Doctor Dissertation 2011

Study of aerenchyma formation in maize roots under waterlogged conditions

（湛水条件下におけるトウモロコシの根の通気組織形成に関する研究）

Imene Rajhi
イメヌ　ラジ

Supervisor: Prof. Mikio Nakazono
指導教員: 中園幹生

Laboratory of Plant Molecular Genetics
Department of Agriculture and Environmental Biology
Graduate School of Agricultural and Life Sciences
University of Tokyo
Japan

CONTENTS

Doctor Dissertation 2011

Study of aerenchyma formation in maize roots under waterlogged conditions

（湛水条件下におけるトウモロコシの根の通気組織形成に関する研究）

Imene Rajhi

イメヌ　ラジ

The University of Tokyo
Japan

Study of aerenchyma formation in maize roots under waterlogged conditions
(湛水条件下におけるトウモロコシの根の通気組織形成に関する研究)

Imene Rajhi
イメヌ　ラジ

Preface

Preface

Waterlogging is the situation of excess water in the root zone. Soil pores that normally would be gas-filled become water-filled. Partial or complete waterlogging or flooding is an environmental stress that affects agricultural production in many regions. In fact, it can inhibit plant growths and can result in premature death of the plants. A primary effect of these conditions is the 10^4 fold slower diffusion of gases dissolved in water as compared in air. As a consequence, levels of oxygen typically decline in submerged tissues and the levels of carbon dioxide increased due to the microbial and root respiration (Colmer and Voesenek, 2009). In addition to the less availability of oxygen under waterlogged conditions, the oxygen will be also depleted by the microorganisms in the soil. This shortage in oxygen levels affects nutrient and water uptake in submerged plants (Bailey-Serres and Voesenek, 2008; Colmer and Greenway, 2011).

Higher plants are aerobic organisms and rely on oxygen to grow. Plants cannot usually survive for long periods under conditions of low oxygen availability but some species have developed strategies to avoid or withstand anaerobiosis. Plants tolerance to low oxygen differs considerably between the species. In fact, only few plants can grow in waterlogged soils, such as rice which is known to be highly tolerant of flooding (Mustroph and Albercht, 2003). Some plants can survive under waterlogged conditions for several days, while others die in a few hours. To escape from the anaerobic environment, numerous adaptations at the anatomical, morphological and metabolic levels took place in submerged tissues. The metabolic shift from aerobic respiration to anaerobic fermentation contributes to a basal energy supply under these conditions. Ethylene, a plant gaseous hormone, plays a central role in hypoxic stress signaling (Watkins, 2006). Indeed, many of the adaptive growth responses occur in response to ethylene, which is accumulated in submerged tissues. This accumulation is due to the reduced diffusion from the plant to the surrounding

water, and the induction of the ethylene biosynthesis under waterlogged conditions (Sairam *et al*., 2008). Ethylene is produced from S-adenosylmethionine (AdoMet). At first, AdoMet is converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). Subsequently, ACC oxidase (ACO) generates ethylene by oxidizing ACC in a reaction that also produces CO_2 and HCN (Gallie *et al*., 2009).

Ethylene regulates a large number of adaptations that help the plant to cope with submergence. It promotes internodal elongation in deepwater rice (Kende *et al*., 1993; Hattori *et al*., 2009) and petiole elongation in *Rumex palustris*. These elongations help the plants to keep part of the leaves above rising water levels (Voesenek *et al*., 2003). In some plants, ethylene promotes growth of adventitious roots, which can replace soil-borne roots and often elongate around the surface of soil to obtain oxygen efficiently (Suge, 1985).

Cell death is also one of important adaptive mechanisms regulated by ethylene. In shoots and roots of a large number of plant species including maize and rice, ethylene promotes death of parenchyma cells, thereby forming gas-filled air spaces (aerenchyma) (He *et al*., 1996b). Aerenchyma is a tissue consisting of longitudinal gas spaces separated by the strands of living cells, which is formed in the root cortex of waterlogged plants (Fig. 1). The function of aerenchyma has been of great interest as it enables the passage of gases in and out of tissues (*e.g*., O_2, CO_2, ethylene and methane), in plant roots, petioles and stems (Armstrong, 1979; Colmer, 2003). Internal transport of oxygen via the aerenchyma from shoots to roots is especially important for survival of plants under waterlogged conditions.

Basically, there are two types of aerenchyma in plants: lysigenous aerenchyma and schizogenous aerenchyma (Jackson and Armstrong, 1999; Seago *et al.,* 2005). Lysigenous aerenchyma is formed by creating gas spaces as a result of programmed cell death (PCD) of certain cells in the cortical region of roots. Lysigenous aerenchyma is observed in many crops such as rice, wheat and maize (Jackson and Armstrong, 1999; Evans, 2003; Mustroph and Albrecht, 2003). Schizogenous aerenchyma is formed by creating gas spaces between cells as a result of highly-

regulated cell separation and differential cell expansion without cell death taking place. Some species, such as *Saggitaria lancifolia*, form both lysigenous aerenchyma and schizogenous aerenchyma in different organs (Schussler and Longstreth, 1996).

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 upon soil waterlogging. On the other hand, lysigenous aerenchyma in non-wetland (dryland) plants including maize, is not 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). Fig. 1 shows cross sections of roots maize demonstrating inducible lysigenous aerenchyma under waterlogged conditions.

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. Evans (2003) proposed five stages for PCD during aerenchyma formation in maize: (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 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). With regard to the stages (1) and (2), the mechanisms for aerenchyma formation were proposed by Shiono *et al*. (2008). Under waterlogged soil or under hypoxic conditions, ethylene accumulates in roots (Drew and Jackson, 1979). Indeed, the expression of ACC synthase (Geisler-Lee *et al*., 2010) and ACC synthase activity (He *et al*., 1994) increase within 3 h and 12 h of hypoxia treatment, respectively. The accumulated ethylene is perceived by ethylene receptors, which is located in the endoplasmic reticulum (ER) (Chen *et al*., 2002). Ethylene responses in maize roots under waterlogged conditions are mediated through a signal transduction cascade,

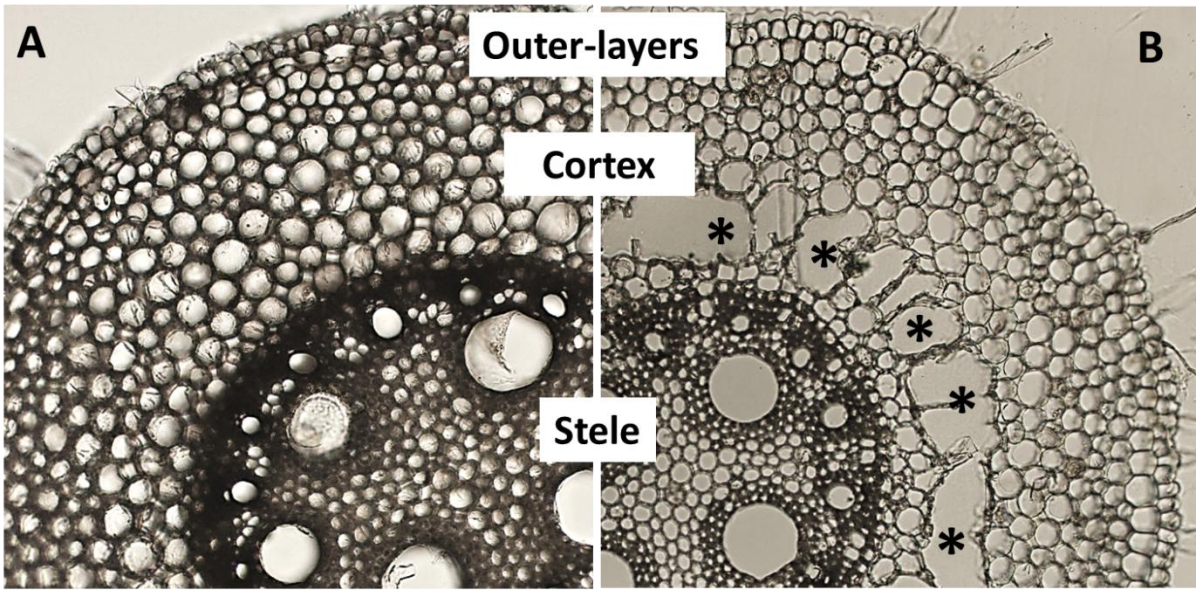

Fig. 1: Cross-sections of roots of maize demonstrating inducible lysigenous aerenchyma. (A) living cells arranged in root cortex of 3-day-old maize before waterlogging treatment (*i.e.* normoxia condition). (B) lysigenous aerenchyma containing internal gas-filled spaces is induced in cortex tissue by 24 h of hypoxic treatment. Asterisk: lysigenous aerenchyma.

which leads to the activation of G-proteins. In higher plants, a possible target for activated G-proteins is phospholipase C (PLC). PLC hydrolyzes phosphatidyl inositol 4,5-bisphoshate and two second messengers, inositol 1,4,5-trisphosphate (IP3) [an inducer of calcium (Ca^{2+}) release from ER or vacuole] and 1,2-diacylglycerol (an activator of protein kinase C) are generated. Furthermore, calcium-dependent signaling pathways are involved in the process of lysigenous aerenchyma formation in maize roots (He *et al*., 1996b, Drew *et al*., 2000). During the stages (3), (4) and (5), cellulase activity (He *et al*., 1994) as well as pectinase and xylanase activities (Bragina *et al*., 2003) are highly enhanced. Expression of a gene encoding xyloglucan endo-transglycosylase is also induced by hypoxia (Saab and Sacks, 1996). This combination of enzymes may enable a stepwise degradation of the cell walls.

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.

Based on these backgrounds, a series of experiments were carried out in this thesis to identify genes involved in aerenchyma formation in maize roots under waterlogged conditions and try to understand how they are regulated. In Chapter 1, the root and shoot growths and aerenchyma formation were investigated in maize seedlings treated under aerobic or waterlogged conditions. In Chapter 2, I grew maize under aerobic or waterlogged conditions with or without pre-treatment with an inhibitor of ethylene perception. Because aerenchyma formation occurs specifically in root cortical cells, I used a laser microdissection (LM; Nakazono *et al*., 2003; Nelson *et al*., 2006) to isolate these cells and then examined their mRNA levels. The extracted RNA will be subjected to a microarray analysis. In Chapter 3, I confirmed the microarray data using semi-quantitative RT-PCR and I determined the tissue specificity of these genes. Mainly I focused on genes that were up- or down-regulated in root cortical cells during aerenchyma formation and discuss their possible roles.

Chapter 1

Root growth and aerenchyma formation in maize seedlings under waterlogged or aerobic conditions

Introduction

Oxygen limitation is the primary plant stress in flood soils. The sudden excess of water due to flooding not only threatens the food supply of human populations but also affects the vegetation. Excess water in soil creates anoxic conditions within a few hours (Gambrell and Patrick, 1978). A slow diffusion rate of oxygen under water pushes the plant (especially roots) towards more anaerobic biochemical acclimation (Armstrong, 1978). Thus plants shift their metabolism at basal metabolism rate to sustain their survival under low energy economy.

To escape the waterlogged conditions, maize plants developed strategies to avoid the oxygen shortage, among them two rooting successful strategies: development of adventitious roots near to the surface and formation of internal gas spaces. Internal gas spaces (aerenchyma) provide a conduit for the transport of oxygen (Armstrong *et al*., 1991). This structural modification in roots is very important for the survival of the plants under low oxygen availability.

In this Chapter, the response of maize roots and shoots to waterlogged conditions was investigated. Aerenchyma formation was also examined at different levels in maize roots during aerobic or waterlogged conditions.

Materials and methods

1. Plant material and growth conditions

Maize (*Zea mays* L. inbred line B73) caryopses were placed on moist chromatography paper (3MM CHR, Whatman, Kent, UK), rolled-up in the paper, placed in a flask half shielded with aluminium foil, and incubated in constant light at 28°C as described by Nakazono *et al.* (2003).

2. Anatomical observations

Three-day-old aerobically-grown seedlings were further grown for 12, 24, 36, and 48 hours (h) under waterlogged conditions or under aerobic conditions. I isolated segments of primary roots at 0-0.5 cm, 0.5-1 cm, 1.5-2.0 cm, and 2.5-3 cm from the root-shoot junction for 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, NIH, MD, Bethesda). Three independent experiments were conducted, each using two primary roots.

3. Marking using a charcoal

Three day-old-seedlings were marked at 0.5, 1, 1.5, 2, 2.5 and 3 cm from the root-shoot junction and at 1 cm behind the root tip. Every 12 hours, the root lengths were examined and the positions at 1 cm behind the root tip were marked.

Results

1. Seedling growth under waterlogged or aerobic conditions

Seeds of maize were germinated under aerobic conditions for 3 days (Fig. 1-1). The water was kept just enough to keep the paper moist but not touch the roots. After 3 days of germination, the root length and the shoot length were ~ 6 cm and ~ 3 cm, respectively (Figs. 1-2 and 1-3).

Three-day-old aerobically grown seedlings were then subjected to waterlogged conditions for 12, 24, 36 and 48 h. For a control, seedlings were grown at the same time under aerobic conditions. Under waterlogged conditions, the root growth was inhibited, because the root length after 48 h of the treatment was ~ 9.77 cm (Figs. 1-4 and 1-5). In contrast, the root length of seedlings grown under aerobic conditions for 5 days was ~ 17 cm. Interestingly, the shoot growth was not affected by the waterlogging treatment (Fig. 1-6).

2. Root elongation and marking using the charcoal

The aim of this study was to identify genes involved in aerenchyma formation. For this purpose, it was important to select one part of the root to collect the samples to perform the microarray experiment. Using the charcoal marking method, I observed the elongation of the root grown under aerobic conditions.

Three-day-old seedlings were marked with a charcoal at 0.5, 1, 1.5, 2, 2.5 and 3 cm from the root-shoot junction, and then investigated the root length at every 12 h (Fig. 1-7). Only the apical part (*i.e.*, elongation zone) of the root was elongated, but no elongation was observed at the basal part of the root. The positions marked with the charcoal at the basal part were not changed between 3 day-old seedlings and 5 day-old seedlings. Thus I decided to use the basal part for investigating the pattern of aerenchyma formation.

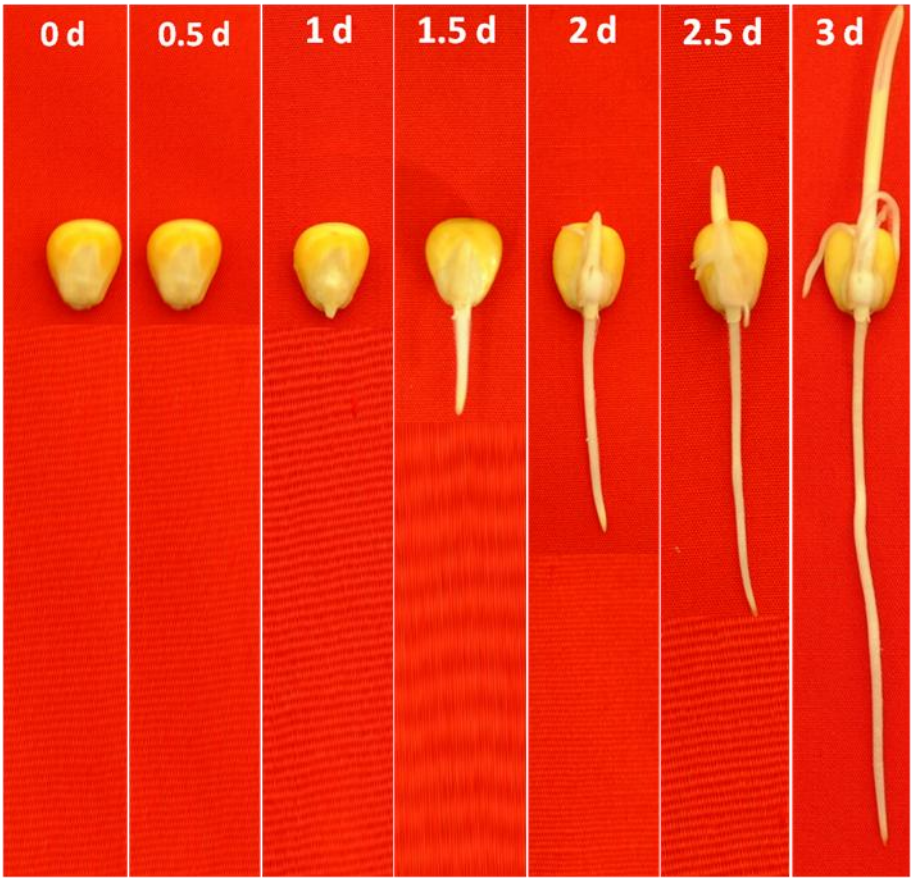

Fig. 1-1: Growth of maize seedlings under aerobic conditions.

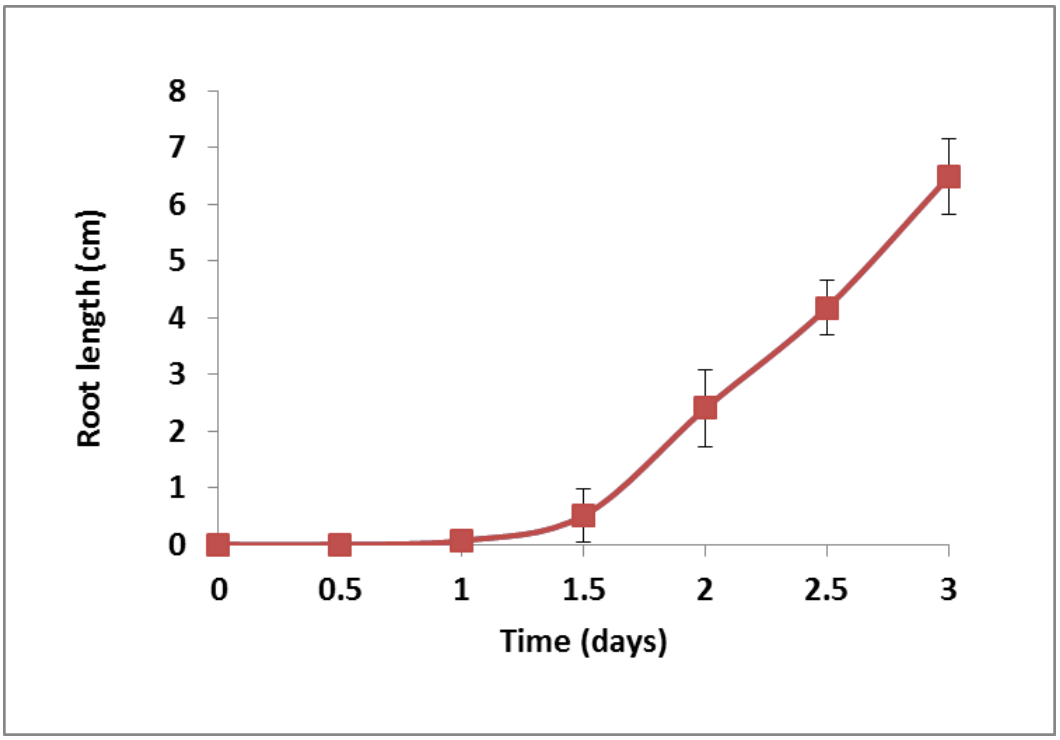

Fig. 1-2: Root lengths of maize seedlings grown under aerobic conditions. The root lengths were measured at each 12 h until 3 days. All values are means (n = 9) ± SD. Three seedlings were subjected to analysis in each of the three experiments.

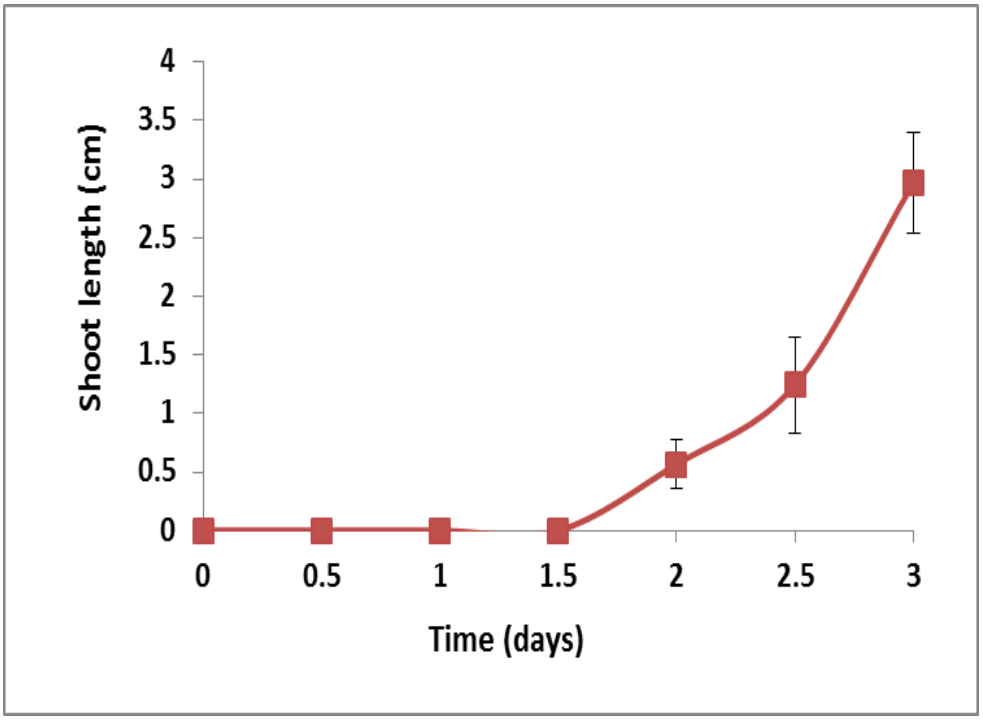

Fig. 1-3: Shoot lengths of maize seedlings grown under aerobic conditions. The shoot lengths were measured at each 12 h until 3 days. All values are means (n = 9) ± SD. Three seedlings were subjected to analysis in each of the three experiments.

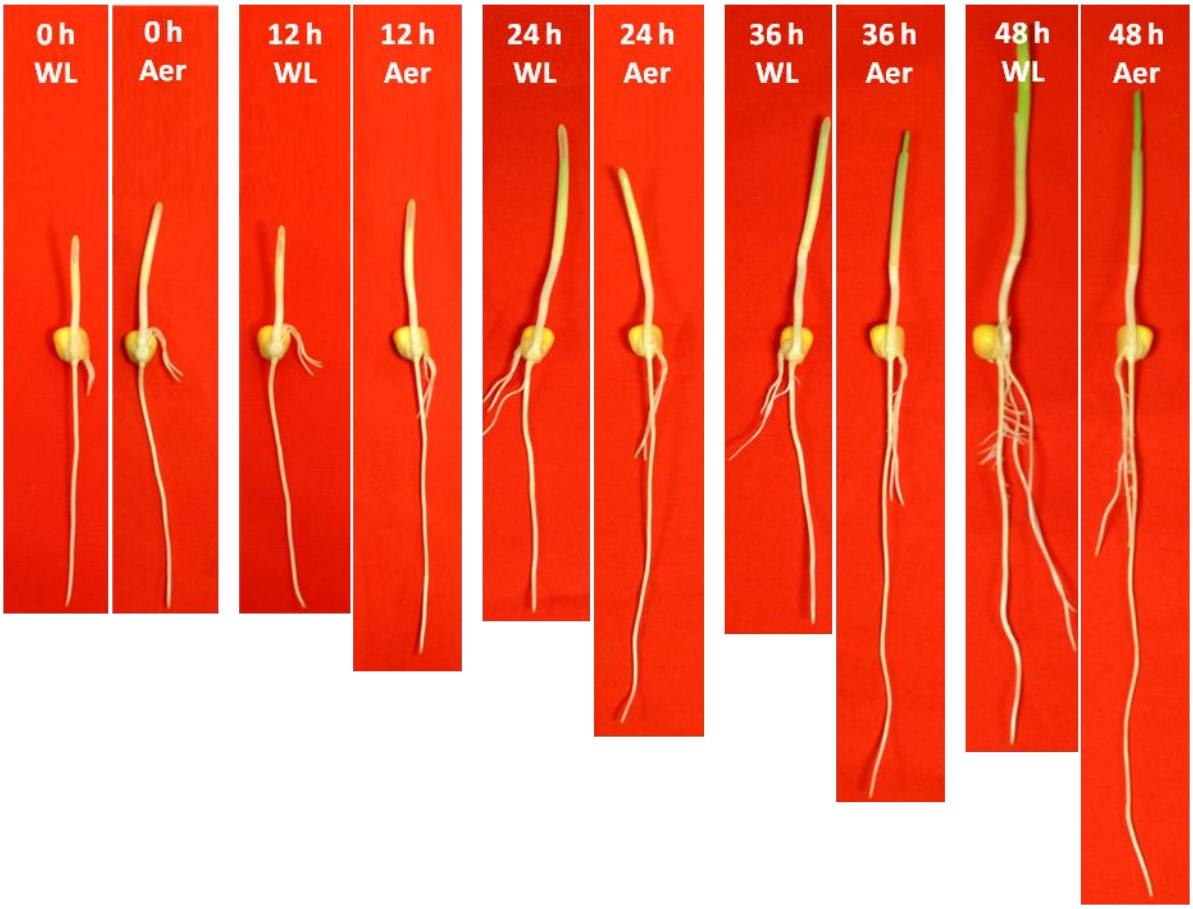

Fig. 1-4: Growth of seedlings under waterlogged or aerobic conditions. Three-day-old seedlings (0 h) were subjected to waterlogged treatment (WL) or aerobic treatment (Aer).

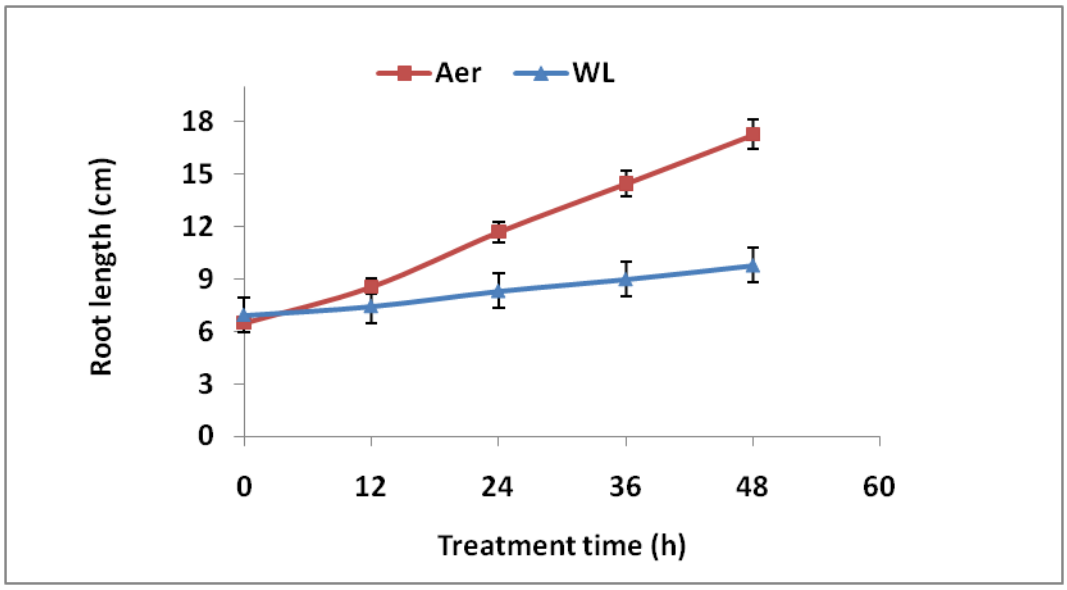

Fig. 1-5: Root lengths of maize seedlings grown under waterlogged conditions (WL) or aerobic conditions (Aer). Three-day-old seedlings were subjected to different treatments. The root lengths were measured at each 12 h until 48 h. All values are means (n = 9) ± SD. Three seedlings were subjected to analysis in each of the three experiments.

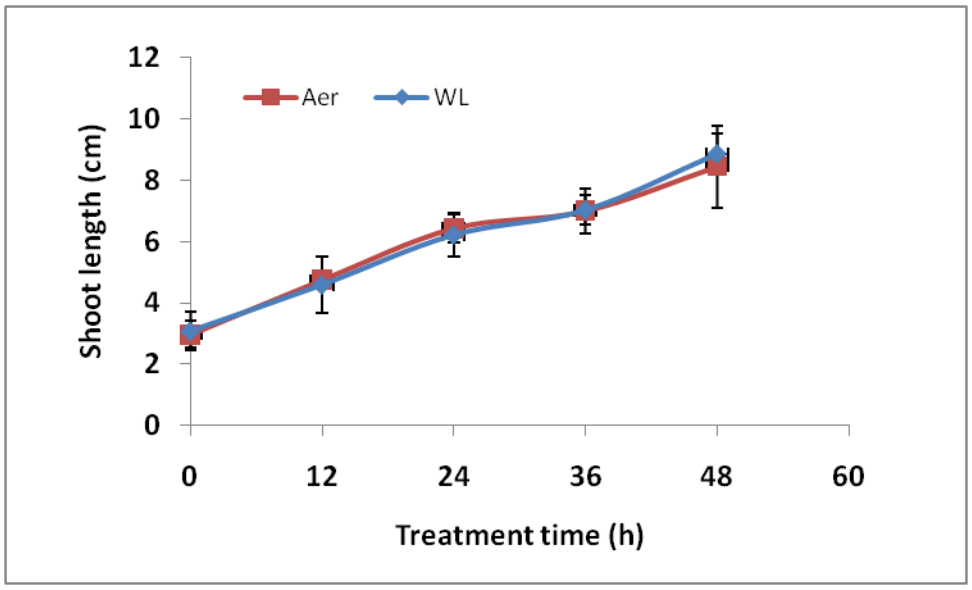

Fig. 1-6: Shoot lengths of maize seedlings grown under waterlogged conditions (WL) or aerobic conditions (Aer). Three-day-old seedlings were subjected to different treatments. The shoot lengths were measured at each 12 h until 48 h. All values are means (n = 9) ± SD. Three seedlings were subjected to analysis in each of the three experiments.

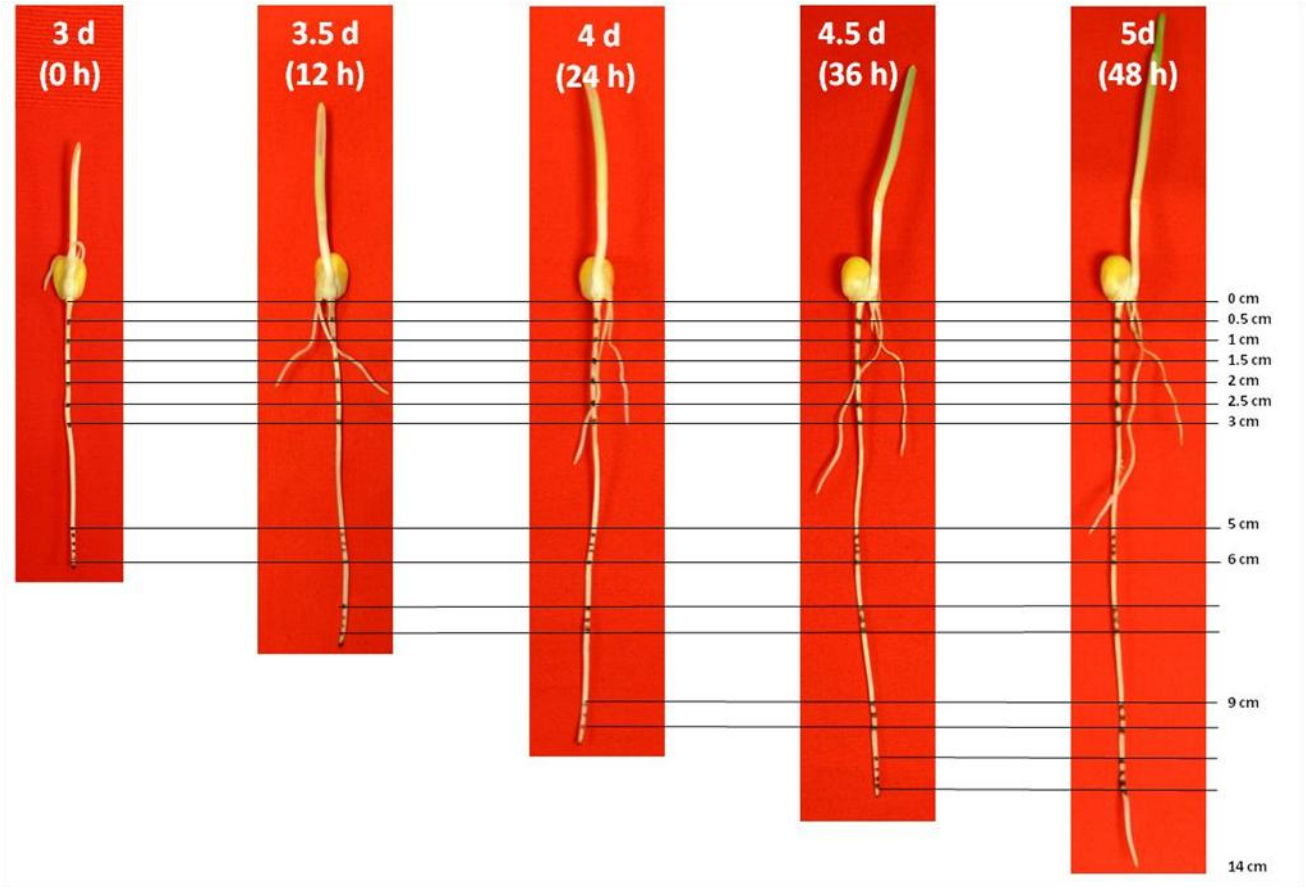

Fig. 1-7: Elongation of the primary root of maize under aerobic conditions. Three-day-old seedlings were marked with the charcoal in the basal part at 0.5 cm, 1 cm, 1.5 cm, 2 cm, 2.5 cm and 3 cm. The root lengths were checked at each 12 h and the root tip was marked each time.

3. Aerenchyma formation

In order to decide the part to collect the samples for the microarray analysis, the area of aerenchyma was measured at 0-0.5 cm, 0.5-1 cm, 1.5-2 cm, and 2.5-3 cm from the root-shoot junction under aerobic or waterlogged conditions. The aerenchyma started forming at the basal part of maize roots between 12 h and 24 h under waterlogged conditions (Figs. 1-8 and 1-9). The area of aerenchyma formation was increased during the waterlogging treatment. The most inducible part was at 1.5-2 cm from the root-shoot junction (Fig. 1-9). The percentage of the area of aerenchyma at 1.5-2 cm was 7.7 % at 24 h and 9.9 % at 48 h after the treatment.

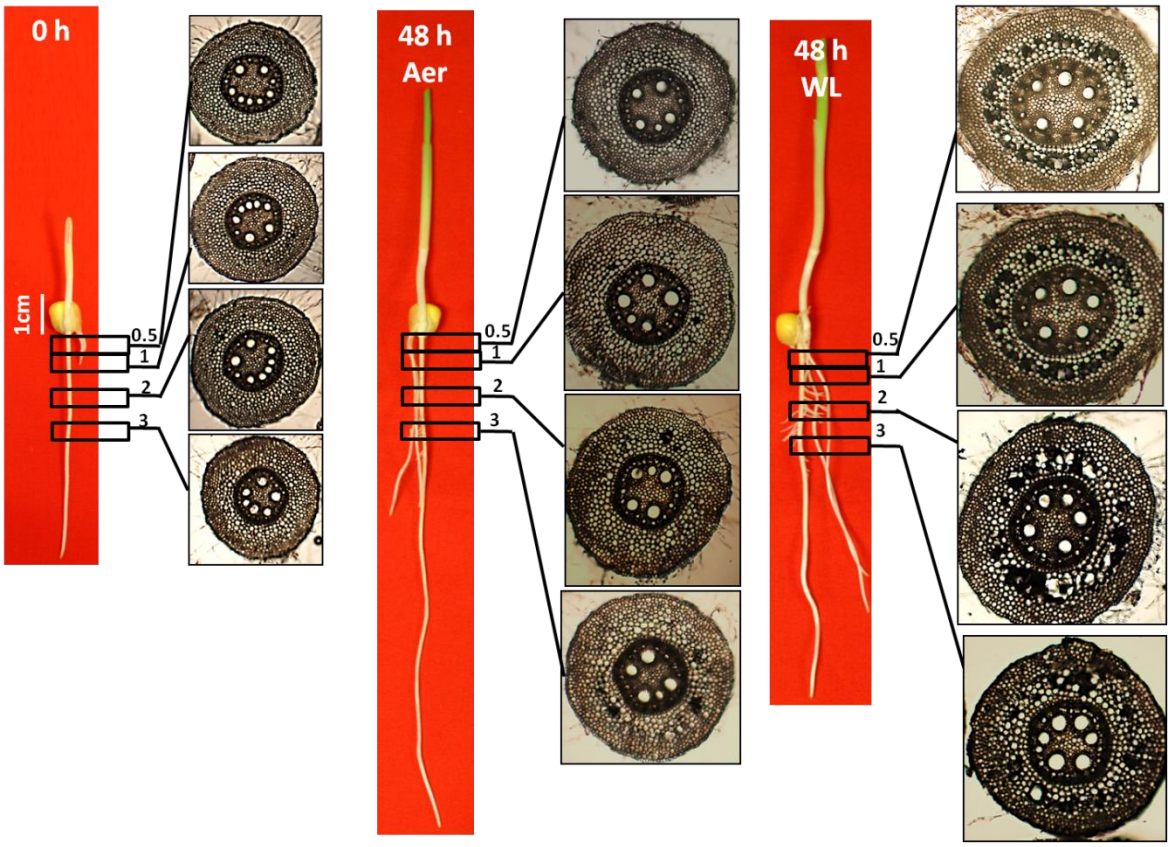

Fig. 1-8: Aerenchyma formation in the maize primary root treated under waterlogged conditions (WL) or aerobic conditions (Aer). Three-day-old seedlings (0 h) were subjected to treatments. The tissue sections were prepared after 48 h of treatments at 0-0.5 cm, 0.5-1 cm, 1.5-2 cm and 2.5-3 cm from the root-shoot junction.

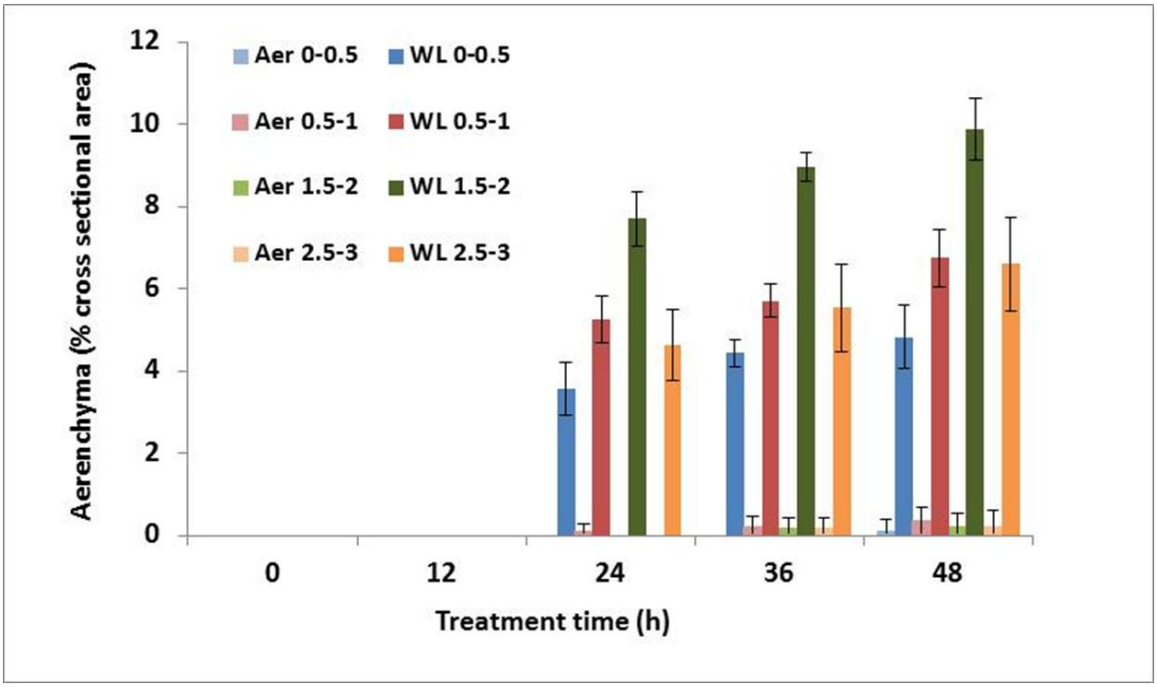

Fig. 1-9: Aerenchyma formation (area of the aerenchyma per area of the whole root on the section) of the maize primary roots under waterlogged conditions (WL) or aerobic conditions (Aer). The aerenchyma formation was analyzed at each 12 h until 48 h after the start of the treatments. Aerenchyma formation was measured at 0-0.5 cm, 0.5-1 cm, 1.5-2 cm and 2.5-3 cm from the root-shoot junction. All values are mean (n = 6) ± SD. Two roots were subjected to analysis in each of the 3 experiments.

Discussion

Plants adapt to waterlogged conditions by morphological changes in the root system. Root is the plant organ that suffers most frequently from low oxygen stress, which is mainly caused by waterlogging. Thus, the root growth is inhibited under waterlogged conditions (Fig. 1-5). However, as shown in Fig. 1-6, there were no differences of the shoot lengths between under aerobic conditions and under waterlogged conditions. It was not surprising that I did not observe any delayed growths or any injuries of the shoots, because the duration of the waterlogging treatment was relatively short (48 h). Vodnik *et al*. (2009) also reported no significant effects of oxygen deprivation for 7 days on the shoot length or on fresh weights of shoots and roots. It is possible that the seeds of maize have enough nutrients to permit the elongation of the shoot under waterlogged conditions. Upon complete submergence, several species such as rice have the capacity to stimulate the elongation of leaves. This fast elongation can restore contact between leaves and air (Bailey-Serres and Voesenek, 2008). It has been shown that in rice, maize and wheat, the root growth was affected more than shoot leading to an increased shoot/root ratio under waterlogged conditions. Maize root length was the most strongly retarded (1/3 of control) to a lesser extent in wheat (1/2 of control) and nearly no effect in rice (Mustroph and Albrecht, 2003).

Waterlogging induces aerenchyma formation in several crops such as rice (Justin and Armstrong, 1991), wheat (Watkin *et al*., 1998), maize (Gunaverdena *et al*., 2001), barley (Arikado and Adachi, 1955) and soybean (Bucanamwo and Purcell 1999). These air spaces facilitate aeration and assure prolonged survival (Drew *et al*., 2000). In maize, the aerenchyma consisted of 19 % of basal part of roots after 4 days of the waterlogging treatment (Mustroph and Albrecht, 2003). In my experimental conditions, the aerenchyma was reached 9 % after 2 days of waterlogging treatment (Fig. 1-8). The most inducible part of the basal root under

waterlogged conditions was at 1.5-2 cm from the root-shoot junction, and thus I decided to collect tissues at this part to study aerenchyma formation in my thesis.

Summary

Three-day-old seedlings were treated under waterlogged conditions or aerobic conditions for 48 h and the shoot and root growths and aerenchyma formation were investigated. The treatment of maize seedlings with waterlogged conditions inhibited the growth of a primary roots. However, no effect was found in the shoot growth. Aerenchyma formation in maize roots was induced by waterlogged conditions. The analysis of the area of aerenchyma in maize roots at different levels showed that the most inducible part was observed at 1.5-2 cm from the junction shoot-root. Therefore, I decided to use the portion at 1.5-2 cm from the root-shoot junction to study aerenchyma formation in maize roots.

Chapter 2

Identification of genes involved in aerenchyma formation in maize roots under waterlogged conditions using a Laser Microdissection and microarray analysis

Introduction

Under waterlogged conditions, a rapid reduction in the partial oxygen pressure triggers a set of adaptive responses. Over several minutes, ethylene production is accelerated (Vartapetian and Jackson, 1997). 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 increased ethylene production in maize roots several fold within 3 hours compared with aerobic conditions. A 5.8 fold was observed at 6 hours after treatment and the maximum of ethylene evolution (10 fold) was detected at 12 hours after the treatment (Geisler-Lee *et al*., 2010). 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.

Based on this information, I used an inhibitor of ethylene perception [1-methylcyclopropene (1-MCP)] to identify the ethylene-inducible genes involved in aerenchyma formation. Indeed, 1-MCP is often used as a tool to further investigate the role of ethylene in different mechanisms of growth in plants such as ripening and senescence, and as a commercial technology to improve maintenance of fruits and vegetables quality and prevent them from the ripening for a while (Watkins, 2006). The 1-MCP is thought to interact with ethylene receptors, thereby preventing ethylene-dependent responses (Sisler and Blankenship, 1996). The powder of 1-MCP is chelated with γ-cyclodextrin, and 1-MCP is easily released as a gas when the powder is dissolved in water. The 1-MCP has a non-toxic mode of action, negligible

residue and is active at very low concentrations (Watkins, 2006). It was also used to investigate the role of ethylene in aerenchyma formation in wetland plant *Juncus effuses* (Visser and Bögemann, 2006). Thus, in this study, I used the 1-MCP to inhibit the action of ethylene during waterlogged conditions.

To monitor genes expressed during aerenchyma formation in maize roots, I combined two technologies: laser microdissection (LM) and a microarray. LM is a new method for isolating specific cells from heterogeneous tissues in one step under direct microscopic visualization with the assistance of a laser beam (Nakazono *et al*., 2003; Nelson *et al*., 2006). LM in combination with microarray analyses can monitor changes in transcript levels in a specific cell types, in which morphological or physiological changes are observed (Ohtsu *et al*., 2007).

In this Chapter, I grew maize plants under aerobic or waterlogged conditions with or without pre-treatment with 1-MCP. As aerenchyma is formed specifically in root cortical cells, I collected the root cortex using the LM and then I examined their mRNA levels with a maize microarray.

Materials and methods

1. Plant material and growth conditions

Three-day-old aerobically-grown maize seedlings, as described in Chapter 1, were then subjected to the following three experimental conditions:

Experiment 1: Effects of waterlogged conditions on aerenchyma formation in a primary root. After 3 days 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. Prior to waterlogging, 2.5-day-old seedlings were pre-treated with 1 ppm gaseous ethylene perception inhibitor 1-MCP for 12 hours (h) in a tightly-closed container. For a control, the same treatment was used except without 1-MCP.

Experiment 3: Effect of ethylene on aerenchyma formation in a primary maize root under aerobic conditions. Three-day-old seedlings were treated with 1 ppm of ethylene gas under aerobic conditions in a tightly-closed container. For a control, the seedlings were grown at the same conditions except without ethylene.

2. Anatomical observations

I used the same method described in Chapter 1.

3. Samples preparation for Laser microdissection

3.1. Sample fixation

Tissues of the basal parts of the primary roots (1.5-2.0 cm from the root-shoot junction) were placed in a vial and fixed in 75% ethanol/ 25% acetic acid under vacuum for 5 min on ice. To ensure full fixation, the vacuum infiltration step was done three times. Then, the fixative solution in the vial was replaced with a new pre-chilled fixative solution. Immediately after the fixation step, the samples were

embedding in paraffin using a microwave method described below. All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.2. Paraffin-embedding using a microwave

Paraffin-embedding was followed by the microwave method reported by Takahashi *et al*. (2010). The fixative solution in the vial was replaced with a new pre-chilled fixative solution before start of the microwave method. The vial was placed on ice and microwaved using a Microwave Processor (Energy Beam Sciences, East Granby, CT) at 250 W at 37°C for 15 min three times. The fixative solution was replaced with a new solution each time. For dehydration and infiltration, the microwave power was changed to 300 W. The fixed samples were dehydrated with 70% ethanol at 58°C for 1 min 30 s, followed by dehydration steps of 1 min 30 s each in 80% ethanol, 90% ethanol, 100% ethanol and absolute ethanol. For infiltration, absolute ethanol was replaced with 50% ethanol and 50% *n*-butanol, followed by 100% *n*-butanol, with each step microwaved at 58°C for 1 min 30 s. Then *n*-butanol was replaced with melted paraffin wax (Paraplast Xtra; Fisher Scientific, Pittsburgh, PA), and specimens in paraffin wax:*n*-butanol (1:1) were microwaved at 300 W at 58°C for 10 min. Subsequently, the specimens were microwaved in 100% paraffin wax at 250 W at 58°C for 10 min, followed by microwaving in 100% paraffin wax at 250 W at 58°C for 30 min for 4 times. The specimens were placed onto a plastic dish filled with new 100% paraffin wax, and the embedded specimens were then cooled to room temperature. After cooling, the paraffin blocks were stored at 4°C.

3.3. Preparation of paraffin-embedded sections and slides for LM

RNase inhibitor solution, RNAsecure™ reagent (Ambion, Austin, TXA) was used for mounting of serial paraffin sections on a slide. The RNAsecure™ reagent was diluted 25 times with RNase-free water and was pre-incubated at 60°C for 5 min. The pre-incubated RNAsecure™ solution was put onto a PEN membrane glass slide (Molecular Devices, Sunnyvale, CA) heated on a hot plate. Serial paraffin sections prepared at 16 μm in thickness were floated on surface of the RNAsecure™, and

were incubated at 57°C for 10 min. Subsequently, the solution was removed with a micropipette, and then residual solution was wiped off with RNase-free paper. The sections were dried at room temperature, and then were kept at 4°C.

To remove paraffin, slides were immersed in 100% of xylene, then in 50% xylene and 50% ethanol, and finally in 100% ethanol for 5 min for each step, followed by air-drying at room temperature. Cortical cells were collected from the root tissue sections using a Veritas Laser Microdissection System LCC1704 (Molecular Devices).

3.4. Extraction, quantification and qualification of RNA

Total RNA was extracted from LM cells with a Pico-Pure^{TM} RNA isolation kit (Molecular Devices) according to the manufacturer's instructions. The extracted total RNA was quantified with a Quant-iT^{TM} RiboGreen RNA reagent and kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The quality of total RNA extracted from LM-collected tissues was assessed using an RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA integrity was judged by RNA Integrity Number (RIN), which was calculated with 2100 Expert Software (Agilent, version B.02.02, eukaryote total RNA pico mode). The software algorithm, developed by Schroeder *et al*. (2006), categorizes total RNA quality on a scale from 1 to 10, in which 10 corresponds to intact RNA and 1 corresponds to highly degraded RNA. RIN calculations are based on RNA electropherograms from non-plant species.

3.5. 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 has 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).

3.6. 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 6 replicates (3 biological replicates and a color swap for each replicate). I 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 less than 0.05. Maize 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 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/data_download.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, I 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/data_download.shtml).

Results

1. Aerenchyma formation in a maize primary root

To determine a time point for identification of ethylene-responsive, aerenchyma formation associated genes by the LM-microarray analyses, 3-day-old aerobically-grown maize seedlings were kept under waterlogged conditions with or without pre-treatment with 1-MCP, an inhibitor of ethylene perception, for 0, 6, 12, 18 and 24 h, and aerenchyma formation (% cross-sectional area) was measured at the basal region of a primary root. Aerenchyma formation started between 18 h and 24 h after waterlogging treatment (the area of aerenchyma was 8% after 24 h of waterlogging treatment), whereas it was suppressed for at least 24 h after waterlogging treatment when the seedlings were pre-treated with 1-MCP (Fig. 2-1). On the other hand, aerenchyma formation was not observed at the basal region of roots of 4-day-old seedlings grown under aerobic conditions (Fig. 2-1). However, aerenchyma is induced by the exogenous ethylene under aerobic conditions. Ethylene-induced aerenchyma started between 6 h and 12 h after the treatment and the area of aerenchyma reached 4 % at 24 h (Fig. 2-1). These results confirm that ethylene works as a trigger for inducible aerenchyma formation. To perform microarray analysis, I decided to collect root cortical cells at 12 h after the treatment under waterlogged conditions with or without pre-treatment with 1-MCP or under aerobic conditions.

2. 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 pre-treatment with 1-MCP or under aerobic conditions, and then the basal parts of primary roots were fixed and tissue sections were prepared for LM. Cortical cells were collected from the tissue sections via LM (Fig. 2-2) and the RNA was extracted with a Pico-Pure^{TM} RNA isolation kit (Molecular Devices). The quality of extracted RNA was

assessed using a RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer. RNA integrity was judged by RNA Integrity Number (RIN). The RINs of the collected RNA were between 6 and 7.3. This quality allowed me to perform microarray analyses with these samples.

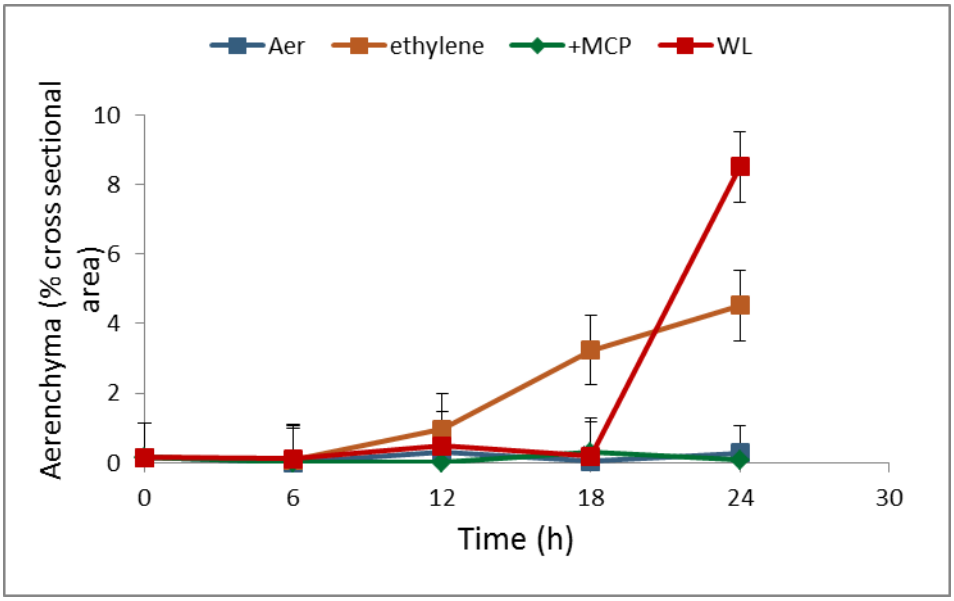

Fig. 2-1: Aerenchyma formation (area of the aerenchyma per area of the whole root on the section) of maize primary roots under waterlogged conditions (WL), waterlogged conditions with the 1-MCP pre-treatment (+MCP) (1 ppm), aerobic conditions (Aer) and treated with ethylene (1 ppm) under aerobic conditions. The aerenchyma formation was analyzed at each 6 h for a period of 24 h after the start of the treatments. All values are mean (n = 9) ± SD. Three roots were subjected to analysis in each of the 3 experiments.

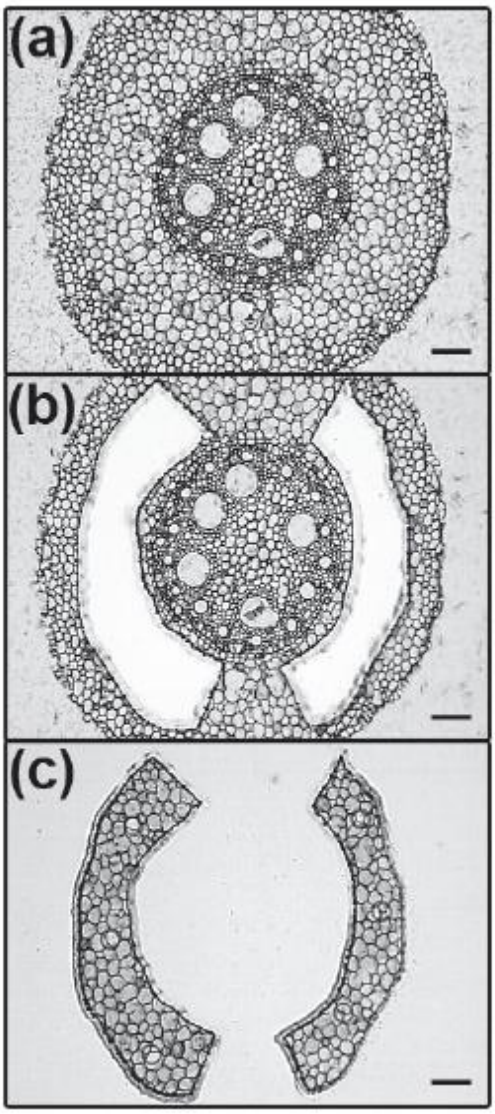

Fig. 2-2: Isolation of cortical cells from paraffin-embedded sections of a maize primary root using laser microdissection (LM). (a) a root tissue section before LM, (b) a root tissue section after LM and (c) LM-isolated cortical cells. Scale bars: 100 μm.

The RNA samples extracted from the LM-isolated cortical cells were labeled with Cy3 or Cy5 dye, and the labeled cDNA from each of 3 biological replications were hybridized to maize oligo-microarrays. To identify genes expressed during aerenchyma formation, gene expressions were compared between the waterlogging treatment and the aerobic control (Experiment 1) or between the waterlogging treatment without the 1-MCP pre-treatment and the waterlogging treatment with the 1-MCP pre-treatment (Experiment 2). The resulting data were analyzed as described in Materials and methods. For each experiment, I selected genes whose intensities were more than 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 (~1.4%) among 42,034 gene probes spotted on a microarray slide were significantly different between the two treatments common in the experiments 1 and 2. Among them, it was likely that 239 genes (~0.6%) were up-regulated and 336 genes (~0.8%) were down-regulated under conditions for inducing aerenchyma formation (*i.e.*, waterlogged conditions) (Fig. 2-3).

3. 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/data_download.shtml) (Fig. 2-4). Approximately 36% of the up-regulated genes and approximately 32% of the down-regulated genes are 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 are also relatively abundant both in 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, are translation-related genes encoding ribosomal proteins, translation initiation factors, and translation elongation factors (Fig. 2-4).

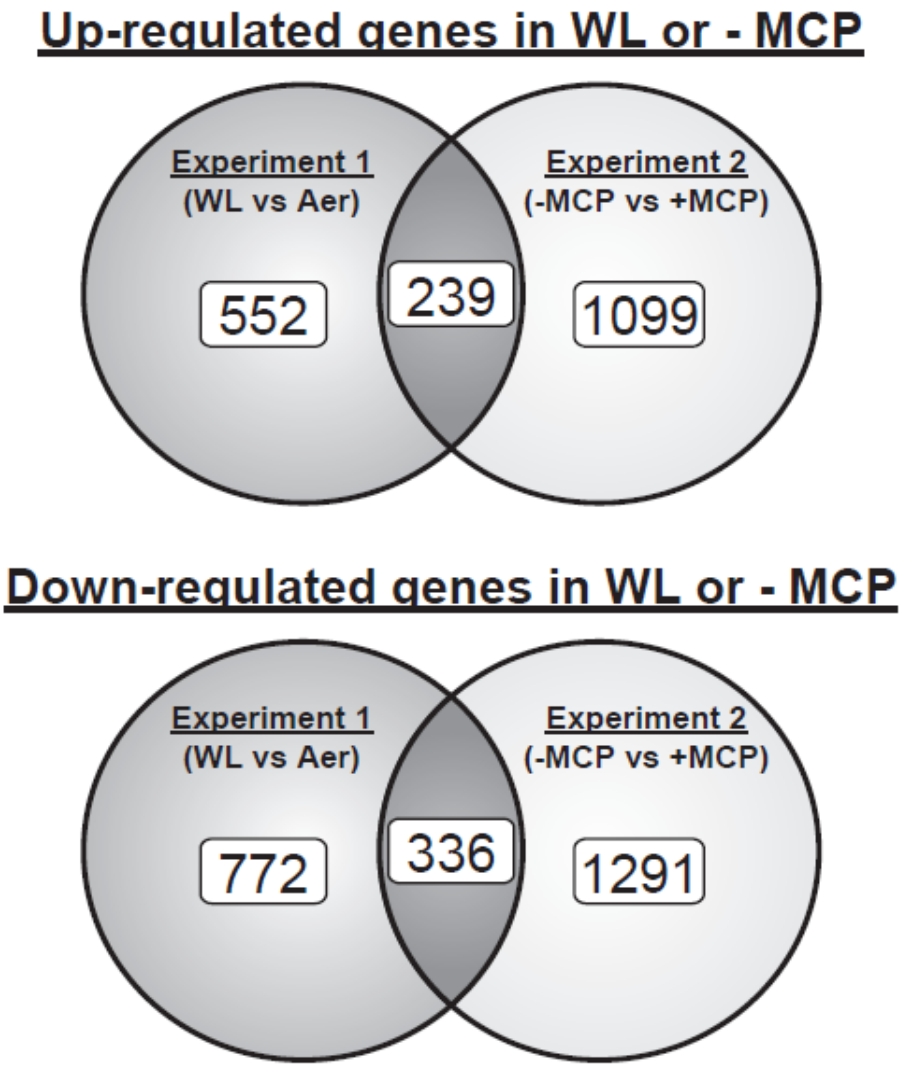

Fig. 2-3: Number of genes up-regulated or down-regulated under waterlogged conditions (without the 1-MCP pre-treatment). Genes whose expression levels differed from the controls by a factor of at least 2 in all three replications and in each color swap were considered to be up-regulated or down-regulated and then genes commonly up-regulated or down-regulated in both experiments were collected. Experiment 1: 12 h waterlogged condition (WL)/12 h aerobic condition (Aer). Experiment 2: 12 h waterlogged condition without the 1-MCP pre-treatment (-MCP)/12 h waterlogged with the 1-MCP pre-treatment (+MCP).

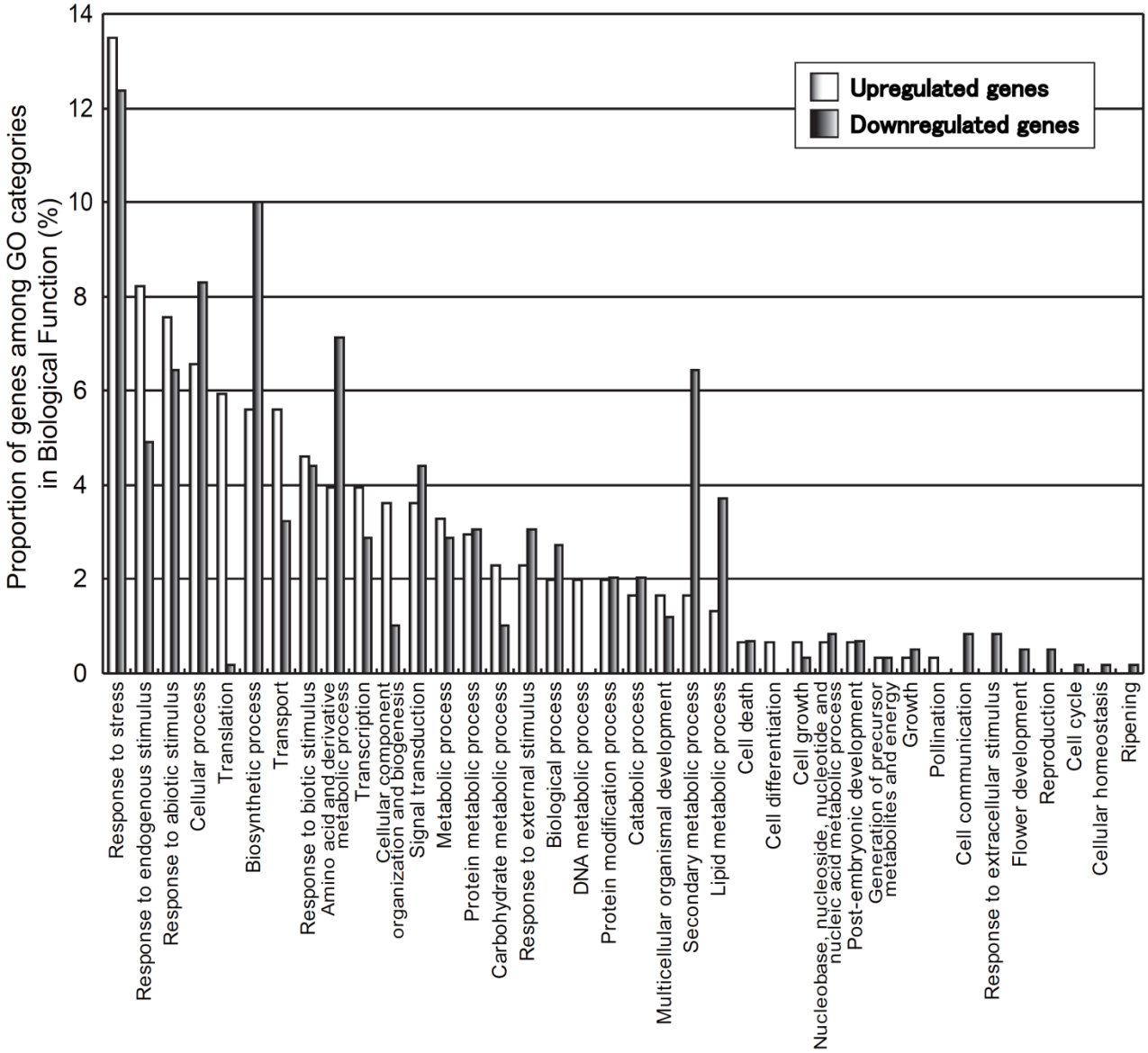

Fig. 2-4: Gene classification based on gene ontology (GO) for genes commonly up-regulated or down-regulated in Experiments 1 and 2. Frequency of GO terms was analyzed using GO Slim Assignment. X-axis and Y-axis indicate names of clusters and the ratio of each cluster, respectively. Only the Biological Processes were used for GO analysis.

4. Analysis of the microarray data

4.1. Reactive oxygen species generation/scavenging-related genes

Production of ROS has been implicated in diverse physiological processes including programmed cell death in plants (Overmyer *et al*., 2003). One of major sources of ROS in plants is a reaction mediated by NADPH oxidase, which is responsible to the conversion of O_2 to superoxide anion (O_2^-), thereby leading to production of hydrogen peroxide (H_2O_2) (Overmyer *et al*., 2003). Here, I found several ethylene-mediated waterlogging-responsive genes related to ROS-generation or ROS-scavenging. The up-regulated genes contain genes encoding respiratory burst oxidase homolog (RBOH), glutathione S-transferase and manganese superoxide dismutase, and the down-regulated genes contain genes encoding RBOH and metallothionein (MT). The *RBOH* gene products are involved in ROS generation, and the *MT* gene is involved in ROS scavenging (Table 2.1).

4.2. 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 expressions were significantly changed under the waterlogged conditions (without 1-MCP pre-treatment) both in experiments 1 and 2, were identified (Table 2.1). They include up-regulated genes encoding EF hand family protein (Calcineurin B like), calmodulin like protein, C2 domain containing protein and EH domain containing protein. The down regulated genes encoding calcineurin B, five calcium/calmodulin dependent protein kinases, OsCML8- Calmodulin-related calcium sensor protein, and two cyclic nucleotide-gated ion channel 2 (Table 2.1).

Table 2.1: 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 accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene Annotation[e]
Reactive oxigen species (ROS) generation/scavenging					
TC289691	117.72	10.72	GRMZM2G300965	Os12g0541300	Respiratory burst oxidase, putative, expressed
TC301460	5.39	20.85	GRMZM2G416632	Os09g0467200	Glutathione S-transferase, putative, expressed
TC301296	3.45	3.16	GRMZM2G160629	Os05g0323900	Superoxide dismutase, mitochondrial precursor, putative, expressed
TC311766	0.27	0.20	GRMZM2G426953	Os01g0835500	Respiratory burst oxidase, putative, expressed
TC298672	0.16	0.39	GRMZM2G164229	Os11g0704500	Metallothionein, putative, expressed
Calcium signaling					
TC294844	6.69	11.56	GRMZM2G125838	Os02g0802400	EF hand family protein, putative, expressed (Calcineurin B like, CBL)
TC313511	5.75	2.91	GRMZM2G052740	Os02g0158100	EH domain-containing protein 1, putative, expressed
TC307198	5.57	3.78	GRMZM2G082199	Os01g0841700	C2 domain containing protein, putative, expressed
TC282752	2.71	15.08	GRMZM2G467184	Os11g0141400	Calmodulin-like protein 1, putative, expressed
DR810794	0.42	0.36	GRMZM2G430600	Os03g0319400	CAMK_KIN1/SNF1/Nim1_like.2 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
TC282050	0.41	0.28	GRMZM2G032852	Os03g0128700	CAMK_CAMK_like.17 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
TC288382	0.38	0.14	GRMZM2G180916	Os10g0564500	CAMK_CAMK_like.41 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
TC298929	0.36	0.42	GRMZM2G173424	Os12g0597000	Calcineurin B, putative, expressed
TC293625	0.33	0.18	GRMZM2G412601	Os09g0418500	CAMK_KIN1/SNF1/Nim1_like.35 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
TC281251	0.27	0.23	GRMZM2G146720	Os11g0134300	CAMK_KIN1/SNF1/Nim1_like.37 - CAMK includes calcium/calmodulin depedent protein kinases, expressed
TC282587	0.27	0.22	GRMZM2G078781	Os01g0782800	Cyclic nucleotide-gated ion channel 2, putative, expressed
BM501148	0.22	0.32	GRMZM2G096228	Os10g0389000	OsCML8 - Calmodulin-related calcium sensor protein, expressed
TC289094	0.06	0.04	GRMZM2G074317	Os03g0758300	Cyclic nucleotide-gated ion channel 2, putative, expressed

Table 2.1 (continued)

Maize EST accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene Annotation
Cell wall modification					
TC315760	10.77	7.35	GRMZM2G043943	Os01g0743200	Pectinesterase, putative, expressed
TC283838	5.86	11.61	GRMZM2G131912	Os04g0137100	Pectate lyase precursor, putative, expressed
TC282363	5.56	2.55	GRMZM2G037431	Os01g0636500	Polygalacturonase, putative, expressed
TC286313	5.47	5.20	GRMZM2G174855	Os07g0529700	Glycosyl hydrolases family 16, putative, expressed (Xyloglucan endotransglucosylase, XET)
TC311007	5.07	23.94	GRMZM2G119471	Os03g0124900	Polygalacturonase, putative, expressed
TC314571	4.36	12.59	GRMZM2G141911	Os02g0123700	Endoglucanase, putative, expressed (Cellulase, CEL)
TC314458	3.00	107.88	AC234190.1[f]	Os01g0249100	Expansin precursor, putative, expressed
TC299614	2.43	4.16	GRMZM2G048430	Os06g0711800	Invertase/pectin methylesterase inhibitor family protein, putative, expressed
TC280317	0.42	0.05	GRMZM2G028353	Os07g0424400	CESA3 - cellulose synthase, expressed
TC287832	0.34	0.10	GRMZM2G424832	Os07g0208500	CESA8 - cellulose synthase, expressed
TC303435	0.24	0.24	GRMZM2G074792	Os01g0766900	CSLC1 - cellulose synthase-like family C, expressed

[a]: Average expression ratio of 3 biological replicates and a color swap for each replicate. [b]: Maize Gene IDs in MaizeSequence Database (http://www.maizesequence.org/).

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

4.3. Cell wall modification-related genes

The last step of aerenchyma formation involves cell wall loosening and degradation, in which many enzymes including xyloglucan endotransglucosylases (XETs), expansins, cellulases and pectinases are involved (He *et al.*, 1994, Saab and Sachs, 1996; Jackson and Armstrong, 1999). In this study, several of the up-regulated genes encode these enzymes, including the genes for pectinesterase, pectate lyase, two polygalacturonases (PG), XET, cellulase (CEL), expansin and invertase/pectin methylesterase inhibitor family protein, and several down-regulated genes encode 2 cellulose synthases and cellulose synthase-like C family protein (Table 2.1).

4.4. Protein kinase, protein phosphatase, and transcriptional regulator genes

Among the waterlogging-sensitive genes, 16 genes were protein kinase genes (4 up-regulated and 12 down-regulated) and 2 genes were protein phosphatase genes (both up-regulated) (Table 2.2). This is consistent with a previous finding that protein phosphorylation and dephosphorylation are important for regulation of aerenchyma formation in maize (He *et al*., 1996b).

Of 34 genes encoding putative transcription regulators including transcription factors of more than 20 different families, 13 genes were up-regulated and 21 genes were down-regulated (Table 2.3). The up-regulated genes included genes encoding MYB family transcription factor, AP2 domain containing protein (Ethylene response factor, ERF), NAC domain transcription factor, NAC domain transcription factor, and others. Three other up-regulated genes are related to histone modification. These include a jmjC domain (jumonj-C-domain)-containing protein and two histone acetyltransferases. Among the down-regulated genes, genes encoding TCP family transcription factor, two MYB family transcription factor, nuclear transcription factor Y subunit, and others (Table 2.3).

Table 2.2: 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 accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene Annotation
TC281230	23.49	6.29	GRMZM2G147051	Os05g0436100	Serine/threonine-protein kinase, putative, expressed
CO458690	18.06	6.11	GRMZM2G092604	Os09g0293500	Serine/threonine-protein kinase BRI1-like 1 precursor, putative, expressed
TC299285	6.39	10.13	GRMZM2G091338	Os01g0789200	Tyrosine protein kinase domain containing protein, putative, expressed
TC282571	4.80	3.18	GRMZM2G113668	Os03g0857600	Receptor protein kinase, putative, expressed
TC293663	3.12	3.12	GRMZM2G126765	Os06g0208700	tyrosine phosphatase family protein, putative, expressed
TC293960	2.52	2.40	GRMZM2G015610	Os04g0449450	protein phosphatase protein, putative, expressed
DR810794	0.42	0.36	GRMZM2G430600	Os03g0319400	CAMK_KIN1/SNF1/Nim1_like.2 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
TC282050	0.41	0.28	GRMZM2G032852	Os03g0128700	CAMK_CAMK_like.17 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
TC306361	0.39	0.38	GRMZM2G074262	Os01g0864700	Protein kinase domain containing protein, expressed
TC288382	0.38	0.14	GRMZM2G180916	Os10g0564500	CAMK_CAMK_like.41 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
TC293625	0.33	0.18	GRMZM2G412601	Os09g0418500	CAMK_KIN1/SNF1/Nim1_like.35 - CAMK includes calcium/calmodulin dependent protein kinases, e
TC308802	0.31	0.18	GRMZM2G119521	Os10g0432001	Tyrosine protein kinase domain containing protein, putative, expressed
TC315230	0.29	0.40	GRMZM2G126858	Os04g0616700	SHR5-receptor-like kinase, putative, expressed
TC281251	0.27	0.23	GRMZM2G146720	Os11g0134300	CAMK_KIN1/SNF1/Nim1_like.37 - CAMK includes calcium/calmodulin dependent protein kinases, e
TC314581	0.25	0.20	GRMZM2G092776	Os03g0113000	Protein kinase domain containing protein, expressed
TC308836	0.23	0.12	GRMZM2G132591	Os10g0174800	OsWAK108 - OsWAK receptor-like protein kinase, expressed
TC312632	0.22	0.12	GRMZM2G135359	Os03g0294800	MRH1, putative, expressed
TC305840	0.15	0.17	AC217293.3[f]	Os12g0615100	Wall-associated receptor kinase-like 22 precursor, putative, expressed
TC302613	0.15	0.20	GRMZM2G158252	Os10g0362300	Histidine kinase, putative, expressed

[a]: Average expression ratio of 3 biological replicates and a color swap for each replicate. [b]: Maize Gene IDs in MaizeSequence Database (http://www.maizesequence.org/). [c]: RAP Os IDs in Rice Annotation Project Database (

Table 2.3: List of genes encoding transcriptional regulation-regulated proteins, whose expression was up-regulated or down-regulated in maize root cortex during aerenchyma formation

Maize EST accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene Annotation
TC289269	30.88	4.33	GRMZM2G053503	Os01g0313300	AP2 domain containing protein, expressed (Ethylene response factor, ERF)
TC288289	25.11	5.39	GRMZM2G417089	Os03g0430400	jmjC domain containing protein, expressed
AI978191	11.66	13.63	GRMZM2G128421	Os03g0633800	OsIAA12 - Auxin-responsive Aux/IAA gene family member, expressed
TC292202	7.89	22.50	GRMZM2G068973	Os01g0816100	NAC domain transcription factor, putative, expressed
CD966549	6.41	9.14	GRMZM2G069886	Os01g0246100	histone acetyltransferase HAC5, putative, expressed
TC293058	4.80	4.82	GRMZM2G106673	Os09g0347800	Acetyltransferase type B catalytic subunit, putative, expressed
TC293056	4.69	10.36	GRMZM2G106673	Os06g0194400	B3 DNA binding domain containing protein, expressed
TC301325	4.42	4.91	GRMZM2G169654	Os01g0693400	B3 DNA binding domain containing protein, expressed (AP2/B3 domain transcription factor RAV1 like)
TC304408	4.39	23.27	GRMZM2G148074	Os10g0561800	Homeobox associated leucine zipper, putative, expressed
TC302970	4.24	44.59	GRMZM2G108865	Os03g0764900	Dof zinc finger domain containing protein, putative, expressed
TC300803	4.21	10.77	GRMZM2G172621	Os07g0679500	bZIP transcription factor domain containing protein, expressed
TC285712	4.16	4.05	GRMZM2G125522	Os05g0449900	MYB family transcription factor, putative, expressed
TC303206	3.74	16.46	GRMZM2G460472	Os11g0158500	Helix-loop-helix DNA-binding protein, putative, expressed
TC300328	2.84	3.60	GRMZM2G003565	Os04g0435700	Regulator of chromosome condensation domain containing protein, expressed (UVB-resistance protein UVR8 like)

Table 2.3 (continued)

Maize EST accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene Annotation
TC283126	0.41	0.42	GRMZM2G093895	Os12g0173125	TCP family transcription factor, putative, expressed
TC288402	0.41	0.29	GRMZM2G142768	Os02g0817600	OsIAA10 - Auxin-responsive Aux/IAA gene family member, expressed
TC299946	0.40	0.39	GRMZM2G154641	Os01g0848400	Homeodomain protein, putative, expressed
TC281170	0.37	0.36	GRMZM2G171365	Os03g0122600	OsMADS50 - MADS-box family gene with MIKCc type-box, expressed
TC312751	0.36	0.33	GRMZM2G104551	Os09g0532900	MYB family transcription factor, putative, expressed
TC313513	0.35	0.02	AC232238.2[e]	Os01g0859500	Transcription factor, putative, expressed
TC295929	0.34	0.27	GRMZM2G037630	Os03g0411100	nuclear transcription factor Y subunit, putative, expressed
TC283299	0.32	0.30	GRMZM2G328481	Os06g0211200	bZIP transcription factor, putative, expressed
TC280481	0.32	0.16	GRMZM2G485184	Os12g0510900	OsWLIM1 - LIM domain protein, putative actin-binding protein and transcription factor, expressed
TC301678	0.31	0.26	GRMZM2G104390	Os01g0343300	GATA zinc finger domain containing protein, expressed
TC292295	0.30	0.25	GRMZM2G180328	Os01g0884300	No apical meristem protein, putative, expressed
TC306650	0.27	0.29	GRMZM2G144196	Os08g0242800	RNA polymerase sigma factor, putative, expressed
TC289804	0.27	0.44	GRMZM2G356439	Os04g0678400	Dof zinc finger domain containing protein, putative, expressed
TC315561	0.26	0.33	GRMZM2G320549	Os01g0922800	OsMADS65 - MADS-box family gene with MIKC* type-box, expressed
TC302957	0.17	0.24	GRMZM2G031094	Os11g0152700	Transcription factor, putative, expressed
TC290696	0.16	0.18	GRMZM2G452178	Os07g0129700	Homeobox domain containing protein, expressed
TC301787	0.16	0.25	GRMZM2G003944	Os11g0175700	TCP family transcription factor, putative, expressed
TC309665	0.16	0.14	GRMZM2G472671	Os08g0543700	helix-loop-helix DNA-binding domain containing protein
TC313975	0.13	0.11	GRMZM2G009406	Os08g0490100	Dof zinc finger domain containing protein, putative, expressed
TC285832	0.13	0.18	GRMZM2G002128	Os05g0449900	MYB family transcription factor, putative, expressed
TC302185	0.09	0.05	GRMZM2G172657	Os07g0586900	SHR, putative, expressed

[a]: Average expression ratio of 3 biological replicates and a color swap for each replicate. [b]: Maize Gene IDs in MaizeSequence Database (http://www.maizesequence.org/).

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

[d]: MSU's Loc_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). [e]: This indicates not gene ID but contig ID.

Discussion

The use of high-throughput technology such as a microarray is a powerful approach to identify genes involved in aerenchyma formation in maize roots. However, microarray analysis of a complete root provides average gene expression levels integrated over all cell types in the isolated root samples. Such an analysis has the potential to mask genes expressed specifically in a particular cell type (Schnable *et al*., 2004). To identify genes specifically expressed in root cortical cells during aerenchyma formation, I used LM for isolation of only cortical cells from sections of root tissues, since isolation of high-quality RNA, which is essential for microarray analysis, from paraffin-embedded plant tissue sections has been succeeded (Takahashi *et al*., 2010) and it has been demonstrated that microarray analyses combined with LM result in a significant increase in specificity and resolution of cell-type specific gene expressions (Nakazono *et al*., 2003; Nelson *et al*., 2006; Ohtsu *et al*., 2007; Jiao *et al*., 2009).

To better understand the molecular mechanism of aerenchyma formation in maize root cortical cells, I screened for genes whose expressions changed in response to ethylene under waterlogged conditions, and found 239 up-regulated genes and 336 down-regulated genes. Unsurprisingly, many of the genes (~36% of the up-regulated genes and ~32% of the down-regulated genes) are known to be responsive to stress or other stimuli (Fig. 2-4). 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 the energy conservation and facilitates the transition to anaerobic metabolism (Branco-Price *et al*., 2008). Thus, to understand 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 the root cortical cells under waterlogged conditions.

The production of ROS under oxygen deprivation is well documented (Fukao and Bailey-Serres, 2004). Here I selected genes related to ROS generation and scavenging which are up-regulated or down-regulated under waterlogged conditions. These genes might be involved in aerenchyma formation in maize roots. 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 expressions of genes responsible for the aerenchyma formation (Subbaiah and Sachs, 2003). Truly, treatments with thapsigargin and caffeine, which increases intracellular Ca^{2+} levels, stimulated cellulase 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 increase of cellulase activity and aerenchyma formation by decreasing cytosolic Ca^{2+} in maize roots even under anaerobic conditions (He *et al.*, 1996b).

It is well known that hydrolytic enzymes including cellulase, xylanase, and pectinase are activated in maize adventitious roots under partial flooding and this activation was accompanied with the augmentation of ethylene concentration (Bragina *et al.*, 2003). The activation of cell wall loosening/degradation-related enzymes is an important event in aerenchyma formation.

Three genes related to histone modification were induced under waterlogged conditions (without 1-MCP pre-treatment) in both experiments 1 and 2 (Table 2.3). One of the histone modification-related genes encodes a jmjC-domain containing protein. Recently, some jmjC-domain containing proteins have been shown to be histone demethylases (Mosammaparast and Shi, 2010). Previously, Tsuji *et al.* (2006) reported that submergence and re-aeration of rice causes dynamic and reversible changes of the histone methylation and acetylation states for genes involved in anaerobiosis. 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 3 maize up-regulated gene products contribute to the changes of histone methylation and acetylation**.**

Summary

Under waterlogged conditions, aerenchyma is formed between 18 h and 24 h in maize cortical roots. The pre-treatment with 1-MCP, an inhibitor of ethylene perception, suppressed the formation of aerenchyma in maize roots under waterlogged conditions.

To identify genes involved in aerenchyma formation, seedlings treated under waterlogged conditions with or without 1-MCP pre-treatment and aerobic conditions were prepared for a microarray analysis. The use of high-throughput technologies such as microarray and laser microdissection is a good tool to understand the mechanism of aerenchyma formation, which is formed specifically in the root cortex.

I selected 239 up-regulated genes and 336 down-regulated genes under waterlogged conditions from the microarray experiment. Genes related to ROS generation and scavenging, calcium signaling, transcription factors, protein kinases and cell wall degradation were selected and it seems that they are likely regulated by ethylene under waterlogged conditions.

Further analysis is needed to confirm the result of microarray. In Chapter 3, genes, which may involve in aerenchyma formation, were selected for tissue-specific expression analysis.

Chapter 3

Confirmation of genes selected from microarray experiment using a semi quantitative RT-PCR

Introduction

Under waterlogged conditions, lysigenous aerenchyma contributes to the ability of plants to tolerate low-oxygen soil environments, by providing an internal aeration system for the transfer of oxygen from the shoot. In maize roots, aerenchyma is formed through programmed cell death (PCD) especially in the root cortex.

PCD is a genetically controlled suicide that occurs as an integral part of the life of the multicellular organisms with the aim to eliminate undesirable cells. In plants, it occurs in response to biotic or abiotic stress and plays an essential role in plant development and survival (Greenberg, 1996). The hypersensitive response (HR) describes local cell death in response to pathogen attack and helps to limit the spread of the pathogen (Zurbriggen *et al*., 2010). Epidermal cell death precedes the emergence of adventitious roots at the nodes of submerged rice plants. While cell death is inducible by various internal and external signals, hormones frequently mediate these signals and regulate the processes leading to PCD (Steffens and Sauter, 2005). As in the case with aerenchymal cell death, epidermal cell death is induced by ethylene (Mergemann and Sauter, 2000). In maize roots and other cereal plants, PCD was shown to be orchestrated by ethylene under waterlogged conditions (He *et al*., 1996a, 1996b). The ethylene-induced aerenchyma formation is affected by chemical inhibitors or stimulators of PCD and other signaling pathways (He *et al*., 1996b). The PCD lowers the total water uptake and oxygen consumption per unit surface of the root through the formation of air cavities in place of living cells (Armstrong *et al*., 1994). These air cavities (aerenchyma) will be the main oxygen reservoir and the major system of ventilation in the submerged root (Armstrong, 1979).

In maize, hypoxia stimulates ethylene production, which induces the expression of genes related to aerenchyma formation. So far, it is unclear what genes are involved at the early stage during the aerenchyma formation although some genes (e.g., xyloglucan endo-transglycosylase (*XET*) gene; Saab and Sachs, 1996)

expressed at the later stage were identified. At the late stage of lysigenous aerenchyma formation, the cell wall is enzymatically degraded. At first, the localizations of esterified pectin and de-esterified pectin in cell wall of the maize cortex are changed during cell death (Gunawardena *et al*., 2001), and subsequently the cell wall is degraded by the combined actions of pectolytic, xylanolytic, and cellulosolytic enzymes (Jackson and Armstrong, 1999). Indeed, the activities of cellulase, xylanase and pectinase, all of which are involved in loosening or degrading the cell wall, are enhanced in maize roots under waterlogged conditions (Jackson and Armstrong, 1999; Bragina *et al*., 2003). On the other hand, the expressions of genes encoding expansin, which promotes cell wall extensibility by breaking hydrogen bonds between hemicellulose and cellulose, are induced by ethylene (Rose *et al*., 2000). The *XET* gene is up-regulated in maize roots after 12 h of flooding and the induction was inhibited by treatment with an ethylene biosynthesis inhibitor (Saab and Sachs, 1996).

Ca^{2+} might be involved in the signaling of aerenchyma formation because increasing cytoplasmic Ca^{2+} concentrations enhances aerenchyma development. By contrast, aerenchyma formation was suppressed by decreasing cytosolic Ca^{2+} with EGTA (a Ca^{2+} chelator) (He *at al*., 1996b).

The recent findings (Overmeyer *et al*., 2000; Steffens and Sauter, 2009; Torres *et al*., 2002; Yoshioka *et al*., 2009; Foreman *et al*., 2003; Overmeyer *et al*., 2003; Woltering *et al*., 2005; Bouranis *et al*., 2006; Evans, 2003) show that ROSs play an important role in plant cell death, defense and growth. ROS include molecules [hydrogen peroxide (H_2O_2) and ozone], ions (hypochlorites), and radicals [hydroxyl radical and superoxide radical (O_2^-)]. The major source of ROS in plants is the NADPH oxidase catalyzed the conversion of dioxygen (O_2) to the O_2^-, which leads finally to the production of H_2O_2. The H_2O_2 has been shown recently to act as a signal that is required for ethylene-induced epidermal cell death and is sufficient to promote it (Steffens and Sauter, 2005, 2009). Steffens *et al*. (2010) showed that H_2O_2 promotes aerenchyma in the rice stem. ROSs have also been recognized as important

signaling molecules in the HR (Lamb and Dixon, 1997). HR is characterized by an oxidative burst during which high levels of H_2O_2 are produced.

In this Chapter, I selected genes from microarray experiment and confirmed the result using a semi quantitative RT-PCR. Furthermore, the tissue-specific expression analysis of these genes was performed. Here I discussed the possible roles of these genes in aerenchyma formation in maize roots.

Materials and methods

1. Plant material and growth conditions

I grew the seedlings as described in Chapter 2. To confirm the result of microarray using semi quantitative RT-PCR, 2 independent replicates were prepared for each experiment treated under the same conditions used for the microarray.

To perform the tissue specificity experiment, 2 different replicates were prepared. Three-day-old seedlings were treated under waterlogged conditions for 12 hours (h). For a control, the seedlings were grown under aerobic conditions for the same time (12 h).

2. Sample preparation for LM

The method for the samples preparation was described in Chapter 2. For the tissue specificity, the cortical or the stelar cells were separately collected from the embedded paraffin sections using the LM.

3. Extraction, quantification and qualification of RNA

Total RNA was extracted from the LM-isolated cells with a Pico-PureTM RNA Isolation Kit (Molecular Devices) according to the manufacturer's instructions. The extracted total RNA was quantified with a Quant-iT^{TM} RiboGreen RNA Reagent and Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA quality was assessed using an RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

4. Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR (sqRT-PCR) analysis was performed to confirm the expression pattern of selected genes identified by the microarray analysis. First-strand cDNA was synthesized using Superscript III (Invitrogen) from10 ng total RNA

extracted from root cortical cells or stelar cells as described in Chapter 2. KOD FX (TOYOBO, Tokyo, Japan) was used for subsequent PCR amplification with appropriate primers (Table 3-1): initial denaturation (94°C for 2 min) and 29-45 cycles of denaturation (94°C for 30 sec), annealing (56-62°C for 30 sec), extension (68°C for 30 sec), and final extension (68°C for 6 min).

5. Diphenylene iodonium treatment

Diphenylene iodonium (DPI) is a chemical used to inhibit NADPH oxidase (Steffens and Sauter, 2009), which normally produces O_2^- that is rapidly converted to H_2O_2 by superoxide dismutase (Hung and Kao, 2007). DPI (ENZO, Butler Pike USA) was dissolved in DMSO at 70°C. The DPI was added to water in the flask and the level was just enough to keep the paper moist during 12 h of pre-treatment with the indicated concentrations. Three-day-old-seedlings pre-treated with DPI were subjected to waterlogging treatment with different concentrations of DPI. For a control, seedlings were treated under the same conditions with the DPI treatment except that the DPI was replaced by DMSO or water. The percentage of aerenchyma in maize sections was calculated using the software ImageJ from root sections (at 1.5-2 cm from the root-shoot junction), as described in Chapter 2.

Table 3-1: List of primers used for a sqRT-PCR analysis

Gene name	Maize EST accession	Forward primer sequence	Reverse primer sequence
Reactive Oxygen Species			
RBOH	TC289691	5'-ACGCCTGTCGCAGGACTTCT-3'	5'-ACGCCTGTCGCAGGACTTCT3'
MnSOD	TC301296	5'-GCTGTTGGGGATTGATGTCT-3'	5'-ATGGTCAAACAGCCGAAAAC-3'
MT	TC298672	5'-GTGGCCCCGGAGAAGAAG -3'	5'-TGTTCAACCACCACCACAAGC-3'
Calcium signaling			
CBL	TC294844	5'-GGGATTACGCATTCTCGCTTG-3'	5'-ACGGTCATTGCCCTGCTACA-3'
CML	TC282752	5'-CGTCTGCTTCCACGACTTCT-3'	5'-TCCCTGATTGGAAAAGATGAA-3'
CNGC	TC289094	5'-CGTGGCGCAGGTATAGGG-3'	5'-CCCCTCCTGATCACTCTAGG-3'
Cell wall modification			
XET	TC286313	5'-ACTACTGCGACGACCGCAAG-3'	5'-CCAACAATCAGCCCGGTTTT-3'
PG	TC282363	5'-TGCTGAAACACTCAAAGTGC-3'	5'-CTCATCAGCAAGCAATGGTG-3'
CEL	TC314571	5'-GCGACAACTACGCGCATACA-3'	5'-TGCAGCAGCAGGGGCTAATA-3'
Transcriptional regulation			
ERF	TC289269	5'-CGCCGAAGAATCGCAGGT-3'	5'-CCGACTACGACGAGCAGGAA-3'
RAV1L	TC301325	5'-GCGTTCAAGAAGCAGCTCGT-3'	5'-GCTCGGTGATGCTACCATCTGA-3'
UVR8L	TC300328	5'-GCGTGCAGTCGTCGTAGTAGG-3'	5'-ACCGCTACCATCTCAACACCAG-3'
Transporter			
H+ATPase	TC305342	5'-CTGCACGGCCTCAACCAG-3'	5'-AGGCCCTTGAGCTTGACGAC-3'
UBQ	TC298342	5'-GTTGAAGCTGCTGCTGTATCTGG-3'	5'-GCGGTCGCACGATAGTTTTG-3'

Results

1. Selected genes from microarray experiment and confirmation with 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 sqRT-PCR to confirm the change in expression. For this purpose, I selected 13 genes (11 up-regulated and 2 down-regulated), which included 3 reactive oxygen species (ROS) generation/scavenging-related genes, 3 calcium signaling-related genes, 3 cell wall modification-related genes, 1 transporter gene and 3 transcriptional regulation-related genes (Table 3-2).

1.1. ROS generation/scavenging-related genes

Among the up-regulated genes related to ROS, selected from microarray, the gene encoding Respiratory Burst Oxidase Homolog *RBOH* (GRMZM2G300965) showed a 117-fold higher expression level under waterlogged conditions than under aerobic conditions, and the induction was partially suppressed by pre-treatment with 1-MCP. In fact the expression of this gene under waterlogged conditions without 1-MCP pre-treatment was 10-fold higher than under waterlogged conditions with 1-MCP pre-treatment (Table 3-2). The analysis of the expression of this gene with a sqRT-PCR showed that its expression is induced in roots treated under waterlogged conditions and it is repressed in roots treated under waterlogged conditions with the 1-MCP pre-treatment or under aerobic conditions (Fig. 3-1).

The second up-regulated selected gene related to ROS scavenging was *Superoxide dismutase* (*MnSOD*) (GRMZM2G160629). The fold changes in expression of this gene in experiments 1 and 2 were 3.45 and 3.16, respectively (Table 3-2). This result was confirmed with the sqRT-PCR analysis (Fig. 3-1).

Table 3-2: selected genes from microarray experiment

Gene Name	Maize EST accession	Exp. 1[a] WL/Aer	Exp. 2[a] -MCP/+MCP	Maize Gene IDs[b]	RAP Os IDs[c]	Gene annotation
Reactive oxygen species (ROS) generation/scavenging						
RBOH	TC289691	117.72	10.72	GRMZM2G300965	Os12g0541300	Respiratory burst oxidase, putative, expressed
MnSOD	TC301296	3.45	3.16	GRMZM2G160629	Os05g0323900	Superoxide dismutase, mitochondrial precursor, putative, expressed
MT	TC298672	0.16	0.39	GRMZM2G164229	Os11g0704500	Metallothionein, putative, expressed
Calcium signaling						
CBL	TC294844	6.69	11.56	GRMZM2G125838	Os02g0802400	EF hand family protein, putative, expressed (Calcineurin B like, CBL)
CML	TC282752	2.71	15.08	GRMZM2G467184	Os11g0141400	Calmodulin-like protein 1, putative, expressed
CNGC	TC289094	0.06	0.04	GRMZM2G074317	Os03g0758300	Cyclic nucleotide-gated ion channel 2, putative, expressed
Cell wall modification						
XET	TC286313	5.47	5.20	GRMZM2G174855	Os07g0529700	Glycosyl hydrolases family 16, putative, expressed (Xyloglucan endotransglucosylase, XET)
PG	TC282363	5.56	2.55	GRMZM2G037431	Os01g0636500	Polygalacturonase, putative, expressed
CEL	TC314571	4.36	12.59	GRMZM2G141911	Os02g01j23700	Endoglucanase, putative, expressed (Cellulase, CEL)
Transporter						
H+ATPase	TC305342	18.07	186.62	GRMZM2G450055	Os02g0797300	ATPase 8, plasma membrane-type, putative, expressed (Plasma membrane H+-ATPase)
Transcriptional regulation						
ERF	TC289269	30.88	4.33	GRMZM2G053503	Os01g0313300	AP2 domain containing protein, expressed (Ethylene response factor, ERF)
RAV1L	TC301325	4.42	4.91	GRMZM2G169654	Os01g0693400	B3 DNA binding domain containing protein, expressed (AP2/B3 domain transcription factor RAV1 like)
UVR8L	TC300328	2.84	3.60	GRMZM2G003565	Os04g0435700	Regulator of chromosome condensation domain containing protein, expressed (UVB-resistance protein UVR8 like)

[a]: Average expression ratio of 3 biological replicates and a color swap for each replicate.
[b]: Maize Gene IDs in MaizeSequence Database (http://www.maizesequence.org/).
[c]: RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/download/index.html). The gene annotation was done according to MSU's Putative Function in Rice Genome Annotation Project Database. Manually improved annotations are described within parentheses.

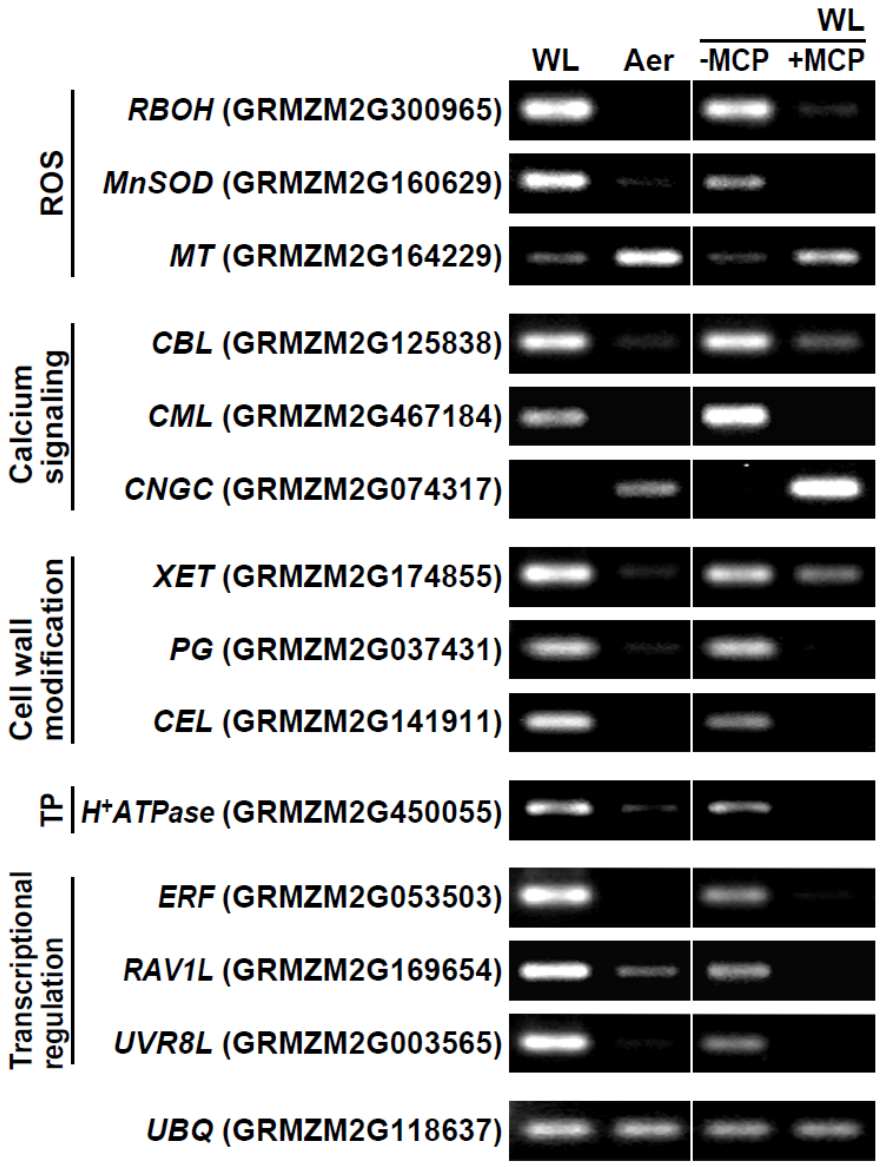

Fig. 3-1: Validation of expressions for the genes selected from microarray analysis with semi-quantitative RT-PCR. Semi-quantitative RT-PCR analysis of the selected genes was performed with appropriate primers. The alphanumeric symbols in parentheses indicate the Maize Gene IDs of the Maize sequence database. *Ubiquitin* gene (*UBQ*) was used as a control. WL, waterlogged conditions; Aer, aerobic conditions; -MCP, waterlogged conditions without 1-MCP pre-treatment; +MCP waterlogged conditions with 1-MCP pre-treatment; TP, transporter; ROS, reactive oxygen species generation/scavenging.

A down-regulation of *Metallothionein* (*MT*) (GRMZM2G164229) gene, which works as a reactive oxygen scavenger, was also detected when the roots were treated under waterlogged conditions in compared with aerobic conditions (Fig. 3-1). Indeed, the fold changes in the expression level of this gene, detected in the microarray experiment, in experiments 1 and 2 are 0.16 and 0.39, respectively (Table 3-2).

1.2. Calcium signaling-related genes

The up-regulated genes included genes encoding calcineurin B-like protein (CBL), Ca^{2+}-binding domain containing proteins and calmodulin-like protein (CML) and down-regulated genes included genes encoding cyclic nucleotide-gated ion channel (CNGC) and CML (Table 3-2). The result of the sqRT-PCR confirmed the up-regulation expression of *CBL* and *CML* genes and the down-regulation of *CNGC* and *CML* genes (Fig. 3-1).

The waterlogging treatment enhanced the expression of *CBL* gene (GRMZM2G125838) by 6.69 fold compared with aerobic conditions (Table 3-2). However, the pre-treatment with 1-MCP inhibited the up-regulation of this gene under waterlogged conditions (Fig. 3-1).

The *CML* gene (GRMZM2G467184) was expressed 15.08 fold more in hypoxic roots than in hypoxic roots pre-treated with 1-MCP. In addition, its expression was 2.71 fold more under waterlogged conditions than under aerobic conditions.

The *CNGC* gene (GRMZM2G074317) was selected as a down-regulated gene. The fold changes of its expression in experiments 1 and 2 were 0.06 and 0.04, respectively (Table 3-2).

1.3. Cell wall modification-related genes

The genes encoding xyloglucan endotransglycosylase (XET), polygalacturonase (PG), and cellulase (CEL) were selected to conduct a sqRT-PCR.

The result was similar to the microarray (Fig. 3-1). The transcript levels of *XET* (GRMZM2G174855), *PG* (GRMZM2G037431) and *CEL* gene (GRMZM2G141911) were 5.47, 5.56 and 4.36 times higher under waterlogged conditions than under aerobic conditions, respectively (Table 3-2). Furthermore, the 1-MCP pre-treatment inhibited their expressions (Table 3-2).

1.4. Protein kinase, protein phosphatase, and transcriptional regulator genes

Among these genes, the gene encoding an *AP2* domain-containing protein (GRMZM2G053503), which is similar to ethylene response factor (ERF), showed a strong (~30-fold) increase in expression under waterlogged conditions and the induction was partially suppressed by pre-treatment with 1-MCP (Table 3-2). These changes in expression between waterlogged and aerobic conditions were confirmed by sqRT-PCR (Fig. 3-1).

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 factor RAV1, was also induced under waterlogged conditions and seemed to be controlled by ethylene (Fig. 3-1 and Table 3-2).

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. The fold changes in the expression of this gene in experiments 1 and 2 were 2.84 and 3.6, respectively (Table 3-2). The up-regulation of this gene under waterlogged conditions and the down-regulation under aerobic or under waterlogged conditions with 1-MCP pre-treatment were confirmed by sqRT-PCR (Fig. 3-1).

2. Tissue-specific gene expression analysis

To examine whether the ethylene-mediated waterlogging-responsive expressions of the selected genes are associated with aerenchyma formation, I used LM to collect

cortical cells (aerenchyma-forming tissue) and stelar cells (non-aerenchyma-forming tissue) from cross sections of primary roots that had been exposed to waterlogged or aerobic conditions for 12 h (Fig. 3-2) and performed sqRT-PCR (Fig. 3-3). Under waterlogged conditions, all of the 13 selected genes (Table 3-2) were up-regulated or down-regulated in cortical cells. Eight of them (*RBOH*, *MnSOD*, *CBL*, *CML*, *CNGC*, *XET*, *ERF* and *UVR8L*) were also up-regulated or down-regulated in stelar cells, but the difference of their mRNA levels between under waterlogged and aerobic conditions was higher in cortical cells than in stelar cells (Fig. 3-3).

2.1. ROS related genes

As shown in Fig. 3-3, the 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 was up-regulated preferentially in cortical cells under waterlogged conditions. Interestingly, the *MT* gene 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. 3-3).

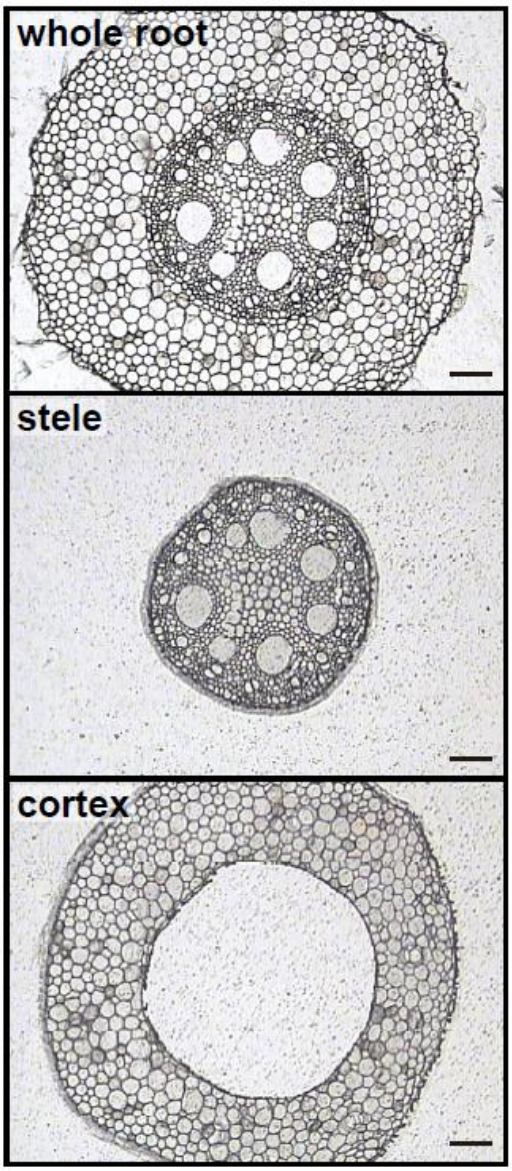

Fig. 3-2: Isolation of stelar cells and cortical cells from paraffin-embedded tissue sections of a maize primary root using laser microdissection (LM). Whole root: a root tissue section before LM. Stele and cortex parts were selected separately. Scale bars: 100 μm.

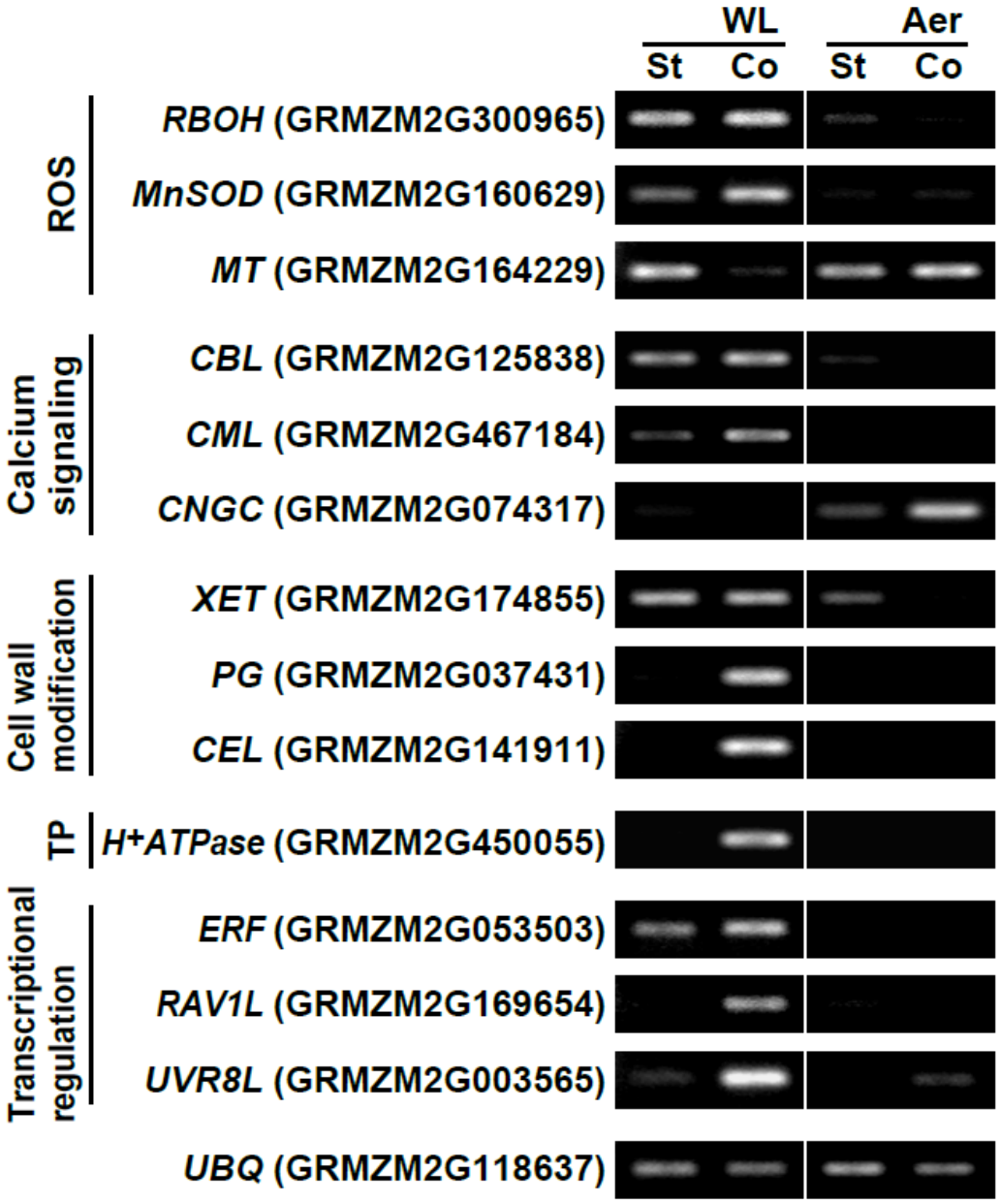

Fig. 3-3: Tissue-specific expression analysis of genes selected from the microarray analysis. Semi-quantitative RT-PCR analysis of the selected genes. The alphanumeric symbols in parentheses indicate the Maize Gene IDs of the Maizesequence database. *Ubiquitin* gene (*UBQ*) was used as a control. WL, waterlogged conditions; Aer, aerobic conditions; St, stele; Co, cortex; TP, transporter; ROS, reactive oxygen species generation/scavenging.

These results suggest that the scavenging of ROS in stelar cells will be more pronounced than in cortical cells under waterlogged conditions. The expression of *MT* gene is inhibited under waterlogged conditions in cortical cells and that may promotes the generation of ROS in maize roots.

2.2. Calcium related genes

As shown in Fig. 3-3, 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 under waterlogged conditions, but the changes in expression were more pronounced in cortical cells than in stelar cells (Fig. 3-3).

2.3. Cell wall modification-related genes

Under waterlogged conditions, 2 of the 3 selected cell wall modification-related genes (*PG* and *CEL*) were specifically up-regulated in cortical cells, whereas the *XET* gene was up-regulated in both cortical cells and stelar cells (Fig. 3-3).

2.4. Transcription factors genes

Under waterlogged conditions, the *ERF* and *UVR8L* genes were mainly up-regulated in cortical cells and the *RAV1L* gene was up-regulated only in cortical cells (Fig. 3-3).

3. Effect of DPI treatment on aerenchyma formation

DPI binds strongly to flavoproteins and thus is a powerful and specific inhibitor of several important enzymes, including the plasma membrane NADPH oxidase (Morre, 2002). In order to confirm that NADPH oxidase (RBOH) is involved in aerenchyma formation, I used DPI to inhibit this activity and to analyze its effect on aerenchyma formation in maize roots.

Aerobically-grown seedlings (2.5-day-old) were pre-treated with DPI using different concentrations for 12 h under aerobic conditions and then were treated with DPI under waterlogged conditions for 48 h. When the roots are treated with water under waterlogged conditions, the percentage of aerenchyma formation reached around 17.6% (Fig. 3-4). The treatment with DMSO (negative control) did not significantly affect aerenchyma formation, because the percentage of aerenchyma formation was 15.6% after 48 h of treatment. In contrast, the percentage of aerenchyma formation in roots treated with 1 μM and 5 μM DPI solutions were 9.7% and 8.5%, respectively (Fig. 3-4). This result showed that DPI reduces aerenchyma formation, suggesting that aerenchyma formation is partly regulated by ROS produced by NADPH oxidase.

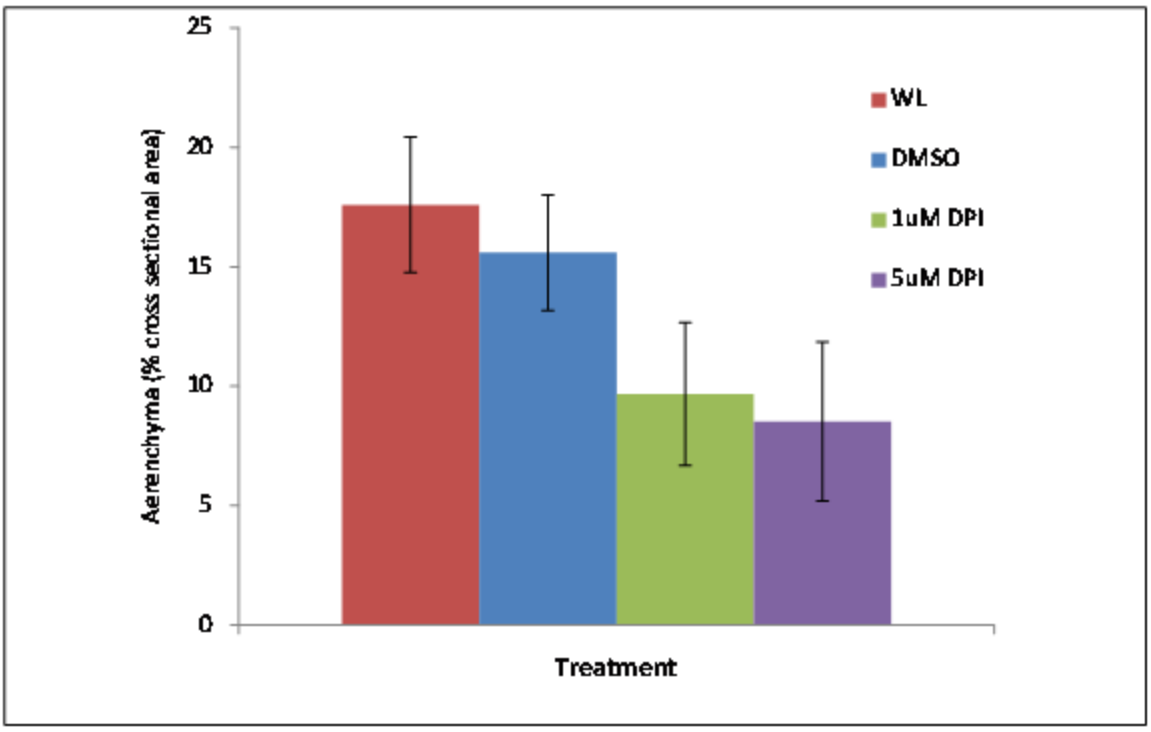

Fig. 3-4: Effect of DPI treatment on maize aerenchyma formation. The roots were aerobically pre-treated with different concentrations of DPI and then were treated using the same DPI solutions under waterlogged conditions for 48 h. The volume of DMSO was the same in all treatments except the waterlogging with water (WL). WL: waterlogging treatment with water; DMSO: waterlogging treatment with 0.05 % DMSO; 1 µM and 5 µM DPI: waterlogging treatments with 1 µM and 5 µM DPI solutions, which contain 0.05 % DMSO, respectively. All values are mean (n = 6) ± SD. Three roots were subjected to analysis in each of the 2 experiments.

Discussion

NADPH oxidase has an important role in ROS-mediated signaling such as 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), and RBOH is a plant homolog of gp91phox in mammalian NADPH. Indeed, Rop (RHO-like small G-protein of plants)-dependent H_2O_2 production mediated by NADPH oxidase, activity of which is stimulated by Ca^{2+}, contributes to induction of expressions 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 emergence of adventitious roots, is regulated by NADPH oxidase (Steffens and Sauter, 2005; 2009). Here, I 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 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 (HWC) from *Phytophthora infestans* causes a rapid and transient oxidative burst (*i.e*., H_2O_2 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 Ser/Thr protein kinase inhibitor K252a or the extracellular Ca^{2+}-chelator EGTA (Kobayashi *et al*., 2007). In epidermal cells, the use of DPI reduced the rates of cell death and that is in part mediated by ROS (Steffens and Sauter, 2009). In carrot (*Daucus carota*) cells, DPI was shown to inhibit O_2^- production from NADPH oxidase (RBOH) and cell death under carbon starvation (Chae and Lee, 2001). In rice roots, DPI reduced Pb^{2+}-induced cell death (Huang and Huang, 2008).

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. Indeed, the treatment of maize roots with different concentrations of DPI partially inhibited the aerenchyma formation (Fig. 3-4). This result indicates that aerenchyma formation is, in part, mediated by ROS released from NADPH oxidase. On the other hand, I found that expression of the gene encoding metallothionein (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. 3-1, Table 3-2). 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 and Sauter, 2009). Similarly, the cortical cell-specific down-regulation of the maize *MT* gene (Fig. 3-3) 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. 3-3), which might reduce the amount of H_2O_2 produced by RBOH. Indeed, it was recently demonstrated that the down-regulation of *MT2b* or application of H_2O_2 promoted aerenchyma formation in internodes of rice stems under submergence (Steffen *et al*., 2010). Based on these results, it is possible that similar mechanisms 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, calmodulin-like protein 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 pre-treatment) (Table 3-2). It seems that Ca^{2+} can directly bind 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 and Fluhr, 2001; Oda *et al*., 2010). In fact, all RBOH activities isolated to date contain a conserved N-terminal extension that possess two putative EF-hand motifs giving the evidence of the regulatory effect of calcium ions (Torres *et al.*, 1998). On the other hand, it was reported that calcium-dependent protein kinase activates RBOH by phosphorylation of its N-terminal region (Kobayashi *et al*., 2007). Besides, the cell death, which is mediated by the production of ROS generated by *Os*RBOHA, was completely inhibited by chelation of extracellular calcium (Yoshie *et al.*, 2005). Based on these results, an interaction between the Ca^{2+} signaling and the RBOH-mediated H_2O_2 production might be important for programmed cell death in the 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 to environmental adaptation processes (Luan, 2009; Weinl and Kudla, 2009), implying that the up-regulated CBL (GRMZM2G125838) and the down-regulated CBL (GRMZM2G173424) 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 calcium Ca^{2+} and potassium 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* (*Psg*) (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 inductions were controlled by ethylene. I 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. 3-1 and 3-3). Previously, Saab and Sachs (1996) reported that

XET mRNA was highly accumulated in maize seedlings under flooding. Treatment with an ethylene biosynthesis inhibitor, aminooxyacetic acid (AOA), under flooded conditions prevented the development of aerenchyma in maize roots and totally suppressed the accumulation of *XET* mRNA, suggesting that the ethylene-responsive *XET* induction is involved in aerenchyma formation through the cell wall loosening and degradation (Saab and Sachs, 1996). The *XET* gene identified in this study is not the same as the *XET* gene reported by Saab and Sachs (1996), suggesting that at least two ethylene-responsive *XET* genes are highly 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, polygalacturonase (PG) and cellulase (CEL)] may also contribute to activations of hydrolytic enzymes including cellulase, xylanase and pectinase in the maize roots under waterlogged conditions (Jackson and Armstrong, 1999). Indeed, the expressions of genes encoding PG (GRMZM2G037431) and CEL (GRMZM2G141911) were up-regulated specifically in cortical cells under waterlogged conditions (Fig. 3-1). On the other hand, I found that 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. I also identified a gene (GRMZM2G450055) encoding plasma membrane (PM) H^+-ATPase as the cortical cell-specific up-regulated gene (Figs. 3-1 and 3-3, Table 3-2). It is proposed that extrusion of an intracellular H^+ into the cell wall by PM H^+-ATPase results in a decrease of apoplastic pH, which induces cell wall loosening, possibly mediated by low-pH activated expansins and XETs (Frias *et al*., 1996; Shieh and Cosgrove, 1998). Thus, 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 PM 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.

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. 3-1 and 3-3). The up-regulation of these gene expressions was suppressed by the 1-MCP pre-treatment (Fig. 3-1, Table 3-2). Recently, Licausi *et al.* (2010) identified two Arabidopsis hypoxia-inducible *ERF* genes, *HRE1* and *HRE2*, which belong to the group VII of the ERF family in Arabidopsis (Nakano *et al*., 2006), and proposed that HRE1 and HRE2 play a partially redundant role in tolerance of the plants to anaerobic stress by enhancing anaerobic gene expressions and ethanol fermentation. The ERF family of group VII also contains Arabidopsis RAP2.2 (Hinz *et al*., 2010), rice SUB1A (Fukao *et al*., 2006; Xu *et al.*, 2006; Fukao and Bailey-Serres, 2008), 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 Arabidopsis HRE2 protein, suggesting that the maize *ERF* gene, like the Arabidopsis *HRE2* gene, is involved in adaptation of the plants to waterlogged conditions. However, so far, it is not clear whether transcriptional regulation by this ERF affect to aerenchyma formation in maize roots, and thus further functional analysis of the *ERF* gene is necessary to examine it. On the other hand, it has been reported that expression of the Arabidopsis *RAV1* gene is induced by treatment with ACC (a precursor of ethylene biosynthesis) and 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-specifically induced RAV1L protein may be positively involved in programmed cell death (*i.e*., in aerenchyma formation) in the root cortical cells under waterlogged conditions.

Summary

Thirteen genes were selected from microarray experiment for the validation of the gene expression patterns by sqRT-PCR. These genes, which are related to many kinds of molecular functions (*e.g.*, ROS generation or scavenging, Ca^{2+}-signaling and cell wall modification), were up-regulated or down-regulated in the root cortical cells under waterlogged conditions and their expressions were likely to be regulated by ethylene. Moreover, the pre-treatment with 1-MCP, an inhibitor of ethylene perception, inhibits the expression of these genes.

The sqRT-PCR experiment confirmed the result of microarray analysis. The tissue-specific expression analysis of these genes was also performed. The expression of genes related to cell wall modification such as *CEL* and *PG* genes are specifically expressed in cortical cells under waterlogged conditions. The expression level of *RBOH* gene was detected in both cortical and stelar cells but the expression in cortical cells was slightly higher than in stelar cells. However, *MT* gene was constitutively expressed in both cortical and stelar cells under aerobic and hypoxic conditions and its mRNA levels were decreased specifically in cortical cells under waterlogged conditions. In addition, aerenchyma formation was repressed in maize roots pre-treated with DPI, an inhibitor of NADPH oxidase, under waterlogged conditions.

The data should provide a basis for understanding the molecular mechanism of the inducible lysigenous aerenchyma formation in plants.

Summary

Summary

To escape low oxygen conditions, numerous adaptations at the anatomical, morphological and metabolic level took place in plants suffering from waterlogged conditions. One of the structural adaptations is aerenchyma formation. Aerenchyma is a tissue consisting of longitudinal gas spaces separated by the strands of living cells, found in the root cortex of waterlogged plants. It enables the passage of gases in and out of tissues in plant roots. Internal transport of oxygen via the aerenchyma from shoots to roots is especially important for survival under waterlogged conditions. However, the molecular mechanism of lysigenous aerenchyma formation remains to be elucidated. 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.

1. Root growth and aerenchyma formation of maize under waterlogged or aerobic conditions

The growths of roots and shoots and aerenchyma formation were investigated. Three-day-old seedlings were treated under waterlogged conditions for 48 hours (h). The growth of the roots was retarded under waterlogged conditions compared with the roots grown under aerobic conditions. No effect was found of the waterlogged treatment on the elongation of the shoots. Three-day-old aerobically grown seedlings were then subjected to waterlogging treatment for 48 h and aerenchyma formation was investigated at different levels at the basal part of the root. I found that the gas spaces increased significantly during the treatment and the most inducible part was at 1.5-2 cm from the root-shoot junction.

2. Identification of genes involved in aerenchyma formation in maize roots using a laser microdissection and microarray analysis

Ethylene is known to be involved in aerenchyma formation in maize roots. It has been reported that aerenchyma is formed through programmed cell death (PCD). Three-day-old aerobically grown seedlings were then treated with ethylene (1 ppm) under aerobic conditions. The result showed that ethylene induced aerenchyma formation, which started between 6 h and 12 h after the ethylene treatment. On the other hand, aerenchyma formation was not observed at the basal region of roots of 4-day-old seedlings grown under aerobic conditions. The use of 1-methylcyclopropene (1-MCP; 1 ppm), an inhibitor of ethylene perception, completely blocked the formation of aerenchyma in the maize cortical cells when treated under waterlogged conditions for 24 h. Whereas the treatment of seedlings under waterlogged conditions without the 1-MCP pre-treatment induced aerenchyma formation starting between 18 h and 24 h after the treatment. Because aerenchyma is formed in the cortical cells of the root, cortical cells of roots treated under waterlogged conditions with or without pre-treatment with 1-MCP or aerobic conditions were isolated by laser microdissection and their mRNA levels were examined with a microarray. As a result, the signal intensities of 575 genes among 42,034 gene probes spotted on a microarray slide were significantly different between the two conditions for inducing and not inducing aerenchyma formation. Among them, it was likely that 239 genes were up-regulated and 336 genes were down-regulated under conditions for inducing aerenchyma formation (waterlogged conditions). The differentially expressed genes included genes related to generation or scavenging of reactive oxygen species (ROS), cell wall loosening and degradation, and Ca^{2+} signaling.

3. Confirmation of microarray result using a semi quantitative RT-PCR and analysis of tissue specificity of the selected genes

Production of ROS has been implicated in diverse physiological processes including PCD in plants. One of 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 production of hydrogen peroxide (H_2O_2). Several ethylene-mediated waterlogging-responsive genes related to ROS-generation or ROS-scavenging were found. The up-regulated genes contain gene encoding respiratory burst oxidase homolog (RBOH) and the down-regulated genes contain gene encoding metallothionein (MT). The *RBOH* gene is involved in ROS generation and the *MT* gene is involved in ROS scavenging. To determine the tissue specificity of these genes, the stelar cells and the cortical cells of maize roots, treated or not under waterlogged conditions, were collected separately using the laser microdissection and the expression of these genes were analyzed using a semi quantitative RT-PCR. The 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. Interestingly, the *MT* gene 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. In addition, the use of diphenylene iodonium (DPI), an inhibitor of RBOH activity, reduced the aerenchyma formation in the root cortical cells treated under waterlogged conditions.

The last step of aerenchyma formation involves cell wall loosening and degradation, in which many enzymes are involved. Genes related to cell wall degradation were also selected from the microarray experiment. The up-regulated genes included genes encoding xyloglucan endo-transglycosylase (XET), cellulase (CEL) and polygalacturonase (PG). These genes are related to cell wall loosening and degradation. The tissue specificity analysis showed that, *XET* and *CEL* genes were specifically expressed in the root cortical cells under waterlogged conditions. However, the *XET* was up-regulated in both cortical cells and stelar cells. A gene encoding plasma membrane (PM) *H^+-ATPase* was identified as the cortical cell-specific up-regulated gene. It is proposed that extrusion of an intracellular H^+ into the

cell wall by PM H^+-ATPase results in a decrease of apoplastic pH, which induces cell wall loosening, possibly mediated by low-pH activated XETs.

Many studies have suggested that the cytosolic calcium ion (Ca^{2+}) functions as a second messenger for signaling pathways in response to oxygen deprivation. Ca^{2+} signaling may also be involved in aerenchyma formation in maize roots. In this study, several genes implicated in calcium signaling such as genes encoding calcineurin B-like protein (CBL), Ca^{2+}-binding domain containing proteins and calmodulin-like protein (CML), were selected. The expressions of *CBL* and *CML* were observed in both cortical cells and stelar cells under waterlogged conditions, but the changes in expression were more pronounced in cortical cells than in stelar cells. However, the expression of the cyclic nucleotide-gated ion channel 2 (*CNGC*) gene was repressed under waterlogged conditions.

In conclusion, in this study, genes related to many types of molecular function (*e.g.* ROS generation or scavenging, cell wall modification, and Ca^{2+} signaling) were found as up-regulated or down-regulated in root cortical cells under waterlogged conditions, and their expression was likely to be regulated by ethylene. The data should provide a basis for an understanding of the molecular mechanism of inducible lysigenous aerenchyma formation in plants.

Acknowledgement

Acknowledgement

I would like to express my deep appreciation to Professor Mikio Nakazono, Graduate School of Agricultural and Life Sciences at the University of Tokyo for his continuous supervision, advices, encouragement and cordial support. I also thank Professor Nobuhiro Tsutsumi and Assistant Professor Shin-ichi Arimura for their fruitful discussions and their endeavor to improve my thesis.

Moreover I would like to thank Hirokazu Takahashi, graduate student at Laboratory of Plant Molecular Genetics, for his kind advice, technical assistance and his support throughout this study.

Special thanks to Dr. Takaki Yamauchi, Post doctoral research fellow in Laboratory of Plant Molecular Genetics for his assistance, technical support and fruitful discussions to improve this study. I would like to thank Drs. Katsuhiro Shiono, Tomomi Abiko and Shunsaku Nishiuchi, Post doctoral research fellows at Laboratory of Plant Molecular Genetics, for their kind support, continuous encouragement and stimulating discussions.

I also thank Dr. Al Imran Malik for his discussion and his continuous support. I would like to thank Hisae Kamakura, technician at Laboratory of Plant Molecular Genetics, for her help with the laser microdissection experiments. Thanks to my colleague Shradha Roy for her understanding, support and continuous encouragement during my PhD thesis. In addition, I would like to thank all the members of Laboratory of Plant Molecular Genetics, the University of Tokyo and without forgetting the university administration stuffs.

I would like to thank Professor Wahid Ghorbel and Dr Ahmed Mliki for their supports and Tunisian Government for giving me a scholar ship to complete my PhD study in Japan.

Thanks to those professors and friends especially my friend Lobna Mkaouar for the continuous moral support I could complete my study. Therefore, someday I have to return their favors and have to be the person who could help others like them.

I dedicate this thesis to my family especial to my father and my mother for their continuous moral support, advices and their pray.

References

References

- **Armstrong W. 1979.** Aeration in higher plants. *Advances in Botanical Research* **7**: 225-332.

- **Armstrong W, Justin SHFW, Beckett PM and Lythe S**. **1991**. Root adaptation to soil waterlogging. *Aquatic Botany* 39: 57-73.

- **Armstrong W, Brandle R, Jackson MB. 1994**. Mechanisms of flood tolerance in plants. *Acta Botanica. Neerl* **43**: 307-358.

- **Arikado H, Adachi Y. 1955.** Anatomical and ecological responses of barleyand some forage crops to the flooding treatment. Bull Fac Agr Univ **11**: 1-29.

- **Bailey-Serres J. 1999.** Selective translation of cytoplasmic mRNAs in plants. *Trends in Plant Science* **4**: 142-148.

- **Bailey-Serres J, Voesenek LACJ. 2008**. Flooding stress: Acclimations and Genetic Diversity. *Annu Rev Plant Biology* **59***:* 313-339.

- **Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J. 2002.** RopGAP4-dependent RopGTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. *Science* **296**: 2026-2028.

- **Bragina TV, Rodionova NA, Grinieva GM. 2003.** Ethylene production and activation of hydrolytic enzymes during acclimation of maize seedlings to partial flooding. *Russian Journal of Plant Physiology* **50**: 794-798.

- **Branco-Price C, Kaiser KA, Jang CJH, Larive CK, Bailey-Serres J. 2008.** Selective mRNA translation coordinates energetic and metabolic adjustments to cellular oxygen deprivation and reoxygenation in *Arabidopsis thaliana*. *The Plant Journal* **56**: 743-755.

- **Breitling R, Armengaud P, Amtmann A, Herzyk P. 2004.** Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Letters* **573**: 83–92.

- **Bucanonamwo M, Purcell LC. 1999.** Soybeen root morphology and anatomical traits associated bwith acclimation to flooding. *Crop Science* **39**: 143-149.

- **Campbell R, Drew MC. 1983.** Electron microscopy of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to oxygen shortage. *Planta* **157**: 350-357.

- **Chae SH, Lee WS. 2001.** Ethylene- and enzyme-mediated superoxide production and cell death in carrot cells grown under carbon starvation. *Plant Cell Report* **20**: 256–261.

- **Chen YF, Randlett MD, Findell JL, Schaller GE. 2002.** Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. *The Journal of Biological Chemistry* 277: 19861–19866.

- **Chung HJ, Ferl RJ. 1999.** Arabidopsis alcohol dehydrogenase expression in both shoots and roots is conditioned by root growth environment. *Plant Physiology* **121**: 429-436.

- **Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK, Bent AF. 2000.** The *Arabidopsis dnd1* ‘‘*defense, no death*’’ gene encodes a mutated cyclic nucleotide-gated ion channel. *Proceedings of the National Academy of Sciences of the USA*. **97**: 9323-9328.

- **Colmer TD. 2003.** Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell and Environment* **26**: 17-36.

- **Colmer TD, Voesenek LACJ. 2009.** Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology* 36: 665-681.

- **Dolferus R, Klok EJ, Delessert C, Wilson S, Ismond KP, Good AG, Peacock WJ, Dennis ES. 2003.** Enhancing the anaerobic response. *Annals Botany* **91**: 111-117.

- **Drew MC, Jackson MB, Giffard S. 1979.** Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptive responses to flooding in Zea Mays L. *Planta* **147**: 83-88.

- **Drew MC, Jackson MB, Giffard SC, Campbell R. 1981.** Inhibition by silver ions of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to exogenous ethylene or to oxygen deficiency. *Planta* **153**: 217-224.

- **Drew MC, He CJ, Morgan PW. 2000.** Programmed cell death and aerenchyma formation inroots. *Trends in Plant Science* **5**: 123-127.

- **Evans DE. 2003.** Aerenchyma formation. *New Phytologist* **161**: 35-49.

- **Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P,Costa S, Brownlee C, Jones JDG, Davies JM, Dolan L. 2003.** Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**: 442-446.

- **Frias I, Caldeira MT, Pdcez-Castineira JR, Navarro-Avino JP, Culianez-Macia FA, Kuppinger O, Stransky H, Pages M, Hager A, Serrano R. 1996.** A major isoform of the maize plasma membrane H +9 -ATPase: Characterization and induction by auxin in coleoptiles. *The Plant Cell* 8: 1533-1544.

- **Freeling M. 1973.** Simultaneous induction by anaerobiosis or 2,4-D of multiple enzymes specified by two unlinked genes: differential *ADH1-ADH2* expression in maize. *Molecular and General Genetics* **127**: 215-227.

- **Fukao T, Bailey-Serres J. 2004.** Plant responses to hypoxia – is survival a balancing act? *Trends in Plant Science* **9**: 449-456.

- **Fukao T, Xu K, Ronald PC, Bailey-Serres J. 2006.** A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. *The Plant Cell* **18**: 2021-2034.

- **Fukao T, Bailey-Serres J. 2008.** Submergence tolerance conferred by *Sub1A* is mediated by SLR1 and SLRL1 restriction of gibberellin responses in rice.

Proceedings of the National Academy of Sciences of the USA **105**: 16814-16819.

- **Gambrell RP, Patrick WH. 1978.** Chemical and microbiological properties of anaerobic soils and sediments. In: Hook DD, Crawford RMM Plant Life in Anaerobic Environments. Ann Arbor Sci Pub, Ann Arbor, pp 375-423.

- **Gallie DR, Geisler-Lee J, Chen J, Jolley B. 2009.** Tissue-specific expression of the ethylene biosynthetic machinery regulates root growth in maize. *Plant Molecular Biology* **69**: 195-211.

- **Geisler-Lee J, Caldwell C, Gallie DR. 2010.** Expression of the ethylene biosynthetic machinery in maize roots is regulated in response to hypoxia. *Journal of Experimental Botany* **61**: 857–871.

- **Greenberg JT. 1996.** Programmed cell death: a way of life of plants. *Proceedings of the National Academy of Sciences* USA **93**: 12094-12097.

- **Gunawardena AHLAN, Pearce DME, Jackson MB, Hawes CR, Evans DE. 2001.** Rapid changes in cell wall pectic polysaccharides are closely associated with early stages of aerenchyma formation, a spatially localized form of programmed cell death in roots of maize (*Zea mays* L.) promoted by ethylene. *Plant, Cell and Environment* **24**: 1369-1375.

- **Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, MatsumotoT, Yoshimura A, Kitano H, Matsuoka M, Mori H, Ashikari M. 2009.** The ethylene response factors *SNORKEL1* and *SNORKEL2* allow rice to adapt 1 to deep water. *Nature* **460**: 1026-1030.

- **He CJ, Morgan PW, Drew MC. 1992.** Enhanced sensitivity to ethylene in nitrogen- or phosphate-starved roots of *Zea mays* L. during aerenchyma formation. *Plant Physiology* 98: 137-142.

- **He CJ, Drew MC, Morgan PW. 1994.** Induction of enzymes associated with lysigenous aerenchyma formation in roots of *Zea mays* during hypoxia or nitrogen starvation. *Plant Physiology* **105**: 861-865.

- **He CJ, Finlayson SA, Drew MC, Jordan WR, Morgan PW. 1996a.** Ethylene biosynthesis during aerenchyma formation in roots of maize subjected to mechanical impedance and hypoxia. *Plant Physiology* **112**: 1679-1685.

- **He CJ, Morgan PW, Drew MC. 1996b.** Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. *Plant Physiology* **112**: 463-472.

- **Hinz M, Wilson WI, Yang J, Buerstenbinder K, Llewellyn D, Dennis ES, Sauter M, Dolferus R. 2010.** Arabidopsis RAP2.2: An ethylene response transcription factor that is important for hypoxia survival. *Plant Physiology* **153**: 757-772.

- **Huang TL, Huang HJ. 2008.** ROS and CDPK-like kinasemediated activation of MAP kinase in rice roots exposed to lead. *Chemosphere* **71**: 1377–1385.

- **Hung KT, Kao CH. 2007.** The participation of hydrogen peroxide in methyl jasmonate-induced NH_4^+ accumulation in rice leaves. *Journal of Plant Physiology* **164**: 1469-1479.

- **Irfan M, Hayat S, Hayat Q, Afroz S, Ahmad A. 2010.** Physiological and biochemical changes in plants under waterlogging. *Protoplasma* **241**: 3-17.

- **Jackson MB, Fenning TM, Drew MC, Saker LR. 1985.** Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L. by small partial pressures of oxygen. *Planta* **165**: 486-492.

- **Jackson MB, Armstrong W. 1999.** Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biology* **1**: 274-287.

- **Jurkowski GI, Smith RK, Yu IC, Ham JH, Sharma SB, Klessig DF, Fengler KA, Bent AF. 2004.** *Arabidopsis DND2*, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "*defense, no death*" phenotype. *Molecular Plant-Microbe Interactions* **17**: 511-520.

- **Justin SHFW, Armstrong W. 1991.** Evidence for the involvement of ethylene in aerenchyma formation in adventitious roots of rice (*Oryza sativa*). *New Phytologist* **118**: 49-62.

- **Kende H, Van der Knaap E, Cho HT. 1998**. Deepwater rice: A model plant to study stem elongation. *Plant Physiology* **118**:1105-10.

- **Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C.** 1998. A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca2+ 4 binding motifs. *Plant Cell* **10**: 255–266.

- **Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H. 2007.** Calcium-Dependent Protein Kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *The Plant Cell* **19**: 1065-1080.

- **Konings H. 1982.** Ethylene-promoted formation of aerenchyma in seedling roots of *Zea mays* L. under aerated and non-aerated conditions. *Physiologia Plantarum* **54**: 119-124.

- **Leng Q, Mercier RW, Yao W, Berkowitz GA. 1999.** Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. *Plant Physiology* **121**: 753-761.

- **Licausi F, van Dongen JT, Giuntoli B, Novi G, Santaniello A, Geigenberger P, Perata P. 2010.** *HRE1* and *HRE2*, two hypoxia-inducible ethylene response factors, affect anaerobic responses in *Arabidopsis thaliana. The Plant Journal* **62**: 302-315.

- **Luan S, Lan W, Lee SC. 2009.** Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL–CIPK network. *Current Opinion in Plant Biology* **12**: 339-346.

- **Lin F, Ding H, Wang J, Zhang H, Zhang A, Zhang Y, Tan M, Dong W, Jiang M. 2009.** Positive feedback regulation of maize NADPH oxidase by mitogen-activated protein kinase cascade in abscisic acid signalling. *Journal of Experimental Botany* **60**: 3221-3228.

- **Mosammaparast N, Shi Y. 2010.** Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. *Annual Review of Biochemistry* **79**: 155-179.

- **Mustroph A and Albercht G. 2003**. Tolerance of crop plants to oxygen deficiency stress: fermenative activity and photosynthetic capacity of entire seedlings under hypoxia and anoxia. *Physiologia Plantarum* 117: 508-520.

- **Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006.** Genome-wide 1 analysis of the ERF gene family in Arabidopsis and Rice. *Plant Physiology* **140**: 411-432.

- **Nakazono M, Qiu F, Borsuk LA, Schnable PS. 2003.** Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: Identification of genes expressed differentially in epidermal cells or vascular tissues of maize. *The Plant Cell* **15**: 583-596.

- **Nelson T, Tausta SL, Gandotra N, Liu T. 2006.** Laser microdissection of plant tissue: what you see is what you get. *Annual Review of Plant Biology* **57**: 181-201.

- **Mergemann H, Sauter M. 2000.** Ethylene induces epidermal cell death at the site of adventitious root emergence in rice. *Plant Physiology* **124**: 609-614.

- **Mühlenbock P, Plaszczyca M, Plaszczyca M, Mellerowicz E, Karpinski S. 2007.** Lysigenous aerenchyma formation in Arabidopsis is controlled by LESION SIMULATING DISEASE1. *Plant Cell* **19**:3819-3830.

- **Perata P and Alpi A**. 1993.Plant responses to anaerobiosis. *Plant Science* **93**: 1-17.

- **Ohtsu K, Takahashi H, Schnable PS, Nakazono M** .**2007**. Cell type specific gene expression profiling in plants by using a combination of laser microdissection and high-throughput technologies. *Plant Cell Physiol* **48**:3-7.

- **Oda T, Hashimoto H, Kuwabara N, Akashi S, Hayashi K, Kojima C, Wong HL, Kawasaki T, Shimamoto K, Sato M, Shimizu T. 2010.** Structure of the N-terminal regulatory domain of a plant NADPH oxidase and its functional implications. *Journal of Biological Chemistry* 285:1435-1445.

- **Overmeyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann HJ, Kangasjarvi J. 2000.** Ozone-sensitive arabidopsis *rcd*1 mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide dependant cell death. *Plant Cell* 12: 1849-1862.

- **Overmyer K, Brosché M, Kangasjärvi J. 2003.** Reactive oxygen species and hormonal control of cell death. *Trends in Plant Science* **8**: 335-342.

- **Rose JKC, Cosgrove DJ, Albersheim P, Darvill AG, Bennett AB. 2000.** Detection of expansin proteins and activity during tomato fruit ontogeny. *Plant Physiology* **123**:1583-1592.

- **Saab IN, Sachs MM. 1996.** A flooding-induced xyloglucan endo-transglycosylase homolog in maize is responsive to ethylene and associated with aerenchyma. *Plant Physiology* **112**: 385-391.

- **Sachs MM. 2008.** Flooding-Stress in Plants. In: Encyclopedia of Life Sciences (ELS). John Wiley and Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0001317.pub2

- **Sachs MM, Freeling M, Okimoto R. 1980.** The anaerobic proteins of maize. *Cell* **20**: 761-767.

- **Sairam RK, Kumutha D, Ezhilmathi K, Deshmukh PS, Srivastava GC. 2008**. Physiology and biochemestry of waterlogging tolerance in plants. *Biologia Plantarum* **52**: 401-412.

- **Sagi M, Fluhr R. 2001.** Superoxide production by plant homologues of the gp91phox NADPH oxidase. Modulation of activity by calcium and by Tobacco Mosaic Virus Infection. *Plant Physiology* **126**: 1281–1290.

- **Schussler EE, Longstreth DJ. 1996.** Aerenchyma develops by cell lysis in roots and cell separation in leaf petioles in *Sagittaria lancifolia* (Alismataceae). *American Journal of Botany* **83**: 1266–1273.

- **Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. 2006.** The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology* **7**:3.

- **Seago JL Jr, Marsh LC, Stevens KJ, Soukup A, Votrubova** 1 **O, Enstone DE. 2005.** A re-examination of the root cortex in wetland flowering plants with respect to aerenchyma. *Annals of Botany* **96**: 565-579.

- **Shieh MW, Cosgrove DJ. 1998.** Expansins. *Journal of Plant Research* **111**: 149-157.

- **Shiono K, Takahashi H, Colmer TD, Nakazono M. 2008.** Role of ethylene in acclimations to promote oxygen transport in roots of plants in waterlogged soils. *Plant Science* **175**: 52-58.

- **Sisler EC, Blankenship SM. 1996.** Methods of counteracting an ethylene response in plants, U.S.

- **Steffens B, Sauter M. 2005.** Epidermal cell death in rice is regulated by ethylene, gibberellin, and abscisic acid. *Plant Physiology* **139**: 713-721.

- **Steffens B, Sauter M. 2009.** Epidermal cell death in rice is confined to cells with a distinct molecular identity and is mediated by ethylene and H2O2 through an autoamplified signal pathway. *The Plant Cell* **21**: 184-196.

- **Steffens B, Sauter M. 2010.** G proteins as regulators in ethylene–mediated hypoxia signaling. *Plant Signaling and Behaviour* **5**: 375-378.

- **Steffens B, Sauter M. 2010.** Aerenchyma formation in the rice stem and its promotion by H_2O_2. *New phytologist*. In press.

- **Subbaiah CC, Zhang J, Sachs MM. 1994.** Involvement of intracellular calcium in anaerobic gene expression and survival of maize seedlings. *Plant Physiology* **105**: 369-376.

- **Subbaiah CC, Sachs MM. 2003.** Molecular and cellular adaptations of maize to flooding stress. *Annals of Botany* **90**: 119-127.

- **Suge H. 1985.** Ethylene and gibberellin: regulation of internodal elongation and nodal root development in floating rice. *Plant Cell Physiology* **26**: 607-14.

- **Takahashi H, Kamakura H, Sato Y, Shiono K, Abiko T, Tsutsumi N, Nagamura Y, Nishizawa NK, Nakazono M. 2010.** A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. *Journal of Plant Research,* **123**: 807–813.

- **Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L. 2008.** Local positive feedback regulation determines cell shape in root hair cells. *Science* 319: 1241-1244.

- **Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG. 1998.** Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (gp91phox) *The Plant Journal* **14**: 365-370.

- **Torres MA, Dangl JL, Jones JDG. 2002.** *Arabidopsis* gp911 phox homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences of the USA* **99**: 517-522.

- **Tsuji H, Nakazono M, Saisho D, Tsutsumi N, Hirai A. 2000.** Transcript levels of the nuclear-encoded respiratory genes in rice decrease by oxygen deprivation: evidence for involvement of calcium in expression of the alternative oxidase 1a gene. *FEBS Letters* **471**: 201-204.

- **Tsuji H, Saika H, Tsutsumi N, Hirai A, Nakazono M. 2006.** Dynamic and reversible changes in histone H3-Lys4 methylation and H3 acetylation

occurring at submergence-inducible genes in rice. *Plant and Cell Physiology* **47:** 995-1003.

- **Vartapetian BB, Jackson MB. 1997**. Plant anaerobic stress. *Annalsof Botany* **79**: 3-20.

- **Visser EJW, Bögemann GM. 2006.** Aerenchyma formation in the wetland plant *Juncus effuses* is independent of ethylene. *New Phytologist* **171**: 305–314.

- **Voesenek LA, Benschop JJ, Bou J, Cox MC, Groeneveld HW, Millenaar FF, et al. 2003.** Interactions between plant hormones regulate submergence induced shoot elongation in the flooding-tolerant dicot *Rumex palustris*. *Annals of Botany* **91**: 205-11.

- **Vodnik D, Strajnar P, Jemc S, Macek I. 2009.** Respiratory potential of maize (*Zea mays* L) roots exposed to hypoxia. *Environmental and Experimental Botany* **65**: 107-110.

- **Watkin ELJ, Campbell CJ, Greenway H. 1998.** Root development and aerenchyma formation in two wheat cultivars and one Triticale cultivar grown in stagnant agar and aerated nutrient solution. *Annals Botany* **81**: 349-354.

- **Watkins CB. 2006.** The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances* 2: 389-409.

- **Weinl S, Kudla J. 2009.** The CBL-CIPK Ca2+12 -decoding signaling network: function and perspectives. *New Phytologist* **184**: 517-528.

- **Woltering EJ, Jong AD, Hoeberichts FA, Iakimova E, Kapchina V. 2005.** Plant programmed cell death, Ethylene and flower senescence. *Acta Horticulturae* **669:** 159-169.

- **Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T, Shimamoto K. 2007.** Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. *The Plant Cell* **19**: 4022-4034.

- **Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K. 2004.** Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. *Plant Physiology* **135**: 1447–1456.

- **Woo HR, Kim JH, Kim J, Kim J, Lee U, Song IJ, Kim JH, Lee HY, Nam HG, Lim PO. 2010.** The RAV1 transcription factor positively regulates leaf senescence in *Arabidopsis*. *Journal of Experimental Botany* **61**: 3947–3957.

- **Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ. 2006.** *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance 1 to rice. *Nature* **442**: 705-708.

- **Yamamizo C, Kuchimura K, Kobayashi A, Katou S, Kawakita K, Jones JDG, Doke N, Yoshioka H. 2006.** Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance. *Plant Physiology* **140**: 681-692.

- **Yoshie Y, Goto K, Takai R, Iwano M, Takayama S, Isogai A, Che FS. 2005.** Function of the rice gp91^{phox} homologs *OsrbohA* and *OsrbohE* genes in ROS-dependent plant immune responses. *Plant Biotechnology* 22: 127-135.

- **Yoshioka H, Sugie K, Park HJ, Maeda H, Tsuda N, Kawakita K, Doke N. 2001.** Induction of plant gp91phox homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato. *Molecular Plant-Microbe Interactions* **14**: 725-736.

- **Yoshioka H, Asai S, Yoshioka M, Kobayashi M. 2009.** Molecular mechanisms of generation for nitric oxide and reactive oxygen species, and role of the radical burst in plant immunity. *Molecules and Cells* **28**: 321-329.

- **Zurbriggen MD, Carrillo N, Hajirezaei MR. 2010**. ROS signaling in the hypersensitive response, where and what for? *Plant Signaling and Behaviour* 5: 393-396.

- **Zeng Y, Avigne WT, Koch KE. 1999.** Rapid repression of maize invertase by low oxygen. Invertase/sucrose synthase balance, sugar signaling potential and seedlings survival. *Plant physiology* **121**: 599-608.