Metabolic process	28.1	26.4
	Binding	20.2	23.4	Membrane	15.4	9.3	Macromolecule metabolic process	16.6	17.8
	Kinase activity	1.9	2.9	Extracellular region	3.1	4.9	Nucleic acid metabolic process	5.0	3.7
	Ion transporter activity	4.2	1.2	Nucleus	3.1	1.3	Transport	7.0	3.1
	Uncategorized	48.4	51.5	Uncategorized	55.4	56.0	Uncategorized	43.2	49.1
II (592 genes) in leaves; VI (234 genes) in roots	Catalytic activity	20.8	21.1	Intracellular	30.1	25.0	Metabolic process	26.0	29.4
	Binding	23.3	29.7	Membrane	7.4	13.1	Macromolecule metabolic process	18.3	16.7
	Kinase activity	3.3	3.1	Extracellular region	1.6	1.2	Nucleic acid metabolic process	5.7	6.9
	Ion transporter activity	1.5	0.8	Nucleus	4.2	7.1	Transport	4.7	3.9
	Uncategorized	51.3	45.3	Uncategorized	56.6	53.6	Uncategorized	45.3	43.1
VI (248 genes) in leaves; VIII (136 genes) in roots	Catalytic activity	22.2	17.6	Intracellular	25.8	31.8	Metabolic process	23.9	28.1
	Binding	19.9	28.6	Membrane	11.7	9.1	Macromolecule metabolic process	17.1	12.5
	Kinase activity	2.8	2.2	Extracellular region	0.8	0	Nucleic acid metabolic process	6.3	6.3
	Ion transporter activity	2.3	2.2	Nucleus	4.7	9.1	Transport	4.4	1.6
	Uncategorized	52.8	49.5	Uncategorized	57.0	50.0	Uncategorized	48.3	51.6
VII (321 genes) in leaves; VII (170 genes) in roots	Catalytic activity	26.3	15.5	Intracellular	22.7	29.4	Metabolic process	29.3	25.4
	Binding	21.1	21.6	Membrane	18.2	7.4	Macromolecule metabolic process	16.7	19.3
	Kinase activity	4.0	1.7	Extracellular region	1.1	1.5	Nucleic acid metabolic process	4.7	6.6
	Ion transporter activity	2.3	3.4	Nucleus	5.7	5.1	Transport	6.7	3.9
	Uncategorized	46.3	57.8	Uncategorized	52.3	56.6	Uncategorized	42.7	44.8
VIII (443 genes) in leaves; II (349 genes) in roots;	Catalytic activity	19.0	23.5	Intracellular	30.4	27.8	Metabolic process	27.9	29.4
	Binding	24.8	23.5	Membrane	9.5	9.3	Macromolecule metabolic process	17.8	19.6
	Kinase activity	2.1	2.6	Extracellular region	0.8	4.1	Nucleic acid metabolic process	4.4	3.9
	Ion transporter activity	1.8	1.3	Nucleus	3.8	6.2	Transport	4.7	5.2
	Uncategorized	52.1	49.0	Uncategorized	55.5	52.6	Uncategorized	45.2	41.8

I, II, VI, VII and VIII indicate the expression category in Fig. 5. Genes under each expression pattern and in each functional group can be obtained from Supplementary Tables S5 and S6

Table 2 Major WD-affected sub-functional groups of gene expression categories with a distinct expression pattern in leaves and roots

Functional category	Expression category and gene percentage (% of genes in the expression category) in each sub-functional group					
	Leaves			Roots		
	III (676 genes)	IV (311 genes)	V (245 genes)	III (528 genes)	IV (76 genes)	V (76 genes)
Cellular function						
Catalytic activity	23.1	23.0	22.9	19.6	20.4	31.5
Binding	25.4	24.6	22.2	22.0	22.2	16.7
Kinase activity	3.7	3.8	2.6	1.7	3.7	7.4
Ion transporter activity	1.1	1.6	0.7	2.5	5.6	0
Uncategorized	46.8	47.0	51.6	54.2	48.2	44.4
Cellular location						
Intracellular	23.3	25.4	28.4	29.2	26.3	31.3
Membrane	11.9	15.3	11.0	9.6	15.8	9.4
Extracellular region	3.1	0.8	0	0.9	0	0
Nucleus	9.7	6.8	6.4	3.9	5.3	3.1
Uncategorized	52.0	51.7	54.1	56.3	52.6	56.3
Cellular process						
Metabolic process	27.1	21.7	27.4	26.7	27.8	29.9
Macromolecule metabolic process	15.7	19.3	16.2	18.1	18.1	20.9
Nucleic acid metabolic process	6.3	5.7	5.6	5.5	5.5	1.5
Transport	5.6	6.1	2.5	5.5	5.5	6.0
Uncategorized	45.4	47.2	48.2	44.2	44.4	41.8

III, IV and V indicate the expression category in Fig. 5. Genes under each expression pattern and in each functional group can be obtained from Supplementary Tables S5 and S6

differentially regulated genes were the same as those for judging WD-regulated genes. Consequently, 1,717 (14.48%) of the arrayed genes were identified as RW-regulated genes (Fig. 4c; Supplementary Table S6). Of these genes, 380 were also WD responsive, and the remaining 1,337 were differentially regulated only under RW. On the whole, the expression of RW-regulated genes was characterized by down-regulation (Supplementary Table S6), as opposed to the characteristic up-regulation of WD-regulated gene expression in leaves (Fig. 4b).

Of the RW-regulated genes, 461 (26.9%) could be functionally categorized (Supplementary Table S6). The major functional groups impacted by RW were the same as those affected by WD.

Expression of photosynthesis-related genes under WD

A total of 24 photosynthesis-related genes were regulated by WD in leaves, of which 12 (50%) were significantly down-regulated (Supplementary Table S7), including RuBisCO protein (EC856616, EC868434 and EC862541), triose phosphate translocators (EC858337, EC863241, EC863458 and EC867958) and orthophosphate dikinase (EC869944) (Supplementary Table S6).

Discussion

PEG treatment

PEG is extensively used to impose WD in solution culture to mimic dry soil (Verslues et al. 1998; van den Berg and Zeng 2006). However, a potential disadvantage is that PEG treatment causes root oxygen deficiency because of its high viscosity, thereby inhibiting plant growth. For maize seedlings, a long-term (13–23 h) and lower WP (less than −0.3 MPa) treatment by PEG can significantly limit elongation of the roots of the seedling (Verslues et al. 1998). In this study, the duration of PEG treatment was much shorter (not more than 2 h) and did not cause inhibition of root elongation (Fig. 1b).

The number and functional annotation of unique genes

The maize cDNA library used in this study was constructed with a mix of tissues from different maize plants grown under normal conditions and under a combined stress including WD, salt and alkalinization. Therefore, the occurrence of a number of novel genes seems reasonable. As in some previous studies (Casu et al. 2004), the unique

genes in this study were estimated on the basis of EST sequences. In this case, the number of potential unique genes arrayed was likely overestimated, because some of the EST sequences were shorter in length.

Gene function annotations often depend on domain identifications. Even in well-studied model species, a part of the genes are not yet assigned biochemical or structural functions due to lack of specific domains (Lin et al. 1999). In this study, a part of the genes were not annotated, partly because analyzed EST sequences were also shorter in length.

Expression and functional categories of WD-regulated genes

It is not possible to, only according to expression changes, assign roles to responsive genes (Bray 2002, 2004). Nevertheless, gene expression pattern can give a clue for further investigation of the functions of WD-regulated, but functionally unknown, genes with the same expression pattern as the functionally known genes that were documented to function in drought tolerance.

It is very difficult, only on the basis of gene expression profile, to define the roles of ER and LR genes in maize in response to WD. Our gene expression data suggested that some of the genes regulated throughout WD likely function as both signal sensing and defense to WD. The indications for this were: up-regulated expression of some stress-responsive genes such as protein kinase (EC863363) and putative sterol-C5(6)-desaturase (EC863569), heat stress transcription factor (EC869317) and ethylene-insensitive 3-like protein (EC870944) in leaves under WD; putative pathogenesis-related protein (EC860685) and low temperature and salt responsive protein (EC865066) in roots under WD (Supplementary Table S6).

Differences and coordination of gene expression between leaves and roots in the response of whole maize plant to WD

Genes differentially regulated specifically in tissues under WD (Fig. 4a; Supplementary Table S6) show change in regulated gene number with WD treatment (Fig. 4a). Gene expression patterns (Fig. 5) and gene number in the same sub-functional groups (Tables 1, 2) indicated considerable differences between leaves and roots in transcriptional responses to WD. The number of WD-regulated genes common to leaves and roots and the expression of the same gene in both tissues, either in the same or in an opposite manner, strongly suggest that there are mechanisms for the coordination of gene expression between leaves and roots in the response of whole maize plant to WD.

A scene of protein biosynthesis under WD

As well known, metabolic processes and/or catalytic activities depend greatly on a number of enzymes composed of proteins. The expression of protein synthesis-related genes under WD clearly depicted a scene of protein biosynthesis that was inhibited in WD-stressed leaves, but enhanced in WD-stressed roots. This partially explains why the major effects of WD concentrates on metabolic processes coupled with catalytic activity, as indicated in Tables 1 and 2, and also gives a very useful clue to future studies of proteomics of maize under WD.

Correlation of growth elongation with expression of growth-related genes

It is impossible to parallel maize growth elongation with all gene expression data. Plant growth and development are very complex, but at least associated with the production of phytohormones such as auxin. Higher levels of auxin can lead to inhibiting effects on leaf and root elongation (Keller et al. 2004; Woodward and Bartel 2005). Significant up-regulation of expression of auxin genes (EC863209, EC856213 and EC869856) in WD-stressed leaves and roots (Supplementary Table S6) may in part explain the inhibition of leaf elongation during WD stress (Fig. 1b).

Correlation of photosynthesis with expression of photosynthesis-related genes under WD

The C_4 plants are characterized by a biochemical CO_2-concentrating mechanism operated by Rubisco in the bundle sheath (von Caemmerer et al. 2005). CO_2 fixation needs some acceptors, such as orthophosphate dikinase (Matsuoka et al. 1988). Export of the fixed carbons from the chloroplasts into the cytosol involves the triose phosphate translocator (Fischer et al. 1994). Activation and maintenance of Rubisco's activity involve proteins of the RuBisCO family (von Caemmerer et al. 2005). The down-regulated expression of RuBisCO, triose phosphate translocator genes and orthophosphate dikinase gene in WD-stressed leaves (Supplementary Table S6) suggests that WD probably affected CO_2 uptake, CO_2 partitioning and transport of fixed carbons in maize leaf cells.

Expression of WD-regulated genes after RW treatment, genes co-regulated by WD and RW, and the roles of RW-specific responsive genes

Expression of a considerable number of WD-regulated genes recovered to the control level after WD treatment, but a great number of RW-specific responsive genes occurred (Supplementary Table S6), suggesting that

growth recovery of WD-stressed maize plants from WD was also involved in re-construction of some cellular activities through some growth recovery-specific signaling circuits. Genes co-regulated by WD and RW are, maybe, important links between maize WD tolerance to, and growth recovery from, WD, indicating that maize can likely also employ some mechanisms to respond to both WD and RW.

Charting plant molecular responsive mechanisms under WD conditions is yet a great challenge to scientists. The significance of our microarray data lies in raising the curtain on some differences and commonalities between leaves and roots when whole maize plant copes with WD at the seedling stage. The data analyses allow us to conclude that: (1) whole maize plant tolerance to WD as well as growth recovery from WD depends at least in part on transcriptional coordination between leaves and roots; (2) WD effects on the maize concentrate on the basal metabolism; (3) WD could probably affect CO_2 uptake, and partitioning, and transport of fixed carbons; (4) WD could likely influence nuclear activity and genome stability; and (5) maize growth recovery from WD is likely involved in some specific signaling pathways related to RW-specific responsive genes.

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