Identification of genes expressed in maize root cortical cells during lysigenous aerenchyma formation using laser microdissection and microarray analyses

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Summary

- To adapt to waterlogging in soil, some gramineous plants, such as maize (*Zea mays*), form lysigenous aerenchyma in the root cortex. Ethylene, which is accumulated during waterlogging, promotes aerenchyma formation. However, the molecular mechanism of aerenchyma formation is not understood.
- The aim of this study was to identify aerenchyma formation-associated genes expressed in maize roots as a basis for understanding the molecular mechanism of aerenchyma formation. Maize plants were grown under waterlogged conditions, with or without pretreatment with an ethylene perception inhibitor 1-methylcyclopropene (1-MCP), or under aerobic conditions. Cortical cells were isolated by laser microdissection and their mRNA levels were examined with a microarray.
- The microarray analysis revealed 575 genes in the cortical cells, whose expression was either up-regulated or down-regulated under waterlogged conditions and whose induction or repression was suppressed by pretreatment with 1-MCP.
- The differentially expressed genes included genes related to the generation or scavenging of reactive oxygen species, Ca^{2+} signaling, and cell wall loosening and degradation. The results of this study should lead to a better understanding of the mechanism of root lysigenous aerenchyma formation.

Introduction

The aerenchyma is a specialized tissue consisting of longitudinal gas spaces, which enables the internal movement of gases (e.g. O_2, CO_2, ethylene and methane) in plant roots, petioles and stems (Armstrong, 1979; Colmer, 2003). The internal transport of oxygen via the aerenchyma from shoots to roots is especially important for survival under waterlogged conditions. In general, aerenchyma can be classified into two main types: schizogenous aerenchyma and lysigenous aerenchyma (Jackson & Armstrong, 1999; Seago *et al.*, 2005). Schizogenous aerenchyma is formed by the creation of gas spaces between cells as a result of highly regulated cell separation and differential cell expansion, without cell death taking place. Lysigenous aerenchyma is formed by the creation of gas spaces as a result of death and the subsequent lysis of some cells (e.g. root cortical cells), and is observed in many crops, such as barley, maize, rice and wheat (Jackson & Armstrong, 1999; Evans, 2003).

Many wetland plant species (e.g. rice and *Juncus effusus*) constitutively form lysigenous aerenchyma in roots under well-drained soil conditions, and its formation is enhanced on soil waterlogging. On the other hand, lysigenous aerenchyma in nonwetland plants, including maize, is not

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normally formed under well-drained soil conditions, but is induced by waterlogging, hypoxia, mechanical impedance and even under aerobic conditions by nutrient deficiency (Drew *et al.*, 1979; He *et al.*, 1992, 1996a). Because aerenchyma formation can be induced in maize roots by external stimuli, maize has often been used as a model plant for understanding the mechanism of aerenchyma formation.

Ethylene has been implicated in lysigenous aerenchyma formation in maize and rice (Drew *et al.*, 1979; Jackson & Armstrong, 1999; Shiono *et al.*, 2008). In maize roots, ethylene biosynthesis is stimulated by enhancing the activities of two ethylene biosynthetic enzymes (1-aminocyclopropene-1-carboxylic acid (ACC) synthase and ACC oxidase) under hypoxic conditions (He *et al.*, 1996a). Indeed, hypoxic treatment increases the production of ethylene in maize roots by several fold within 3 h (Geisler-Lee *et al.*, 2010). The treatment of maize roots with inhibitors of ethylene action (e.g. silver ions) or ethylene biosynthesis (e.g. aminoethoxyvinylglycine (AVG), aminooxyacetic acid (AOA) and cobalt chloride) effectively blocks aerenchyma formation under hypoxic conditions (Drew *et al.*, 1981; Konings, 1982; Jackson *et al.*, 1985). Moreover, aerenchyma can be induced by treatment with ethylene, even under aerobic conditions (Jackson *et al.*, 1985). These observations indicate that ethylene works as a trigger for inducible aerenchyma formation in maize roots. Ethylene-responsive aerenchyma formation is affected by chemical inhibitors or stimulators of programmed cell death and other signaling pathways (He *et al.*, 1996b). These analyses suggest that heterotrimeric G-protein-, phospholipase C (PLC)-, inositol 1,4,5-trisphosphate (IP_3)- and calcium-dependent signaling pathways are involved in the process of lysigenous aerenchyma formation in maize roots (He *et al.*, 1996b; Drew *et al.*, 2000).

In the late stage of lysigenous aerenchyma formation, the cell wall is degraded enzymatically. Initially, the location of esterified pectin and de-esterified pectin in the cell wall of the maize cortex is changed during cell death (Gunawardena *et al.*, 2001), and subsequently the cell wall is degraded by the combined action of pectolytic, xylanolytic and cellulosolytic enzymes (Jackson & Armstrong, 1999). Indeed, the activities of cellulase (CEL), xylanase and pectinase, all of which are involved in the loosening or degradation of the cell wall, are enhanced in maize roots under waterlogged conditions (Jackson & Armstrong, 1999). On the other hand, the expression of genes encoding expansin, which promotes cell wall extensibility by the breaking of hydrogen bonds between hemicellulose and cellulose, is induced by ethylene (Rose *et al.*, 2000). A gene encoding xyloglucan endo-transglycosylase (XET) is up-regulated in maize roots after 12 h of flooding, and induction is inhibited by treatment with an ethylene biosynthesis inhibitor (Saab & Sachs, 1996).

On the basis of these results, Evans (2003) proposed that selective cell death in the maize root cortex occurs in five stages: (1) perception of hypoxia and initiation of ethylene biosynthesis; (2) perception of an ethylene signal by cells of the mid-cortex; (3) initiation of cell death with loss of ions to the surroundings, plasma membrane invagination and the formation of small vesicles; (4) chromatin condensation, increased activities of cell wall hydrolytic enzymes and the surrounding of organelles by membranes; (5) cell wall degradation, cell lysis and absorption of cell contents and water by the surrounding cells, thereby forming gas spaces (i.e. aerenchyma).

So far, these results have mainly been obtained by morphological, anatomical and pharmacological studies, and thus the molecular mechanism of lysigenous aerenchyma formation remains to be elucidated. To better understand the mechanism of lysigenous aerenchyma formation, it is necessary to identify the genes involved and to determine how they are regulated. In this study, we grew maize under aerobic or waterlogged conditions, with or without pretreatment with an inhibitor of ethylene perception. Because aerenchyma formation occurs specifically in root cortical cells, we used laser microdissection (LM; Nakazono *et al.*, 2003; Nelson *et al.*, 2006) to isolate these cells, and then examined their mRNA levels with a microarray and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). As a result, we identified genes that were up-regulated or down-regulated in root cortical cells during aerenchyma formation, and discuss their possible roles.

Materials and Methods

Plant material and growth conditions

Maize (*Zea mays* L. inbred line B73) caryopses were placed on moist chromatography paper (3MM CHR; Whatman, Maidstone, Kent, UK), rolled up in the paper, placed in a flask half shielded with aluminum foil and incubated in constant light at 28°C as described by Nakazono *et al.* (2003). Three-day-old aerobically grown seedlings were then subjected to the following two experimental conditions.

Experiment 1: effects of waterlogged conditions on aerenchyma formation in a primary root

After 3 d of growth, the underground part (i.e. roots) of seedlings was submerged in distilled water to create waterlogged conditions. For an aerobic control, the chromatography paper was kept moist, but never submerged.

Experiment 2: effect of ethylene on aerenchyma formation in a primary root under waterlogged conditions

Before waterlogging, 2.5-d-old seedlings were pretreated with 1 ppm of a gaseous ethylene perception inhibitor,

1-methylcyclopropene (1-MCP), for 12 h in a tightly closed container. For a control, the same treatment was used but without 1-MCP.

Anatomical observations

Three-day-old aerobically grown seedlings were further grown for 24 h under waterlogged conditions with or without pretreatment with 1-MCP, or under aerobic conditions. We isolated segments of primary roots at 1.5–2.0 cm from the root–shoot junction for the observation of aerenchyma formation. Transverse sections of primary roots were used to determine the extent of aerenchyma formation (defined as the area of the aerenchyma per area of the whole root on the section). Each section was photographed using a light microscope (ECLIPSE E600; Nikon, Tokyo, Japan) with a CCD camera (DIGITAL SIGHT DS-L1; Nikon). Areas were measured with Image J software (Ver. 1.39u; National Institutes of Health, Bethesda, MD, USA). Three independent experiments were conducted, each using three primary roots.

Laser microdissection (LM)

The basal parts of the primary roots (1.5–2.0 cm from the root–shoot junction) were fixed in 75% ethanol : 25% acetic acid; after dehydration in a graded ethanol series, the tissues were embedded in paraffin and sectioned at a thickness of 16 μm. Serial sections were placed onto PEN membrane glass slides (Molecular Devices, Toronto, ON, Canada) for LM as described by Takahashi *et al.* (2010). To remove paraffin, slides were immersed in 100% xylene for 5 min, and then in 50% xylene and 50% ethanol for 5 min, and finally in 100% ethanol for 5 min, followed by air drying at room temperature. Cortical cells or stelar cells were collected from the root tissue sections using a Veritas Laser Microdissection System LCC1704 (Molecular Devices).

RNA extraction

Total RNA was extracted from the LM-isolated cortical cells or stelar cells using a PicoPure™ RNA isolation kit (Molecular Devices) according to the manufacturer's instructions. The extracted total RNA was quantified with a Quant-iT™ RiboGreen RNA reagent and kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of total RNA was assessed using a RNA 6000 Pico kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as described by Takahashi *et al.* (2010).

Microarray experiment

Total RNAs (10 ng each) were labeled with a Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy5-labeled and Cy3-labeled cRNA (750 ng each) were used for hybridization in a 4 × 44k Maize Gene Expression Microarray (Agilent Technologies). The array contains 42, 034 60-mer oligo probes to maize genes. Three biological replicates and a color swap for each replicate were analyzed. The hybridized slides were scanned using a DNA microarray scanner G2505C (Agilent Technologies), and signal intensities were extracted by Feature Extraction software (Version 10.5.1.1; Agilent Technologies). A complete set of microarray data was deposited to the Gene Expression Omnibus (GEO) repository under accession number GSE22943.

Microarray data analysis

For inter-array normalization, a global median normalization was applied across all microarrays to achieve the same median signal intensities for each array, and the false discovery rate (FDR) estimation method was used to obtain *P* values corrected for multiple testing using R software (http://www.r-project.org/) and the RankProduct package (Breitling *et al.*, 2004). The fold change of each probe between two conditions was calculated using an average of six replicates (three biological replicates and a color swap for each replicate). We identified the genes for which there was more than a 2.0-fold change in expression between the two conditions on average (at least 1.5-fold change in each replicate) and whose FDR *P* value was < 0.05.

Maize expressed sequence tag (EST) sequences were downloaded from the Dana-Farber Cancer Institute (DFCI) Maize Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=maize). Maize Gene IDs were identified from the Maizesequence Database (http://www.maizesequence.org/) by BLASTN similarity searches using the maize EST sequences as queries. The maximum *E* value was set at 0.0001. The top hit rice genes were selected using homology-based searches against the Michigan State University's (MSU's) Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/) and the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/download/index.html). The maximum *E* value was set at 0.0001. The putative functions were identified from the MSU Rice Genome Annotation Project Data Download (http://rice.plantbiology.msu.edu/downloads_gad.shtml). The annotations were manually improved using BLASTX searches for sequences matching the maize EST sequences (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html).

For gene ontology (GO) analysis, we merged the same IDs and analyzed the frequency of GO terms of up-regulated and down-regulated genes using GO Slim Assignments (http://rice.plantbiology.msu.edu/downloads_gad.shtml).

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis was performed to confirm the expression pattern of selected genes identified by the microarray analysis. Two replicate samples were used for RNA extraction. First-strand cDNA was synthesized using Superscript III (Invitrogen) from 10 ng of total RNA extracted from root cortical cells or stelar cells as already described. KOD FX (TOYOBO, Tokyo, Japan) was used for subsequent PCR amplification with appropriate primers (Supporting Information Table S1): initial denaturation (94°C for 2 min) and 29–45 cycles of denaturation (94°C for 30 s), annealing (56–62°C for 30 s), extension (68°C for 30 s) and final extension (68°C for 6 min).

Results

Aerenchyma formation in a maize primary root

To determine a time point for the identification of ethylene-responsive, aerenchyma formation-associated genes by the LM microarray analyses, 3-d-old aerobically grown maize seedlings were kept under waterlogged conditions, with or without pretreatment with 1-MCP, an inhibitor of ethylene perception, for 0, 6, 12, 18 and 24 h, and aerenchyma formation (percentage cross-sectional area) was measured at the basal region of a primary root. Aerenchyma formation started between 18 and 24 h after waterlogging treatment, whereas it was suppressed for at least 24 h after waterlogging treatment when the seedlings were pretreated with 1-MCP (Fig. 1). These results confirm that ethylene works as a trigger for inducible aerenchyma formation under waterlogged conditions. On the other hand, aerenchyma formation was not observed at the basal region of roots of 4-d-old seedlings grown under aerobic conditions (Fig. 1). To perform microarray analysis, we decided to collect root cortical cells at 12 h after the treatment under waterlogged conditions, with or without pretreatment with 1-MCP, or under aerobic conditions.

Microarray analyses combined with LM

Three-day-old aerobically grown maize seedlings were further grown for 12 h under three conditions: under waterlogged conditions, with or without pretreatment with 1-MCP, or under aerobic conditions; then, the basal parts of the primary roots were fixed and tissue sections were prepared for LM. Cortical cells were collected from the tissue

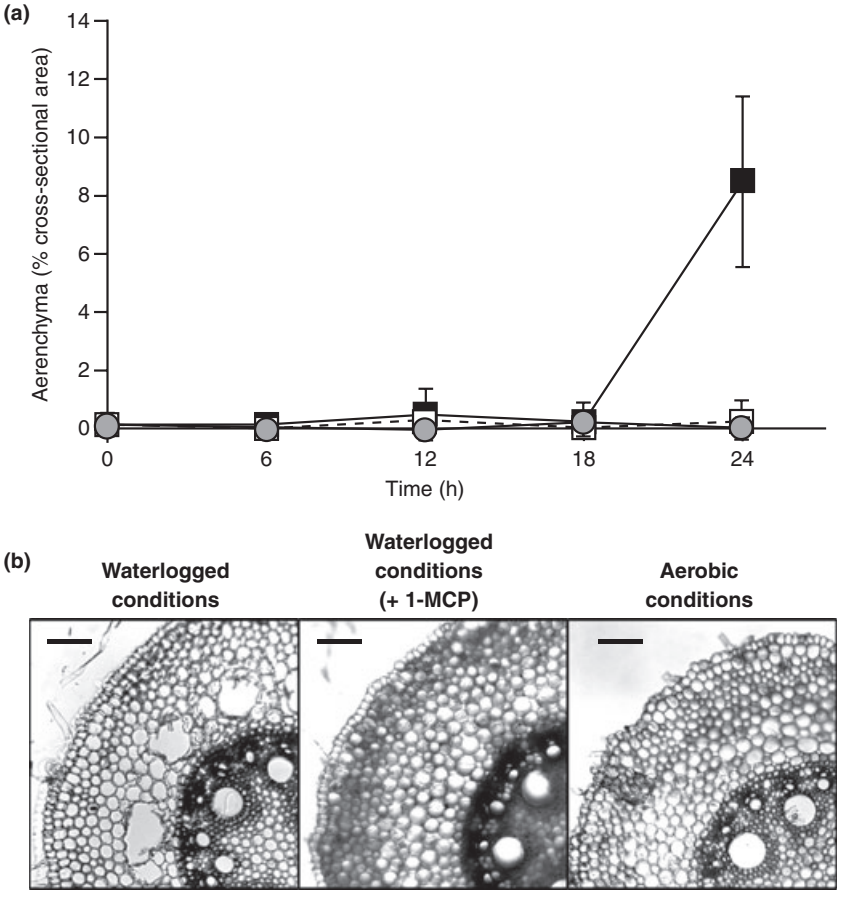

Fig. 1 Aerenchyma formation of maize (*Zea mays*) primary roots under waterlogged conditions (closed squares), waterlogged conditions with 1-methylcyclopropene (1-MCP) pretreatment (circles) and aerobic conditions (open squares). (a) The extent of aerenchyma formation (area of the aerenchyma per area of the whole root on the section) was analyzed at each 6 h for a period of 0–24 h after the start of treatment. All values are means (n = 9) ± SD. Three roots were subjected to analysis in each of the three experiments. (b) Tissue sections of maize root at 24 h after the start of treatment. Bar, 100 μm.

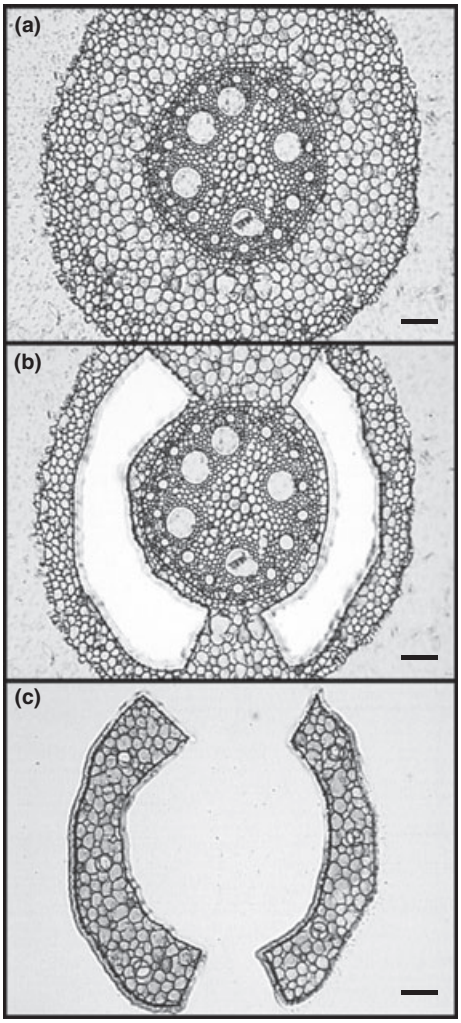

Fig. 2 Isolation of cortical cells from paraffin-embedded sections of a maize (*Zea mays*) primary root using laser microdissection (LM). (a) A root tissue section before LM. (b) A root tissue section after LM. (c) LM-isolated cortical cells. Bars, 100 μm.

sections via LM (Fig. 2). The RNA samples extracted from the LM-isolated cortical cells were labeled with Cy3 or Cy5 dye, and the labeled cDNA from each of three biological replications was hybridized to maize oligo-microarrays. To identify the genes expressed during aerenchyma formation, gene expressions were compared between the waterlogging treatment and the aerobic control (Expt 1) or between the waterlogging treatment without 1-MCP pretreatment and the waterlogging treatment with 1-MCP pretreatment (Expt 2). The resulting data were analyzed as described in the Materials and Methods section. For each experiment, we selected genes whose intensities were > 2.0-fold higher or lower under one condition than under another condition (FDR P value < 0.05). As a result, the signal intensities of 575 genes (*c.* 1.4%) among the 42,034 gene probes spotted on a microarray slide were significantly different between the two treatments common in Expts 1 and 2. Among

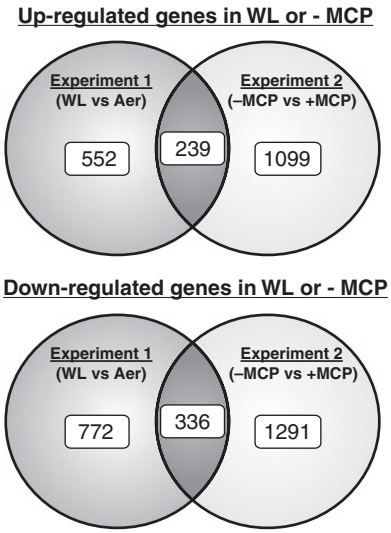

Fig. 3 Number of genes up-regulated or down-regulated under waterlogged conditions [without 1-methylcyclopropene (1-MCP) pretreatment]. Genes whose signal intensities were > 2.0-fold higher or lower under one condition than under another condition (FDR P value < 0.05) were considered to be up-regulated or down-regulated, and the genes commonly up-regulated or down-regulated in both experiments were collected. Experiment 1: 12 h waterlogged conditions (WL)/12 h aerobic conditions (Aer). Experiment 2: 12 h waterlogged conditions without 1-MCP pretreatment (–MCP)/12 h waterlogged conditions with 1-MCP pretreatment (+MCP).

them, it was likely that 239 genes (*c.* 0.6%) were up-regulated and 336 genes (*c.* 0.8%) were down-regulated under the conditions inducing aerenchyma formation (i.e. waterlogged conditions) (Fig. 3, Tables S2, S3).

Characterization of specific gene clusters based on GO

The up-regulated and down-regulated genes were classified into several categories based on their allocated GO terms using GO Slim Assignments (http://rice.plantbiology.msu.edu/downloads_gad.shtml) (Fig. 4). Approximately 36% of the up-regulated genes and *c.* 32% of the down-regulated genes were categorized to genes responsive to stress and several stimuli (e.g. abiotic, biotic, endogenous, external and extracellular stimuli). The genes related to cellular process, biosynthetic process and transport were also relatively abundant in both the up-regulated and down-regulated genes. On the other hand, 5.9% of the up-regulated genes, but only 0.002% of the down-regulated genes, were translation-related genes encoding ribosomal proteins, translation initiation factors and translation elongation factors (Fig. 4).

Validation of gene expression patterns by semi-quantitative RT-PCR

Some of the genes shown to be up-regulated or down-regulated by the microarray were also analyzed by semi-quantitative

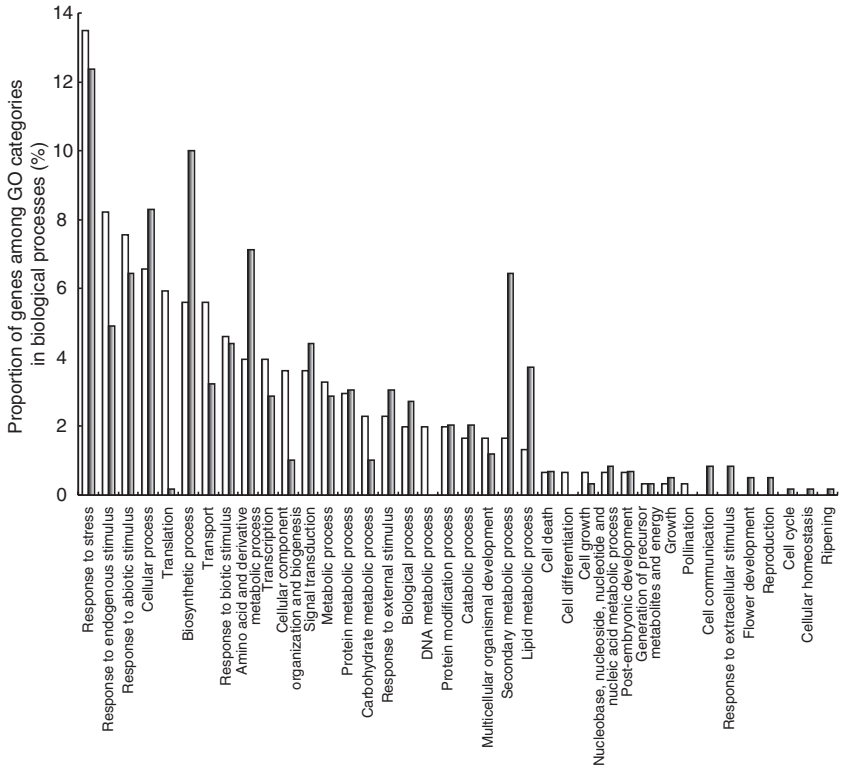

Fig. 4 Gene classification based on gene ontology (GO) for genes commonly up-regulated (open bars) or down-regulated (closed bars) in *Zea mays* in Expts 1 and 2. The frequency of GO terms was analyzed using GO Slim Assignment. The *x*-axis and *y*-axis indicate the names of clusters and the ratio of each cluster, respectively. Only the biological processes were used for GO analysis.

RT-PCR to confirm the change in expression. For this, we selected 13 genes (11 up-regulated and two down-regulated), which included three reactive oxygen species (ROS) generation/scavenging-related genes, three calcium signaling-related genes, three cell wall modification-related genes, one transporter gene and three transcriptional regulation-related genes (Table 1). The semi-quantitative RT-PCR results confirmed the microarray results for each of the genes (Fig. 5).

Tissue-specific gene expression analysis

To examine whether the ethylene-mediated waterlogging-responsive expression of the selected genes was associated with aerenchyma formation, we used LM to collect sections of cortical cells (aerenchyma-forming tissue) and stelar cells (nonaerenchyma-forming tissue) from cross-sections of primary roots that had been exposed to waterlogged or aerobic conditions for 12 h (Fig. 6a), and performed semi-quantitative RT-PCR (Fig. 6b). Under waterlogged conditions, all of the 13 selected genes (Table 1) were up-regulated or down-regulated in cortical cells. Eight (*RBOH*, *MnSOD*, *CBL*, *CML*, *CNGC*, *XET*, *ERF* and *UVR8L*) were also up-regulated or down-regulated in stelar cells, but the difference in the mRNA levels between waterlogged and aerobic conditions was greater in cortical cells than in stelar cells (Fig. 6b).

ROS generation/scavenging-related genes

The production of ROS has been implicated in diverse physiological processes, including programmed cell death, in plants (Overmyer *et al.*, 2003). One of the major sources of ROS in plants is a reaction mediated by NADPH oxidase, which is responsible for the conversion of O_2 to superoxide anion (O_2^-), thereby leading to the production of hydrogen peroxide (H_2O_2) (Overmyer *et al.*, 2003). Here, we found several ethylene-mediated waterlogging-responsive genes related to ROS generation or ROS scavenging (Table 2). The up-regulated genes include those encoding respiratory burst oxidase homolog (RBOH), glutathione *S*-transferase and manganese superoxide dismutase, and the down-regulated genes include those encoding RBOH and metallothionein (MT). The *RBOH* gene products are involved in ROS generation, and the other gene products are involved in ROS scavenging. Among these genes, *RBOH* (GRMZM2G30 0965) showed a 117-fold higher expression level under waterlogged conditions than under aerobic conditions, and the induction was partially suppressed by treatment with 1-MCP (Fig. 5, Table 2). As shown in Fig. 6(b), up-regulation of *RBOH* expression was observed in both cortical cells and stelar cells, but the mRNA levels appeared to be slightly higher in cortical cells than in stelar cells under waterlogged conditions. On the other hand, the *MnSOD* gene (GRMZM2G160629) was up-regulated preferentially in

Table 1 List of genes confirmed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Gene name	Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] −MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
Reactive oxygen species (ROS) generation/scavenging							
RBOH	TC289691	117.72	10.72	GRMZM2G300965	Os12g0541300	LOC_Os12g35610	Respiratory burst oxidase, putative, expressed
MnSOD	TC301296	3.45	3.16	GRMZM2G160629	Os05g0323900	LOC_Os05g25850	Superoxide dismutase, mitochondrial precursor, putative, expressed
MT	TC298672	0.16	0.39	GRMZM2G164229	Os11g0704500	LOC_Os11g47809	Metallothionein, putative, expressed
Calcium signaling							
CBL	TC294844	6.69	11.56	GRMZM2G125838	Os02g0802400	LOC_Os02g55880	EF hand family protein, putative, expressed (calcineurin B-like, CBL)
CML	TC282752	2.71	15.08	GRMZM2G467184	Os11g0141400	LOC_Os11g04560	Calmodulin-like protein 1, putative, expressed
CNGC	TC289094	0.06	0.04	GRMZM2G074317	Os03g0758300	LOC_Os03g55100	Cyclic nucleotide-gated ion channel 2, putative, expressed
Cell wall modification							
XET	TC286313	5.47	5.20	GRMZM2G174855	Os07g0529700	LOC_Os07g34580	Glycosyl hydrolases family 16, putative, expressed (xyloglucan endotransglucosylase, XET)
PG	TC282363	5.56	2.55	GRMZM2G037431	Os01g0636500	LOC_Os01g44970	Polygalacturonase, putative, expressed
CEL	TC314571	4.36	12.59	GRMZM2G141911	Os02g0123700	LOC_Os02g03120	Endoglucanase, putative, expressed (cellulase, CEL)
Transporter							
H^+ATPase	TC305342	18.07	186.62	GRMZM2G450055	Os02g0797300	LOC_Os02g55400	ATPase 8, plasma membrane-type, putative, expressed (plasma membrane H^+-ATPase)
Transcriptional regulation							
ERF	TC289269	30.88	4.33	GRMZM2G053503	Os01g0313300	LOC_Os01g21120	AP2 domain-containing protein, expressed (ethylene response factor, ERF)
RAV1L	TC301325	4.42	4.91	GRMZM2G169654	Os01g0693400	LOC_Os01g49830	B3 DNA-binding domain-containing protein, expressed (AP2/ERF-B3 domain-containing transcription factor RAV1-like)
UVR8L	TC300328	2.84	3.60	GRMZM2G003565	Os04g0435700	LOC_Os04g35570	Regulator of chromosome condensation domain-containing protein, expressed (UVB-resistance protein UVR8-like)

Aer, aerobic conditions; –MCP, waterlogged conditions without 1-methylcyclopropene (1-MCP) pretreatment; +MCP waterlogged conditions with 1-MCP pretreatment; WL, waterlogged conditions.

[a]Maize expressed sequence tags (ESTs) in DFCI Maize Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=maize).

[b]Average expression ratio of three biological replicates and a color swap for each replicate.

[c]Maize Gene IDs in Maizesequence Database (http://www.maizesequence.org/).

[d]RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

[e]MSU's Loc_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).

[f]MSU's Putative Function in Rice Genome Annotation Project Database. Manually improved annotations from Genbank BLASTX search (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html) are described in parentheses.

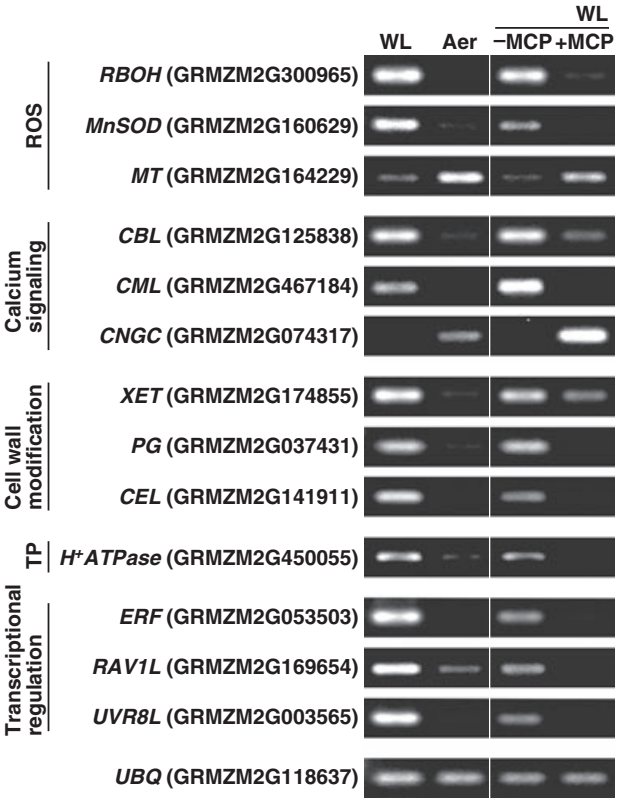

Fig. 5 Validation of expression for genes selected from the microarray analysis with semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Semi-quantitative RT-PCR analysis of the selected genes was performed with appropriate primers (Supporting Information Table S1). The alphanumeric symbols in parentheses indicate the Maize Gene IDs of the Maizesequence Database. The *ubiquitin* gene (*UBQ*) was used as a control. Aer, aerobic conditions; –MCP, waterlogged conditions without 1-methylcyclopropene (1-MCP) pretreatment; +MCP, waterlogged conditions with 1-MCP pretreatment; ROS, reactive oxygen species generation/scavenging; TP, transporter; WL, waterlogged conditions.

cortical cells under waterlogged conditions. Interestingly, the *MT* gene (GRMZM2G164229) was constitutively expressed in both cortical cells and stelar cells under aerobic conditions, but the *MT* mRNA levels were decreased specifically in cortical cells under waterlogged conditions (Fig. 6b).

Calcium signaling-related genes

Many studies have suggested that the cytosolic calcium ion (Ca^{2+}) functions as a second messenger for signaling pathways in response to oxygen deprivation (Subbaiah *et al.*, 1994; Tsuji *et al.*, 2000; Baxter-Burrell *et al.*, 2002). Ca^{2+} signaling may also be involved in aerenchyma formation in maize roots (He *et al.*, 1996b). In this study, several genes implicated in calcium signaling, whose expression was changed significantly under waterlogged conditions (without 1-MCP pretreatment) in both Expts 1 and 2, were identified (Table 2). They included up-regulated genes encoding calcineurin B-like protein (CBL), Ca^{2+}-binding domain-containing proteins and calmodulin-like protein (CML), and down-regulated genes encoding calcium/calmodulin-dependent protein kinases, CBL, cyclic nucleotide-gated ion channel (CNGC) protein and CML (Table 2). As shown in Fig. 6(b), the up-regulation of *CBL* (GRMZM2G125838) and *CML* (GRMZM2G467184) and the down-regulation of *CNGC* (GRMZM2G074317) were observed in both cortical cells and stelar cells, but the changes in expression were more pronounced in cortical cells than in stelar cells.

Cell wall modification-related genes

The last step of aerenchyma formation involves cell wall loosening and degradation, in which many enzymes, including XETs, expansins, CELs and pectinases, are involved (He *et al.*, 1994; Saab & Sachs, 1996; Jackson & Armstrong, 1999). In this study, several of the up-regulated genes encode these enzymes, including the genes for pectinesterase, pectate lyase, polygalacturonase (PG), XET, CEL, expansin and invertase/pectin methylesterase inhibitor family protein, and several down-regulated genes encode cellulose synthase and cellulose synthase-like C family protein (Table 2). Under waterlogged conditions, two of the three selected cell wall modification-related genes, the genes encoding PG (GRMZM2G037431) and CEL (GRMZM2G141911), were specifically up-regulated in cortical cells, whereas the *XET* gene (GRMZM2G174855) was up-regulated in both cortical cells and stelar cells (Fig. 6b).

Protein kinase, protein phosphatase and transcriptional regulator genes

Among the waterlogging-sensitive genes, 16 genes were protein kinase genes (four up-regulated and 12 down-regulated) and two genes were protein phosphatase genes (both up-regulated) (Table 3). This is consistent with a previous finding that protein phosphorylation and dephosphorylation are important for the regulation of aerenchyma formation in maize (He *et al.*, 1996b).

Of the 34 genes encoding putative transcriptional regulators, including transcription factors of > 20 different families, 13 genes were up-regulated and 21 genes were down-regulated (Table 4). Among these genes, the gene encoding an AP2 domain-containing protein (GRMZM2G053503), which is similar to ethylene response factor (ERF), showed a strong (*c.* 30-fold) increase in expression under waterlogged conditions, and the induction was partially suppressed by pretreatment with 1-MCP (Fig. 5, Table 4). The expression of a gene [designated as *RAV1-like* (*RAV1L*) in this study; GRMZM2G169654] encoding a protein containing a B3 DNA-binding domain, which is homologous to an AP2/ERF domain and B3 domain containing transcription

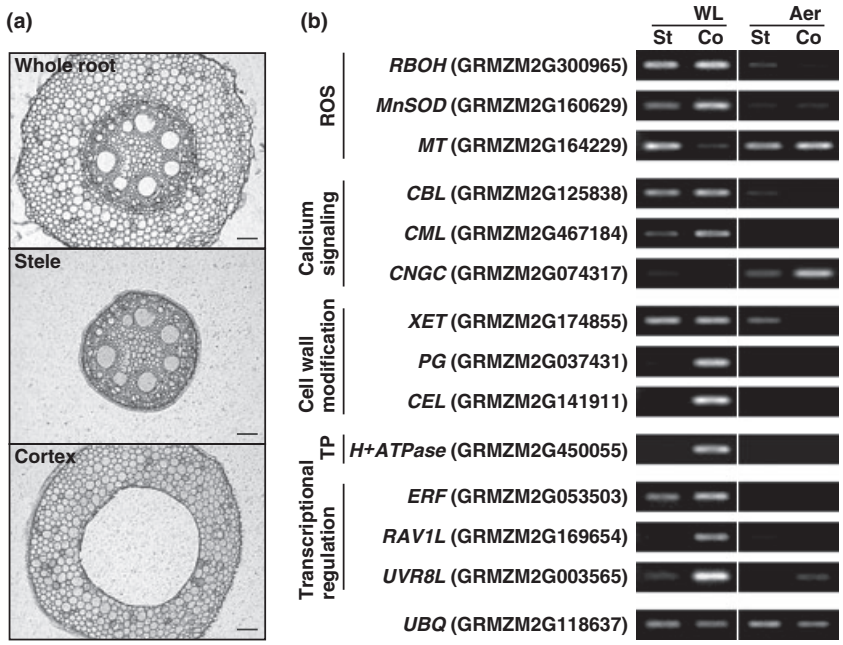

Fig. 6 Tissue-specific expression analysis of genes selected from the microarray analysis. (a) Isolation of stelar cells (stele) and cortical cells (cortex) from paraffin-embedded tissue sections of a maize (*Zea mays*) primary root using laser microdissection. Bars, 100 μm. (b) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of the selected genes. The alphanumeric symbols in parentheses indicate the Maize Gene IDs of the Maizesequence Database. The *ubiquitin* gene (*UBQ*) was used as a control. Aer, aerobic conditions; Co, cortex; ROS, reactive oxygen species generation/scavenging; St, stele; TP, transporter; WL, waterlogged conditions.

factor RAV1, was also induced under waterlogged conditions, and seemed to be controlled by ethylene (Fig. 5, Table 4). Another up-regulated gene is a regulator of chromosome condensation domain-containing protein (GRMZM2G003565). It is a *UVR8-like* gene (*UVR8L*), which is homologous to the Arabidopsis *UVR8* gene (Table 4). Three other up-regulated genes are related to histone modification. These include a jmjC domain (jumonj-C-domain)-containing protein (GRMZM2G-417089) and two histone acetyltransferases (GRMZM2G069886 and GRMZM2G106673) (Table 4). Under waterlogged conditions, the *ERF* (GRMZM2G053503) and *UVR8L* (GRMZM2G003565) genes were mainly up-regulated in cortical cells and the *RAV1L* (GRMZM2G169654) gene was up-regulated only in cortical cells (Fig. 6b).

Discussion

To better understand the molecular mechanism of aerenchyma formation in maize root cortical cells, we screened for genes whose expression changed in response to ethylene under waterlogged conditions, and found 239 up-regulated genes and 336 down-regulated genes. Unsurprisingly, many of the genes (*c.* 36% of the up-regulated genes and *c.* 32% of the down-regulated genes) are known to be responsive to stress or other stimuli (Fig. 4). However, it is not clear why many translation-related genes (5.9%) are included in the up-regulated genes, but not in the down-regulated genes. It is known that translation of many normal cellular mRNAs is extremely limited in maize roots under anoxia, whereas mRNAs for anaerobic proteins (related to anaerobic metabolism, such as glycolysis and fermentation) are selectively translated (Sachs *et al.*, 1980; Bailey-Serres, 1999). The selective translation under oxygen deprivation is important for energy conservation and facilitates the transition to anaerobic metabolism (Branco-Price *et al.*, 2008). Thus, to understand the roles of the differentially expressed genes (including the up-regulated translation-related genes), it is necessary to examine whether their mRNAs are effectively translated in root cortical cells under waterlogged conditions.

RBOH, a plant homolog of $gp91^{phox}$ in mammalian NADPH oxidase, has an important role in ROS-mediated signaling, such as the defense response, programmed cell death and development in plants (Torres *et al.*, 2002; Foreman *et al.*, 2003; Takeda *et al.*, 2008; Yoshioka *et al.*, 2009). Indeed, the Rop (RHO-like small G-protein of plants)-dependent H_2O_2 production mediated by NADPH oxidase, the activity of which is stimulated by Ca^{2+}, contributes to the induction of expression of *ADH* and *RopGAP4* genes in Arabidopsis under oxygen deprivation (Baxter-Burrell *et al.*, 2002). In rice, ethylene-induced, H_2O_2-mediated epidermal cell death, which precedes the emergence of adventitious roots, is regulated by NADPH oxidase (Steffens & Sauter, 2005, 2009). Here, we found that one *RBOH* gene (GRMZM2G300965) was up-regulated and another *RBOH* gene [*ZmRBOHA* (Lin *et al.*, 2009); GRMZM2G426953] was down-regulated during aerenchyma formation in maize roots, implying that the roles of the two RBOH proteins may be different. The maize up-regulated RBOH is homologous to rice OsRBOHH (Wong *et al.*, 2007), Arabidopsis AtRBOHB (Torres *et al.*, 1998) and potato StRBOHB (Yoshioka *et al.*, 2001) (data not shown). In potato, treatment of tubers with hyphal wall components (HWCs) from *Phytophthora infestans* causes a rapid and transient oxidative burst (i.e. H_2O_2

Table 2 List of genes related to reactive oxygen species (ROS) generation/scavenging, calcium signaling and cell wall modification, whose expression was up-regulated or down-regulated in maize root cortex during aerenchyma formation

Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] −MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
Reactive oxygen species (ROS) generation/scavenging						
TC289691	117.72	10.72	GRMZM2G300965	Os12g0541300	LOC_Os12g35610	Respiratory burst oxidase, putative, expressed
TC301460	5.39	20.85	GRMZM2G416632	Os09g0467200	LOC_Os09g29200	Glutathione *S*-transferase, putative, expressed
TC301296	3.45	3.16	GRMZM2G160629	Os05g0323900	LOC_Os05g25850	Superoxide dismutase, mitochondrial precursor, putative, expressed
TC311766	0.27	0.20	GRMZM2G426953	Os01g0835500	LOC_Os01g61880	Respiratory burst oxidase, putative, expressed
TC298672	0.16	0.39	GRMZM2G164229	Os11g0704500	LOC_Os11g47809	Metallothionein, putative, expressed
Calcium signaling						
TC294844	6.69	11.56	GRMZM2G125838	Os02g0802400	LOC_Os02g55880	EF hand family protein, putative, expressed (calcineurin B-like, CBL)
TC313511	5.75	2.91	GRMZM2G052740	Os02g0158100	LOC_Os02g06340	EH domain-containing protein 1, putative, expressed
TC307198	5.57	3.78	GRMZM2G082199	Os01g0841700	LOC_Os01g62430	C2 domain-containing protein, putative, expressed
TC282752	2.71	15.08	GRMZM2G467184	Os11g0141400	LOC_Os11g04560	Calmodulin-like protein 1, putative, expressed
TC282050	0.41	0.28	GRMZM2G032852	Os03g0128700	LOC_Os03g03660	CAMK_CAMK_like.17 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC288382	0.38	0.14	GRMZM2G180916	Os10g0564500	LOC_Os10g41490	CAMK_CAMK_like.41 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC298929	0.36	0.42	GRMZM2G173424	Os12g0597000	LOC_Os12g40510	Calcineurin B, putative, expressed
TC293625	0.33	0.18	GRMZM2G412601	Os09g0418500	LOC_Os09g25100	CAMK_KIN1/SNF1/Nim1_like.35 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC281251	0.27	0.23	GRMZM2G146720	Os11g0134300	LOC_Os12g03810	CAMK_KIN1/SNF1/Nim1_like.37 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC282587	0.27	0.22	GRMZM2G078781	Os01g0782800	LOC_Os01g57370	Cyclic nucleotide-gated ion channel 2, putative, expressed
BM501148	0.22	0.32	GRMZM2G096228	Os10g0389000	LOC_Os10g25010	OsCML8 – calmodulin-related calcium sensor protein, expressed
TC289094	0.06	0.04	GRMZM2G074317	Os03g0758300	LOC_Os03g55100	Cyclic nucleotide-gated ion channel 2, putative, expressed
Cell wall modification						
TC315760	10.77	7.35	GRMZM2G043943	Os01g0743200	LOC_Os01g53990	Pectinesterase, putative, expressed
TC283838	5.86	11.61	GRMZM2G131912	Os04g0137100	LOC_Os04g05050	Pectate lyase precursor, putative, expressed
TC282363	5.56	2.55	GRMZM2G037431	Os01g0636500	LOC_Os01g44970	Polygalacturonase, putative, expressed
TC286313	5.47	5.20	GRMZM2G174855	Os07g0529700	LOC_Os07g34580	Glycosyl hydrolases family 16, putative, expressed (xyloglucan endotransglucosylase, XET)
TC311007	5.07	23.94	GRMZM2G119471	Os03g0124900	LOC_Os03g03350	Polygalacturonase, putative, expressed
TC314571	4.36	12.59	GRMZM2G141911	Os02g0123700	LOC_Os02g03120	Endoglucanase, putative, expressed (cellulase, CEL)

Table 2 (Continued)

Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] −MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
TC314458	3.00	107.88	AC234190.1[g]	Os01g0249100	LOC_Os01g14660	Expansin precursor, putative, expressed
TC299614	2.43	4.16	GRMZM2G048430	Os06g0711800	LOC_Os06g49760	Invertase/pectin methylesterase inhibitor family protein, putative, expressed
TC280317	0.42	0.05	GRMZM2G028353	Os07g0424400	LOC_Os07g24190	CESA3 – cellulose synthase, expressed
TC287832	0.34	0.10	GRMZM2G424832	Os07g0208500	LOC_Os07g10770	CESA8 – cellulose synthase, expressed
TC303435	0.24	0.24	GRMZM2G074792	Os01g0766900	LOC_Os01g56130	CSLC1 – cellulose synthase-like family C, expressed

Aer, aerobic conditions; –MCP, waterlogged conditions without 1-methylcyclopropene (1-MCP) pretreatment; +MCP, waterlogged conditions with 1-MCP pretreatment; WL, waterlogged conditions.

[a]Maize ESTs in DFCI Maize Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=maize).

[b]Average expression ratio of three biological replicates and a color swap for each replicate.

[c]Maize Gene IDs in Maizesequence Database (http://www.maizesequence.org/).

[d]RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

[e]MSU's Loc_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).

[f]MSU's Putative Function in Rice Genome Annotation Project Database. Manually improved annotations from Genbank BLASTX search (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html) are described in parentheses.

[g]AC234190.1 indicates contig ID.

accumulation; phase I), followed by a massive oxidative burst (phase II) (Yoshioka *et al.*, 2001). It is likely that StRBOHA contributes to phase I of the oxidative burst, and that other RBOHs (StRBOHB, StRBOHC and StRBOHD) contribute to phase II (Yamamizo *et al.*, 2006). Both oxidative bursts are inhibited by the serine/threonine protein kinase inhibitor K252a or the extracellular Ca^{2+} chelator ethyleneglycol-bis (β-aminoethylether)-*N,N*′-tetraacetic acid (EGTA) (Kobayashi *et al.*, 2007). These observations raise the possibility that, in maize, waterlogging-induced up-regulation of RBOH is involved in H_2O_2 production, and the H_2O_2 induces cell death (i.e. aerenchyma formation) in root cortical cells. On the other hand, we found that the expression of the gene encoding MT (GRMZM2G164229), which works as an ROS scavenger (Wong *et al.*, 2004), was repressed under waterlogged conditions, and that the repression seemed to be ethylene dependent (Fig. 5, Table 2). Interestingly, the rice *Metallothionein2b* (*MT2b*) gene is down-regulated in response to ethylene and H_2O_2 in epidermal cells, thereby amplifying the accumulation of H_2O_2 produced by NADPH oxidase (i.e. RBOH), to induce cell death (Steffens & Sauter, 2009). Similarly, the cortical cell-specific down-regulation of the maize *MT* gene (Fig. 6b) may contribute to higher accumulation of the RBOH-produced H_2O_2, which induces cell death in the cortical cells for lysigenous aerenchyma formation. In stele, the *MT* gene is constitutively expressed even under waterlogged conditions (Fig. 6b), which might reduce the amount of H_2O_2 produced by RBOH. Indeed, it has been demonstrated recently that the down-regulation of MT2b or application of H_2O_2 promotes aerenchyma formation in internodes of rice stems (Steffens *et al.*, 2010). On the basis of these results, it is possible that similar mechanisms may regulate epidermal cell death in rice, aerenchyma formation in rice internodes and aerenchyma formation in maize root cortex. Further functional analyses of the up-regulated *RBOH* gene and the down-regulated *MT* gene in maize are necessary to examine this possibility.

Genes related to Ca^{2+} signaling, including CBL, CML and calcium/calmodulin-dependent protein kinase, were also identified as the up-regulated or down-regulated genes in response to waterlogging treatment (without 1-MCP pretreatment) (Table 2). It has been proposed that, under oxygen deprivation, Ca^{2+} is released from the apoplast and from mitochondria into the cytoplasm, and the elevated cytosolic Ca^{2+} provokes subsequent activation of kinases and phosphatases, resulting in the activation of the expression of genes responsible for aerenchyma formation (Subbaiah & Sachs, 2003). Treatments with thapsigargin and caffeine, which increase intracellular Ca^{2+} levels, stimulated CEL activity and aerenchyma formation under aerobic conditions, whereas both EGTA (a Ca^{2+} chelator) and ruthenium red (an inhibitor of Ca^{2+} fluxes from organelles) prevented the increase in CEL activity and aerenchyma

Table 3 List of genes encoding protein kinases and protein phosphatases, whose expression was up-regulated or down-regulated in maize root cortex during aerenchyma formation

Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] –MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
TC281230	23.49	6.29	GRMZM2G147051	Os05g0436100	LOC_Os05g36050	Serine/threonine protein kinase, putative, expressed
CO458690	18.06	6.11	GRMZM2G092604	Os09g0293500	LOC_Os09g12240	Serine/threonine protein kinase BRI1-like 1 precursor, putative, expressed
TC299285	6.39	10.13	GRMZM2G091338	Os01g0789200	LOC_Os01g57940	Tyrosine protein kinase domain-containing protein, putative, expressed
TC282571	4.80	3.18	GRMZM2G113668	Os03g0857600	LOC_Os03g64050	Receptor protein kinase, putative, expressed
TC293663	3.12	3.12	GRMZM2G126765	Os06g0208700	LOC_Os06g10650	Tyrosine phosphatase family protein, putative, expressed
TC293960	2.52	2.40	GRMZM2G015610	Os04g0449450	LOC_Os04g37660	Protein phosphatase protein, putative, expressed
TC282050	0.41	0.28	GRMZM2G032852	Os03g0128700	LOC_Os03g03660	CAMK_CAMK_like.17 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC306361	0.39	0.38	GRMZM2G074262	Os01g0864700	LOC_Os01g64490	Protein kinase domain-containing protein, expressed
TC288382	0.38	0.14	GRMZM2G180916	Os10g0564500	LOC_Os10g41490	CAMK_CAMK_like.41 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC293625	0.33	0.18	GRMZM2G412601	Os09g0418500	LOC_Os09g25100	CAMK_KIN1/SNF1/Nim1_like.35 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC308802	0.31	0.18	GRMZM2G119521	Os10g0432001	LOC_Os10g29620	Tyrosine protein kinase domain-containing protein, putative, expressed
TC315230	0.29	0.40	GRMZM2G126858	Os04g0616700	LOC_Os04g52640	SHR5-receptor-like kinase, putative, expressed
TC281251	0.27	0.23	GRMZM2G146720	Os11g0134300	LOC_Os12g03810	CAMK_KIN1/SNF1/Nim1_like.37 – CAMK includes calcium/calmodulin-dependent protein kinases, expressed
TC314581	0.25	0.20	GRMZM2G092776	Os03g0113000	LOC_Os03g02190	Protein kinase domain-containing protein, expressed
TC308836	0.23	0.12	GRMZM2G132591	Os10g0174800	LOC_Os10g09620	OsWAK108 – OsWAK receptor-like protein kinase, expressed
TC312632	0.22	0.12	GRMZM2G135359	Os03g0294800	LOC_Os03g18370	MRH1, putative, expressed
TC305840	0.15	0.17	AC217293.3[g]	Os12g0615100	LOC_Os10g07556	Wall-associated receptor kinase-like 22 precursor, putative, expressed
TC302613	0.15	0.20	GRMZM2G158252	Os10g0362300	LOC_Os10g21810	Histidine kinase, putative, expressed

Aer, aerobic conditions; –MCP, waterlogged conditions without 1-methylcyclopropene (1-MCP) pretreatment; +MCP, waterlogged conditions with 1-MCP pretreatment; WL, waterlogged conditions.

[a]Maize ESTs in DFCI Maize Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=maize).

[b]Average expression ratio of three biological replicates and a color swap for each replicate.

[c]Maize Gene IDs in Maizesequence Database (http://www.maizesequence.org/).

[d]RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

[e]MSU's Loc_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).

[f]MSU's Putative Function in Rice Genome Annotation Project Database. Manually improved annotations from Genbank BLASTX search (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html) are described in parentheses.

[g]AC217293.3 indicates contig ID.

Table 4 List of genes encoding transcriptional regulation-related proteins, whose expression was up-regulated or down-regulated in maize root cortex during aerenchyma formation

Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] –MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
TC289269	30.88	4.33	GRMZM2G053503	Os01g0313300	LOC_Os01g21120	AP2 domain-containing protein, expressed (ethylene response factor, ERF)
TC288289	25.11	5.39	GRMZM2G417089	Os03g0430400	LOC_Os03g31594	jmjC domain-containing protein, expressed
TC292202	7.89	22.50	GRMZM2G068973	Os01g0816100	LOC_Os01g60020	NAC domain transcription factor, putative, expressed
CD966549	6.41	9.14	GRMZM2G069886	Os01g0246100	LOC_Os01g14370	Histone acetyltransferase HAC5, putative, expressed
TC293058	4.80	4.82	GRMZM2G106673	Os09g0347800	LOC_Os09g17850	Acetyltransferase type B catalytic subunit, putative, expressed
TC293056	4.69	10.36	GRMZM2G106673	Os06g0194400	LOC_Os06g09420	B3 DNA-binding domain-containing protein, expressed
TC301325	4.42	4.91	GRMZM2G169654	Os01g0693400	LOC_Os01g49830	B3 DNA-binding domain-containing protein, expressed (AP2/ERF-B3 domain-containing transcription factor RAV1-like)
TC304408	4.39	23.27	GRMZM2G148074	Os10g0561800	LOC_Os10g41230	Homeobox-associated leucine zipper, putative, expressed
TC302970	4.24	44.59	GRMZM2G108865	Os03g0764900	LOC_Os03g55610	Dof zinc finger domain-containing protein, putative, expressed
TC300803	4.21	10.77	GRMZM2G172621	Os07g0679500	LOC_Os07g48180	bZIP transcription factor domain-containing protein, expressed
TC285712	4.16	4.05	GRMZM2G125522	Os05g0449900	LOC_Os05g37730	MYB family transcription factor, putative, expressed
TC303206	3.74	16.46	GRMZM2G460472	Os11g0158500	LOC_Os11g06010	Helix–loop–helix DNA-binding protein, putative, expressed
TC300328	2.84	3.60	GRMZM2G003565	Os04g0435700	LOC_Os04g35570	Regulator of chromosome condensation domain-containing protein, expressed (UVB-resistance protein UVR8-like)
TC283126	0.41	0.42	GRMZM2G093895	Os12g0173125	LOC_Os12g07480	TCP family transcription factor, putative, expressed
TC288402	0.41	0.29	GRMZM2G142768	Os02g0817600	LOC_Os02g57250	OsIAA10 – auxin-responsive Aux/IAA gene family member, expressed
TC299946	0.40	0.39	GRMZM2G154641	Os01g0848400	LOC_Os01g62920	Homeodomain protein, putative, expressed
TC281170	0.37	0.36	GRMZM2G171365	Os03g0122600	LOC_Os03g03100	OsMADS50 – MADS-box family gene with MIKCc type-box, expressed
TC312751	0.36	0.33	GRMZM2G104551	Os09g0532900	LOC_Os09g36250	MYB family transcription factor, putative, expressed
TC313513	0.35	0.02	AC232238.2[g]	Os01g0859500	LOC_Os01g64020	Transcription factor, putative, expressed

Table 4 (Continued)

Maize EST[a] accession	Expt. 1[b] WL/Aer	Expt. 2[b] −MCP/+MCP	Maize gene IDs[c]	RAP Os IDs[d]	MSU LOC_Os IDs[e]	Gene annotation[f]
TC295929	0.34	0.27	GRMZM2G037630	Os03g0411100	LOC_Os03g29760	Nuclear transcription factor Y subunit, putative, expressed
TC283299	0.32	0.30	GRMZM2G328481	Os06g0211200	LOC_Os06g10880	bZIP transcription factor, putative, expressed
TC280481	0.32	0.16	GRMZM2G485184	Os12g0510900	LOC_Os12g32620	OsWLIM1 – LIM domain protein, putative actin-binding protein and transcription factor, expressed
TC301678	0.31	0.26	GRMZM2G104390	Os01g0343300	LOC_Os01g24070	GATA zinc finger domain-containing protein, expressed
TC292295	0.30	0.25	GRMZM2G180328	Os01g0884300	LOC_Os01g66120	No apical meristem protein, putative, expressed
TC306650	0.27	0.29	GRMZM2G144196	Os08g0242800	LOC_Os08g14450	RNA polymerase sigma factor, putative, expressed
TC289804	0.27	0.44	GRMZM2G356439	Os04g0678400	LOC_Os04g58190	Dof zinc finger domain-containing protein, putative, expressed
TC315561	0.26	0.33	GRMZM2G320549	Os01g0922800	LOC_Os01g69850	OsMADS65 – MADS-box family gene with MIKC* type-box, expressed
TC302957	0.17	0.24	GRMZM2G031094	Os11g0152700	LOC_Os12g05680	Transcription factor, putative, expressed
TC290696	0.16	0.18	GRMZM2G452178	Os07g0129700	LOC_Os07g03770	Homeobox domain-containing protein, expressed
TC301787	0.16	0.25	GRMZM2G003944	Os11g0175700	LOC_Os11g07460	TCP family transcription factor, putative, expressed
TC309665	0.16	0.14	GRMZM2G472671	Os08g0543700	LOC_Os08g43070	Helix–loop–helix DNA-binding domain-containing protein
TC313975	0.13	0.11	GRMZM2G009406	Os08g0490100	LOC_Os09g29960	Dof zinc finger domain-containing protein, putative, expressed
TC285832	0.13	0.18	GRMZM2G002128	Os05g0449900	LOC_Os05g37730	MYB family transcription factor, putative, expressed
TC302185	0.09	0.05	GRMZM2G172657	Os07g0586900	LOC_Os07g39820	SHR, putative, expressed

Aer, aerobic conditions; –MCP, waterlogged conditions without 1-methylcyclopropene (1-MCP) pretreatment; +MCP, waterlogged conditions with 1-MCP pretreatment; WL, waterlogged conditions.

[a]Maize ESTs in DFCI Maize Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=maize).

[b]Average expression ratio of three biological replicates and a color swap for each replicate.

[c]Maize Gene IDs in Maizesequence Database (http://www.maizesequence.org/).

[d]RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).

[e]MSU's Loc_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).

[f]MSU's Putative Function in Rice Genome Annotation Project Database. Manually improved annotations from Genbank BLASTX search (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html) are described in parentheses.

[g]AC232238.2 indicates contig ID.

formation by decreasing cytosolic Ca^{2+} in maize roots, even under anaerobic conditions (He *et al.*, 1996b). It seems that Ca^{2+} can bind directly to Ca^{2+}-binding EF-hand motifs in the N-terminal region of RBOH (i.e. NADPH oxidase) and stimulate its activity (Keller *et al.*, 1998; Sagi & Fluhr, 2001; Oda *et al.*, 2010). On the other hand, it has been reported that calcium-dependent protein kinase activates RBOH by phosphorylation of its N-terminal region (Kobayashi *et al.*, 2007). On the basis of these results, an interaction between Ca^{2+} signaling and RBOH-mediated H_2O_2 production might be important for programmed cell death in root cortical cells.

In plants, complexes of Ca^{2+} sensors (CBLs) and their targets [CBL-interacting protein kinases (CIPKs)] form a complex network of Ca^{2+} signaling, and are responsible for environmental adaptation processes (Luan *et al.*, 2009; Weinl & Kudla, 2009), implying that the up-regulated CBL (GRMZM2G125838) and down-regulated CBL (GRMZM 2G173424) might be involved in adaptation (e.g. aerenchyma formation) to waterlogged conditions. The genes (GRMZM2G074317 and GRMZM2G078781) encoding proteins similar to cyclic nucleotide-gated ion channel AtCNGC2 and AtCNGC4, respectively, were included in the down-regulated genes. AtCNGC2 is involved in influxes of Ca^{2+} and K^+ in a cyclic nucleotide-dependent fashion (Leng *et al.*, 1999). It is noteworthy that mutations of *AtCNGC2* and *AtCNGC4* genes [designated *defense, no death 1* (*dnd1*) and *dnd2*, respectively] cause a phenotype that shows reduced ability to produce the hypersensitive response (HR) in response to avirulent *Pseudomonas syringae* pv. *glycinea* (Clough *et al.*, 2000; Jurkowski *et al.*, 2004).

Several genes related to cell wall loosening and degradation were up-regulated under waterlogged conditions, and it is likely that their induction was controlled by ethylene (Table 2). We found that a gene (GRMZM2G174855) encoding XET, a cell wall loosening enzyme, was up-regulated in both cortical cells and stelar cells in response to waterlogging (Figs 5, 6b). Previously, Saab & Sachs (1996) reported that *XET* mRNA was strongly accumulated in maize seedlings under flooding. Treatment with an ethylene biosynthesis inhibitor, AOA, under flooded conditions prevented the development of aerenchyma in maize roots and totally suppressed the accumulation of *XET* mRNA, suggesting that ethylene-responsive *XET* induction is involved in aerenchyma formation through cell wall loosening and degradation (Saab & Sachs, 1996). The *XET* gene identified in this study is not the same as the *XET* gene reported by Saab & Sachs (1996), suggesting that at least two ethylene-responsive *XET* genes are strongly expressed in maize roots under waterlogged conditions. The up-regulation of other genes related to cell wall loosening or degradation (e.g. pectinesterase, pectate lyase, PG and CEL) may also contribute to the activation of hydrolytic enzymes, including CEL, xylanase and pectinase, in maize roots under waterlogged conditions (Jackson & Armstrong, 1999). Indeed, the expression of genes encoding PG (GRMZM2G037431) and CEL (GRMZM2G141911) was up-regulated specifically in cortical cells under waterlogged conditions (Fig. 6b). On the other hand, we found that the genes for cellulose synthase (GRMZM2G028353 and GRMZM2G424832) and cellulose synthase-like C family protein (GRMZM2G074792) were down-regulated, suggesting that this down-regulation promotes cell wall degradation via repression of cellulose synthesis. We also identified a gene (GRMZM2G450055) encoding plasma membrane H^+-ATPase as a cortical cell-specific up-regulated gene (Figs 5, 6b, Table 1). It is proposed that the extrusion of intracellular H^+ into the cell wall by plasma membrane H^+-ATPase results in a decrease in apoplastic pH, which induces cell wall loosening, possibly mediated by low-pH-activated expansins and XETs (Frias *et al.*, 1996; Shieh & Cosgrove, 1998). Thus, the expression of the *XET* gene (GRMZM2G174855) is up-regulated in both cortical cells and stelar cells, but the activity of XET protein might be enhanced preferentially in cortical cells because the gene encoding plasma membrane H^+-ATPase shows cortical cell-specific induction of expression under waterlogged conditions. In this way, the up-regulated *H^+-ATPase* gene might be involved in cell wall loosening in cortical cells during cell death.

Under waterlogged conditions, the *ERF* gene (GRMZM2G053503) was preferentially up-regulated in cortical cells and the *RAV1L* gene (GRMZM2G169654) was specifically up-regulated in cortical cells (Figs 5, 6b, Table 4). The up-regulation of these genes was suppressed by 1-MCP pretreatment (Fig. 5, Table 4). Recently, Licausi *et al.* (2010) have identified two Arabidopsis hypoxia-inducible *ERF* genes, *HRE1* and *HRE2*, which belong to group VII of the ERF family in Arabidopsis (Nakano *et al.*, 2006), and have proposed that HRE1 and HRE2 play a partially redundant role in the tolerance of plants to anaerobic stress by enhancing anaerobic gene expression and ethanol fermentation. Group VII of the ERF family also contains Arabidopsis RAP2.2 (Hinz *et al.*, 2010), rice SUB1A (Fukao *et al.*, 2006; Xu *et al.*, 2006; Fukao & Bailey-Serres, 2008), and rice SNORKEL1 and SNORKEL2 (Hattori *et al.*, 2009), all of which play important and distinct roles in survival under hypoxia or submergence. Interestingly, the maize up-regulated ERF is highly homologous to the Arabidopsis HRE2 protein, suggesting that the maize *ERF* gene, like the Arabidopsis *HRE2* gene, is involved in the adaptation of plants to waterlogged conditions. However, to date, it is unclear whether transcriptional regulation by this ERF affects aerenchyma formation in maize roots, and thus further functional analysis of the *ERF* gene is necessary. On the other hand, it has been reported that the expression of the Arabidopsis *RAV1* gene is induced by treatment with ACC (a precursor of ethylene biosynthesis) and that the RAV1 protein positively controls

leaf senescence, which is a developmentally programmed cell death process (Woo *et al.*, 2010). Similarly, in maize, the cortical cell-specific RAV1L protein may be positively involved in programmed cell death (i.e. in aerenchyma formation) in root cortical cells under waterlogged conditions.

Three genes related to histone modification were induced under waterlogged conditions (without 1-MCP pretreatment) in both Expts 1 and 2 (Table 4). One of the histone modification-related genes (GRMZM2G417089) encodes a jmjC domain-containing protein. Recently, some jmjC domain-containing proteins have been shown to be histone demethylases (Mosammaparast & Shi, 2010). We have reported previously that submergence and re-aeration of rice cause dynamic and reversible changes of the histone methylation and acetylation states for the genes involved in anaerobiosis (Tsuji *et al.*, 2006). Similarly, it is possible that dynamic histone modifications occur in chromatin at particular genes in the maize cortex in response to ethylene under waterlogged conditions, and that the three maize up-regulated gene products contribute to the changes in histone methylation and acetylation**.**

In conclusion, in this study, we found that genes related to many types of molecular function (e.g. ROS generation or scavenging, Ca^{2+} signaling and cell wall modification) were up-regulated or down-regulated in root cortical cells under waterlogged conditions, and their expression was likely to be regulated by ethylene. We are also currently conducting microarray analysis for the identification of the inducible aerenchyma formation-associated genes of rice. By comparison of these microarray data and the identification of the genes up-regulated or down-regulated in common in maize and rice during aerenchyma formation, good candidate genes for functional analyses may be selected. The data should provide a basis for an understanding of the molecular mechanism of inducible lysigenous aerenchyma formation in plants.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of primers used for semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Table S2 List of genes whose expression was up-regulated in maize root cortex during aerenchyma formation

Table S3 List of genes whose expression was down-regulated in maize root cortex during aerenchyma formation

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