Extracellular ATP Signaling is Linked to Endocytic Vesicle Recycling in Root Apex

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Table of Contents

List of Tables

List of Figures

Abbreviations

ABD2	actin-binding domain 2
ADH	alcohol dehydrogenase gene
ADP	adenosine 5'-diphosphate
AMP	adenosin-5'-monophosphat
At	*Arabidopsis thaliana*
ATP	adenosine 5'-triphosphate
AtRBOHC	*Arabidopsis thaliana* respiratory burst oxidase homolog C
BFA	Brefeldin-A
Ca^{2+}	calcium ion
$[Ca^{2+}]_{cyt}$	cytosolic free Ca^{2+} concentration
Col-0	*Arabidopsis thaliana* ecotype Columbia 0
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1
DMSO	dimethyl sulfoxide
DORN1	extracellular ATP receptor in plant, Does not Respond to Nucleotided1
eATP	extracellular ATP
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
EtOH	ethanol
F-actin	filamentous actin
Fig	figure
FM 4-64	(N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino)Phenyl) Hexatrienyl)Pyridinium Dibromide)
GFP	green fluorescent protein
GLRs	glutamate-like receptors
GUS	ß-Glucoronidase
HCl	hydrochloric acid
HSD	honestly significant difference
IAA	Indole-3-acetic acid
KOH	potassium hydroxide
KPB	potassium phosphate buffer
LecRK I.9	legume-like lectin receptor kinase, LecRK-I.9

MAPK	mitogen-activated protein kinase
MES	2-(*N*-morpholino)ethanesulfonic acid
mRFP	monomeric red fluorescent protein
MS	Murashige and Skoog medium
NaClO	sodium hypochlorite
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NBT	nitroblue tetrazolium salt
NMDA	N-methyl-D-aspartate
NO	nitrogen oxide
$O_2^{\bullet-}$	superoxide
$ONOO^-$	peroxynitrite
PIN	PIN-FORMED
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PM	plasma membrane
PR gene	pathogenesis-related gene
RBOHC	respiratory burst oxidase homolog
RNS	reactive nitrogen species
ROS	reactive oxygen species
TM	trans membrane

Summary

Plants are sessile organisms, the roots of which exudate a large number of chemical compounds into the rhizosphere that contain several chemicals and microbes. It is known that at the root apex, the transition zone is located between the apical meristem and basal elongation zone. The transition zone plays a role as an important environmental sensor and controller of the motoric outputs. The high endocytic vesicle recycling found in this root apex region is essential to translocate PIN proteins (PINs) that, presumed auxin efflux carriers. PINs are reported to be major players for many root tropic growth responses, including root gravitropism and phototropism. Therefore, investigations of the transition zone may provide a comprehensive understanding of plants, and especially their adaptation to the sessile life. The aim of this thesis is to investigate the dynamics and activities of endocytic vesicle recycling in the root apex transition zone in response to such environmental stimuli as light, root cultivation medium components (MES), and solvents (DMSO and ethanol) *via* extracellular (eATP).

Chapter 1 describes the activity of endocytic vesicle recycling and PIN2 localization in root cells grown at different durations of light exposure. In this study, dark-grown seedlings showed lower rates of endocytic recycling activities in cells of root apex transition zones, compared to the light-grown roots. Interestingly, light-promoted endocytic recycling activity was attenuated to a level equivalent to dark-grown roots by an additional 24 hours of dark treatment. PIN2-GFP was shown to accumulate in vacuoles both in dark-grown and 24-hour dark treatment seedlings. Moreover, the PIN2-GFP signal found in 24-hour dark-treated roots was stronger than in the dark-grown sample. Here, I am proposing a model for dynamic regulation of PIN2 localization regulated by endocytic vesicle recycling in the transition zone according to light circumstances, which might be important for roots to prepare for upcoming unfavorable light.

Chapter 2 describes the DMSO and EtOH impacts in *Arabidopsis* root on endocytic vesicle recycling and cellular F-actin polarities. These are closely related to membrane conditions. In this study, DMSO and EtOH showed growth inhibition, interrupted endocytic vesicle recycling and disturbance of F-actin polarization in *Arabidopsis* root. Distortion of the plasma membrane shape was shown in plasmolyzed root epidermal cells in the presence of these chemicals. These results suggest that both DMSO and EtOH, in the range known as experimentally effective concentrations, may modify plasma membrane properties, thereby affecting endocytic vesicle recycling and cellular polarity in living cells.

Chapter 3 describes the effects of different concentrations of the MES buffer using growing root apices of *Arabidopsis*. The results show that 1% of MES significantly inhibits root growth, the number of root hairs and the length of the meristem, whereas 0.1% promotes root growth in the root apex area (region spanning from the root tip up to the transition zone). Furthermore, superoxide generation in the root apex disappeared at 1% of MES. These results suggest that MES disturbs normal root morphogenesis by changing the reactive oxygen species' (ROS) homeostasis in the root apex.

Chapter 4 describes the impact of eATP as a signaling molecule on root growth. In this study, the light-grown seedlings showed inhibited root growth with 1 mM eATP, whereas the dark-grown seedlings showed no inhibition. Moreover, BFA treatments indicate that eATP modify activity of endocytic vesicle recycling in root cells. eATP-induced inhibition of root growth and endocytic vesicle recycling requires ROS generation/signaling by NADPH oxidase (AtRBOHC), which was confirmed using the loss-of-function mutant line *rhd2-4*.

Chapter 5 describes the mechanism of the inhibition of root gravitropic response and growth by eATP using confocal microscopy. Five minutes of ATP treatment enhanced the endocytic vesicle recycling, whereas a treatment longer than five min inhibited it. Moreover, eATP-induced inhibition of root elongation and endocytic vesicle recycling require eATP receptor, DORN1, as shown using the point-mutated line *dorn1-1*. DORN1 is relevant for the plasma membrane (PM) rigidity as documented with plasmolysis using mannitol. The PM rigidity is known to be involved in control of the endocytic recycling activity. Next, pH changes were monitored after eATP application to roots of the pHusion (apoplastic pH indicator) transgenic *Arabidopsis* line. As a result, eATP lowered the pH value in the root tip. Moreover, the highest expression level of *DORN1* (At5G60300) was shown at the root apex transition zone. These findings suggest that eATP disturbs the pH value and endocytic recycling activity in the root apex, resulting in inhibition of root growth and gravitropic response.

In conclusion, obtained results indicate that the root apex transition zone responds to environmental stimuli by alteration of the activities of endocytic recycling, ROS generation, membrane rigidity, and root apex zonation. These studies provide the first insights for an understanding of eATP signaling in plant cell physiology, and also have relevance for other research fields, such as agriculture and potentially also pharmaceutical or medical studies.

General Introduction

Plants anchor their bodies within soil with roots, generating and maintaining rhizosphere, a spatially narrow soil area. Besides soil, the rhizosphere contains components and root exudates as well as numerous microbes, some of which are useful whereas others are harmful for plant roots. Some of them, such as mycorrhizae fungi and plant-growing promoting rhizobacteria, establish symbiotic relationships with roots in the soil (Fig. 1). Meanwhile, plant roots fight against pathogens or herbivorous microbes in the soil. Since plants are sessile organisms, plant roots exude numerous compounds attracting useful microbes or fighting against harmful microbes, such as for example sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenols, enzymes, and proteins (Badri et al., 2009). In contrast, root growth is known to be affected by aliphatic acids released from rhizobacteria (*Bacillus subtilis*) and cytokinins from nematodes (Badri et al., 2009). This means that plant roots and microbes communicate with some signaling molecules released into the rhizosphere, which can be regarded as a chemical language. In addition, plants suffer not only from microbes but also physical factors existing in the rhizosphere, such as obstacles, heavy metals, aluminum, salinity, acidic and alkaline soils, conditions caused by strong rain, and accidental light exposure from the surface of the soil (Foy et al., 1978; Koyama et al., 2001; Yokawa et al., 2014). In acidic soils, aluminum toxicity is the most important soil constraint for plant growth and development (Horst et al., 2010). It has also been reported that the number of lateral roots is modified by auxin-analogous compounds indole-3-acetic acid, indole-3-acetaldehyde and indole-3-ethanol produced from a fungus, *Trichoderma virens* (Contreras-Cornejo et al., 2009). Furthermore, plant roots grow downwards along the gravity vector, which is a phenomenon well known as gravitropism. However, the root growth directed by gravity is altered by some factors in the rhizosphere: for example, extracellular adenosine-5'-triphosphate (eATP) released from the root apex by touch stimulus is known to alleviate root gravitropism (Tang et al., 2003). Meanwhile, light has been reported to promote root gravitropism in maize (Burbach et al., 2012). Consequently, plants continuously need to improve their fitness according to the circumstances in the soil.

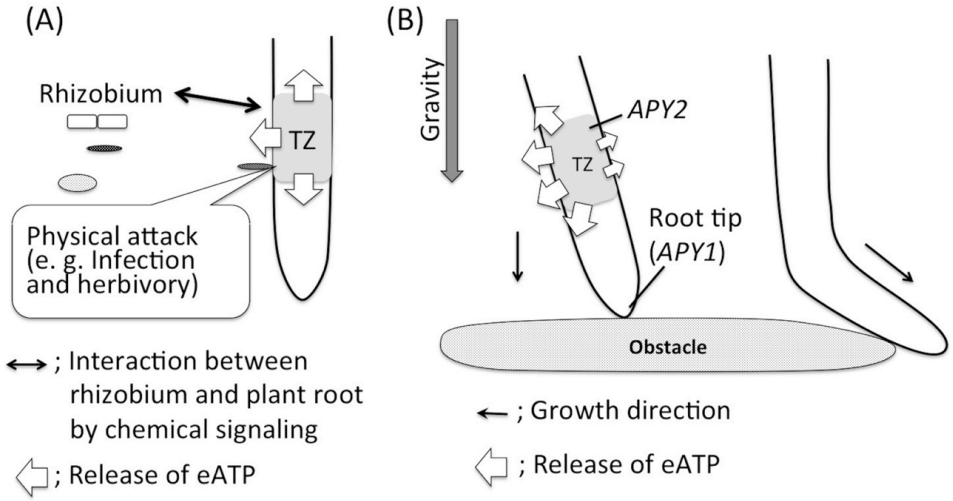

Fig. 1. Models of the eATP signaling in plant roots: A - biocommunication, B - obstacle avoidance. (A) A possible model of the eATP-mediated cross-kingdom communication between rhizobium bacteria and plant roots. Moreover, it is known that a nematode, *Meloidogyne incognita*, invades root tissue from the root transition zone (TZ) (Sijmons et al., 1991). It could physically damage the root transition zone. eATP is known to be released by wounding stress (Dark et al., 2011). Therefore, nematode infection to the root may induce eATP release from root cells. The eATP may contribute to chemical communication between plant roots and rhizobium. (B) Obstacle-avoiding response of plant roots. The left scheme indicates that roots release eATP from the root transition zone when root apices encounter a barrier. The root transition zone is an important environmental sensor and controller of the motoric outputs because this root apex region shows the highest vesicle recycling, which is essential to translocate PIN proteins (Baluška at al., 2010). eATP inhibits indole-3-acetic acid (IAA) transport in roots, resulting in the inhibition of gravitropic response or the change of the elongation rate (Tang et al., 2003). Moreover, the different kinetics of transient eATP release from the touched side (left the scheme) and from the opposed side (right the scheme). This allows the root apex to change the direction of growth with the asymmetric speeding-up of root growth to avoid the obstacles (Weerasinghe et al., 2009).

Transition Zone

In the complex rhizospheric environment, root apices play a role as important environmental sensors and controllers of motoric outputs (Baluška at al., 2010). The root transition zone is recognized at root apices between the apical meristem and distal elongation region (*ca*. 520 to

850 μm from root cap), at *ca*. 200-520 μm from the root tip of *Arabidopsis* (in the case of five-day-old seedlings grown at 23˚C and 16h/8h light/dark cycle, Verbelen et al., 2006). According to Verbelen et al. (2006), the region has the feature of slow growth of cells, and few cells divide due to the expression of cdc2, which is a key player in cell cycle regulation. The authors suggest that the transition zone maintaining competence for cell division in a kind of dynamic reservoir of developmentally plastic cells, allowing for rapid adjustment to environmental changes. Further morphological approaches show that F-actin distributes at nuclear surface in the region, allowing nuclei to keep their central position in the cells; in contrast, F-actin shows a longitudinal and wrinkled/loosened appearance in the elongation zone (Baluška and Mancuso, 2013).

The region shows high sensitivity to environmental stimuli based on high activity of endocytic vesicle recycling. The vesicle recycling found in the region is essential to translocate PIN proteins that is assumed auxin efflux carrier (Baluška et al., 2010). PINs are reported to be major players in root tropic growth, including root gravitropism and phototropism. In addition, recycling vesicles carry structural components of plants such as sterol (Grebe et al., 2003) and rhamnogalacturonan II (RGII)-borate pectins (Baluška et al., 2002). Based on these mechanisms, this highly active vesicle recycling enables cells in transition zones to perceive not only metals abundant in nature, such as aluminum (Sivaguru and Horst, 1998; Illéš et al., 2006), chromium (Eleftheriou et al., 2015) and cadmium (Suzuki, 2005), but also low pH environments (pH 4.5) (Koyama et al., 2001) and external light input (Wan et al., 2012; Yokawa et al., 2013). Furthermore, the root transition zone is reported to release some chemical compounds such as eATP induced by touch stimulus (Weerasinghe et al., 2009), which is involved in cell-to-cell signaling and the obstacle avoidance response of roots. Consequently, the root transition zone is likely a commander of environmental sensors and a determinant of growth direction for adaptation to heterogeneous circumstances (Fig.1). Therefore, investigations of the transition zone may provide a comprehensive understanding of plants' environmental adaptations in their sessile life.

Endocytosis and Brefeldin A

As mentioned above, the root transition zone shows the highest activity of endocytic recycling (Baluška at al., 2010). Endocytosis is a crucial cellular event conserved in all eukaryotic cells. This process allows cells to internalize many membrane-associated compounds as well as extracellular molecules (Šamaj et al., 2004). Plants also apply endocytosis to control growth and

tropisms in response to actual environment. Therefore, a number of studies concerned with root "tropisms" often focus on vesicle recycling activity in the root transition zone. For the study of vesicle recycling, a rate of endocytosis can be estimated with Brefeldin A (BFA), which is macrolide lactone antibiotic deriving from fungal organisms, such as *Eupenicillium brefeldianum* (Wang et al., 2012). BFA blocks a formation of vesicles from the endoplasmic reticulum, thus reversibly preventing the transport of secretory proteins to the Golgi apparatus, as well as aggregating endosomes, resulting in the formation of a round-shaped endocytic structure called the BFA-induced compartment.

While roots respond to environmental changes, dynamic endocytic activities are monitored by observing the BFA compartment under a confocal microscope. For example, a change in the formation of the BFA compartment in root cells by some environmental factors such as aluminum (Illéš et al., 2006), salicylic acid that is an important hormone for pathogen defense (Du et al., 2013), and GR24 that is analogous to the plant hormone strigolactone (Pandya-Kumar et al., 2014).

PIN-FORMED (PIN) protein and P-glycoprotein (PGP) transport are important to auxin flux in plants (Noh et al., 2003). These proteins are localized on the plasma membrane and effuse auxin into extracellular space. Whereas auxin is imported into the cell by AUX1 protein. PIN proteins are transported constantly by endocytic vesicles. This endocytosis activity is subject to some signals from external and internal environment, as mentioned above. Consequently, auxin flux is controlled and adapted to the environmental context.

It has been reported that BFA disturbs the recycling of PIN1 (Steinmann et al., 1999), PIN2 (Abas et al., 2006), and PIN3 (Friml et al., 2002) proteins. Hence, plant growth and tropisms are regulated by BFA-sensitive pathways of endocytic vesicle recycling.

Plants May Utilize Extracellular ATP (eATP) to Probe and Manipulate Rhizosphere

In the rhizosphere a number of signaling chemicals exist, such as for instance strigolactones, which are released by roots from *Lotus japonicm* (Akiyama et al., 2005) and *Sorghum bicolorin* (Besserer et al., 2006). This signal stimulates the germination of parasitic plants and enhances symbiosis between arbuscular mycorrhizal fungi and plants. Interestingly, plants have receptors of strigolactones in their roots (Waters et al., 2012); in the results, these chemicals vary the root growth and the tropisms. Moreover, strigolactones analogous to GR24 increase the size of the PIN2-containing BFA compartment, suggesting that the rate of endocytosis is changed (Pandya-Kumar et al., 2014).

Likewise, ATP may be an important signaling chemical that informs the plant of environmental conditions, and interacts with organisms in the rhizosphere. Some studies have demonstrated that eATP is involved in regulating plants' growth and adaptation to their environment. However, very little is known about how eATP signaling is utilized for organisms in the rhizosphere.

ATP serves not only as an energy source for all living organisms, but also as an important signaling agent in mammal and plant cells if it is released extracellularly (Tanaka et al., 2010). Burnstock (1972) reported the possibility of two types of receptor functions to perceive ATP as a neurotransmitter in mammals. Indeed, two mammalian eATP receptors, P2X (Valera et al., 1994) and P2Y (Webb et al., 1993), were identified. After that, eATP studies were accelerated in mammals. Based on the findings regarding eATP receptors in mammalian cells, some pharmaceutical companies have developed a painkiller that targets the purinoceptors or controls the metabolism of eATP. Although plants do not have homologs of mammal purinoceptors (reviewed in Tanaka et al., 2010), the plant purinoceptor P2K (K for kinase) was recently reported (Choi et al., 2014).

In plants, eATP is known to be involved in regulating plant growth and environmental adaptation. For example, eATP is shown to inhibit root gravitropism and growth in *Arabidopsis* (Tang et al. 2003). Moreover, a root apex shows high activity of eATP release induced by touch stress (Jeter et al., 2004; Weerasinghe et al., 2009), cold stress (Sun et al., 2012a), hypertonic stress (Kim et al., 2009), or some chemical stimuli such as salt (Sun et al., 2012b), NaCl, sorbitol, ABA, and L-glutamate (Dark et al., 2011). Interestingly, eATP is released specifically from the root transition zone, but not from the root tip (Weerasinghe et al., 2009). As mentioned above, the root transition zone shows sensor-like activity to environmental stimuli based on the highest activity of endocytic vesicle recycling. Therefore, the root transition zone may have the function of primary recognition of eATP molecules or signaling. It is reported that eATP changes vesicular trafficking to repair the plasma membrane during cold stress (Deng et al., 2015).

It is suggested that eATP is involved in the control of nodulation in nitrogen-fixing root nodules. Thus, eATP may play an important role as a signaling molecule that conducts environmental conditions to plant and mediates interactions with organisms in the rhizosphere.

In the rhizosphere, besides eATP also extracellular adenosine 5'-diphosphate (eADP) and extracellular adenosine 5'-monophosphate (eAMP) are present. The concentration of these compounds is highly variable, because roots always change their exudation or leaching in

response to cellular physiological conditions, and soil physical conditions also dominate their concentrations.

According to Kim et al. (2006), eATP is released by exocytosis in areas of plant growth by a calcium-dependent mechanism. This means that activity of plant exocytosis changes the concentration of eATP released.

It has been demonstrated that the soluble form of apyrases purified from insect cells catalytically produces ADP as a stable intermediate during the hydrolysis of eATP (Chen and Guidotti, 2001). In *Arabidopsis*, both apyrases, AtAPY1 and AtAPY2, hydrolyzed ATP fourfold more than they did ADP (Steinebrunner et al., 2000). This suggests that eADP can stay in the extracellular matrix longer than eATP can. eADP caused a cell response through a Ca^{2+} influx in the root epidermal cells as well as eATP. However, eADP did not accumulate reactive oxygen species in intracellular space (Demidchik et al., 2011). In plants, eADP receptors are still unknown, although in mammals, eADP, eAMP and adenine receptors are well studied (Burnstock, 2006).

eATP concentration may also be subject to environmental changes such as metals, pH value, and number of rhizobium bacteria. Thus, plant roots in soil may have the ability to decipher changes of the gradation of eATP concentration in the rhizosphere.

For example, it has been demonstrated that ATP binds divalent cations (Mg^{2+}, Ca^{2+}) at middle to alkaline conditions and forms a stable structure (Carvalho and Leo, 1967; Ramirez et al., 1980). It is also known that ATP is easily hydrolyzed under acidic conditions (Zhang et al., 2015), suggesting that although ATP molecules are unstable in acidic pH conditions, they can behave as reactive signaling molecules modulated through environmental physico-chemical factors. For this reason, plant roots might be able to perceive environmental changes through the concentrations of eATP or eADP sensed at their surfaces.

Jaffe and Galston (1966) report that eATP enhanced curvatures of excised pea tendrils in the dark. Both tendrils and roots play an important role in searching for a proper place to support their body. However, roots and shoots show opposite responses to mechanical stimuli. For example, tendrils show positive thigmotropism and phototropism, whereas roots show negative thigmotropism and phototropism. Similarly, roots show positive gravitropism while shoots show negative gravitropism (Table 1). These tropisms are important for the reorientation of plant growth and adaptation to the environment. Thus, in this Thesis it is hypothesized that eATP might have opposite functions in roots and shoots in terms of tropisms.

Table 1. Plant tropisms. (+) indicates positive tropism. (-) indicates negative tropism. The columns indicate experimented plants and references, respectively.

	Shoot	Tendril coiling	Root
Phototropism,	+	+	-
References	e.g. *Phalaris Canariensis* Darwin, (1880)	e.g. Bignonia Darwin, (1880)	e.g. *Sinapis alba* Darwin, (1880)
Thigmotropism,	+	+	-
References	*Phaseolus vulgaris* Huberman and Jaffe, (1986)	*Phaseolus vulgaris* Jaffe and Galston, (1966)	e.g. *Vicia faba* Darwin, (1880)
Gravitropism,	-	?	+
References	e.g. *Cytisus fragrans* Darwin, (1880)		e.g. *Ipomoea leptophylla* Darwin, (1880)

The aim of this thesis is to investigate the dynamics of root adaptation to the environment by comparing the activity of endocytic vesicle recycling in the root transition zone after some environmental stimuli such as light, medium components (MES), solvents (Dimethyl sulfoxide and ethanol), and eATP in *Arabidopsis*.

This thesis consists of five chapters. **Chapter 1** describes the activity of endocytic vesicle recycling and PIN2 localization in seedlings that are grown in different light conditions (16h light/8h dark, 4d light/24h dark, or 5d dark). This result shows dynamic control of PIN2 localization regulated by endocytic vesicle recycling in the transition of light circumstances, which might be important for roots to prepare for upcoming unfavorable light.

Chapter 2 describes the effects of Dimethyl sulfoxide (DMSO) and ethanol (EtOH) on endocytic recycling activity. DMSO and EtOH are essential as solvents for chemicals in many biological experiments, such as BFA and FM4-64 (membrane tracer). In addition DMSO is reported to change plasma membrane properties (Notman et al., 2007; Cheng et al., 2015).

Chapter 3 describes the effects of a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer on growing roots of *Arabidopsis*. MES is generally used as a pH control buffer for media or ATP solutions in plant experiments. MES is reported to interfere with peroxidase activities of oxidizing phenolic compounds because of its chemical structure (Baker et al., 2007). eATP signaling interferes with signaling molecules (NO and ROS) (Song et al., 2006; Jeter et al., 2004;

Demidchik et al., 2009; Shang et al., 2009; Wang et al., 2014). MES could disturb eATP signaling.

Two membrane proteins, NADPH oxidase and the eATP receptor, DORN1, are reported that they are involved in eATP signaling in plants (Song et al., 2006; Jeter et al., 2004; Demidchik et al., 2009; Shang et al., 2009; Wang et al., 2014; Choi et al., 2014). Therefore, this study assessed the eATP effect on endocytic activity with NADPH oxidase (*Arabidopsis thaliana* respiratory burst oxidase homolog C, AtRBOHC) using loss-of-function mutant line *rhd2-4* (**Chapter 4**) or *DORN1* mutant lines *dorn1-1*, point-mutated line or *oxDORN1*, ectopic expression line (**Chapter 5**). **Chapter 4** describes eATP-induced inhibition of root elongation and endocytic vesicle recycling which requires ROS generation by NADPH oxidase (AtRBOHC).

Chapter 5 describes the function of the eATP receptor DORN1 that mediates the eATP signal to regulate endocytic vesicle recycling in root gravitropic response. Furthermore, the Chapter 5 shows that cytosolic pH values were changed by eATP stimuli in the root apex.

Chapter 1

Dynamic Regulation of Endocytic Vesicle Recycling and PIN2 Localization in *Arabidopsis* Roots under Varying Light Regimes

1.1 Abstract

For root tropic behavior, auxin is the essential phytohormone to regulate a cell growth directing root development. It was reported that light promotes the translocation of auxin carrier proteins such as PINs (PIN-FORMED) providing a polarity for roots to complete negative phototropism. These PIN proteins are known to be translocated *via* endocytic vesicle recycling in root cells. However, direct influence of light conditions on endocytic vesicle recycling in *Arabidopsis* root cells is not well assessed. In this study, I compared the activity of endocytic vesicle recycling and PIN2 localization in root cells at root transition zone grown under (1) light regime (16 h light/8 h dark) for 5 d, (2) light regime for initially 4 d followed by 24-h of dark, and (3) continuous dark for 5 d. In the result, dark-grown seedlings showed lower rate of endocytic activities in the root transition zones, compared to the light-grown roots. Interestingly, light-promoted endocytic recycling activity was attenuated to the level equivalent to dark-grown roots after 24-h of dark treatment. PIN2-GFP was shown to accumulate in vacuoles both in dark-grown and 24-h dark treatment seedlings. Moreover, the PIN2-GFP signal found in 24-h dark-treated roots was stronger than in the dark-grown sample. Here I propose a model (Fig. 1-2) for dynamic of PIN2 localization regulated by endocytic vesicle recycling in the transition zone at different durations of light exposure, which might be important for roots to prepare for upcoming unfavorable light.

1.2 Introduction

The sunshine is the most important energy resource to every land-dwelling creature. Plants receive the benefit of light to complete photosynthetic reaction. Since light is directly linked to energy availability, plants have evolved to regulate their growth and development depending on external light conditions. A mechanism that plant can recognize and change a direction of growth toward light is called phototropism. It is an essential movement for plants to effectively collect the energy in the form of photons. Unlike aerial part of plants such as leaves, root system in many plant species have a tendency toward to grow belowground, because roots have roles for anchoring their body and absorbing the necessary water and nutrients from soil. The behavior of roots towards light is completely opposite to that of aboveground half of plants.

As Darwins described in last century, root gravitropism, the ability that roots can sense gravity and grow vertically, is well recognized as one of root tropisms (Darwin, 1880). This tropism is necessary for roots to explore patchy soil environment. In addition to gravity, recent studies in plant physiology have revealed that *Arabidopsis thaliana* expresses all photoreceptors not only in shoot but also in root portion, and therefore roots can sense the external light stimuli. Upon roots are illuminated, tips of roots start growing back into darkness as fast as possible (thus, seeking for the soil). This phenomenon is called negative phototropism (escaping phototropism), which is now regarded as one of important root tropisms.

In *Arabidopsis thaliana*, root growth is accelerated under continuous illuminated condition (with a light intensity in normal growth chamber *ca*. 120 μmol/m^2/s of white light) compared to dark grown roots. The light-enhanced root growth might be representing the light-escape tropism. We have previously demonstrated that short-time blue light illumination to roots promoted the generation of reactive oxygen species (ROS) in root apex region (Yokawa et al., 2011; 2013; 2014). ROS are radical-oxygen molecules acting as important signaling molecules, which drive propagation of secondary cellular signaling molecules. It suggests that ROS generated upon illumination trigger the negative phototropism in the roots (fast root growth).

In the tropic behavior of roots, the major phytohormone, auxin (IAA; indole-3-aceticacid) plays a crucial role by regulating the growth of root cells. As one of auxin functions, it alters the rate of root growth depending on its concentration. During tropism, auxin molecule is transported asymmetrically from one side to the other side of root apex in order to develop a steep gradient in auxin concentration eventually changing the speed of growth. It thus enables

the root apex to bend.

In order to achieve cell-to-cell transport of auxin, several auxin carrier proteins must be aligned on one side of cells in response to a direction of external input of information (such as light stimulus). In root negative phototropism, it was reported that the re-location of auxin efflux carrier (PIN-FORMED) 1, 2 and 3 proteins are involved in asymmetric auxin distribution and negative phototropism in roots mediated by a Brefeldin A (BFA) sensitive-trafficking pathway (Wan et al., 2012; Zhang et al., 2013; 2014; Geldner et al., 2003). PIN2 proteins (PIN-FORMED 2; auxin efflux carrier) in root cells change their distribution upon response to environmental light (Laxmi et al., 2008). In addition, the re-location of PIN2 in root cells upon illumination was reported to be completed within 30 min (Wan et al., 2012). Wan et al. (2012) further demonstrated that blue light promotes the basipetal (shootward) polar auxin transport facilitated by PIN2, which regulates the negative phototropism in roots. Roots grown in partially darkened-Petri dish system (only root part is protected from light) showed weak PIN2 accumulation in cross walls in root meristematic cells. Taken together, based on these mechanisms roots can respond to incoming light and escape from light. In addition to PIN-related reaction, Dyachok et al. (2011) reported that light-activated COP1, E3 ubiquitin ligase, enhances actin polymerization and F-actin bundling in root cells, resulting in fast root growth under light growth conditions. It was also reported that light regulates F-actin bundling in maize coleoptiles (Waller and Nick, 1997), suggesting that both areal and under-ground portions of plants share the same mechanism for light-driven reorganization of cellular skeletons, possible in the course of cellular axis formation. Through self-referring regulatory circuits between polar auxin transport and auxin induced actin reorganization, self-amplification of auxin transport which is central element to auxin-dependent patterning is achieved (Nick et al., 2009). The interplay between F-actin and polar auxin transport is mediated by endocytic vesicle recycling in the transition zone of root apex, and it controls the root tropisms (Baluška et al., 1996; 2004; 2005; 2010; Baluška and Mancuso, 2013).

It is well studied that a re-localization of auxin carrier proteins such as PINs, requires the endocytic vesicle recycling mechanisms (including endocytosis and exocytosis), these are very fundamental and important cellular machineries for transporting mainly membrane-associated proteins or compounds. Therefore, it is thought that endocytic vesicle recycling is essential to almost all tropic behaviors of plants. However, an impact of light on this endocytic vesicle recycling has not been documented in details, although effect of light (especially blue light) on PIN re-localization or actin reformation was reported (Zhang et al., 2013; 2014). In this study, I

report the dynamic of endocytic vesicle recycling modulated in response to different light conditions for root growth.

1.3 Materials and Methods

1.3.1 Plant Growth Condition

$PIN2_{pro}$:PIN2-GFP was kindly provided by Dr. Yinglang Wan (College of Biological Sciences and Biotechnology, Beijing Forestry University). At first, seeds of *Arabidopsis thaliana*, ecotype Col-0 (wild type) and *$PIN2_{pro}$:PIN2-GFP* were sterilized by 2% of sodium hypochlorite (ROTH, Karlsruhe, Germany) in the presence of 0.1% of Triton-X (ROTH, Karlsruhe, Germany) for 5 min. Secondly, these seeds were rinsed out by water for 4 times. These seeds were then planted on solidified 0.4% (w/v) phytagel plates (Sigma, Steinheim, Germany) containing half-strength of Murashige-Skoog nutrient mixture (Duchefa, Haarlem, The Netherlands) and 1% (w/v) sucrose (pH 5.8 adjusted with KOH). These Petri dishes were incubated at 4˚C in dark for 1 d for imbibition and placed vertically at 23-25˚C in the light (under light regime of 16 h light/8 h dark with white light from fluorescent lamp, 120 $\mu mol/m^2/s$) or in the dark. For dark adaptation experiment, 4 day-old seedlings of light grown (16 h light/8 h dark) seedlings were transferred in the dark for 24 h.

1.3.2. Confocal Microscopy

Seedlings of Col-0 (Wild type) were stained with 4 μM FM4-64, membrane-staining fluorescence probe (Sigma, Steinheim, Germany) for 10 min. FM4-64 was prepared from a stock solution at 2000 times higher concentration dissolved in dimethyl sulfoxide (DMSO, Sigma, Steinheim, Germany). The seedlings were then incubated in 0.5x MS medium containing 35 μM BFA (Sigma, Steinheim, Germany). BFA was made from stock solution at 1000 times higher concentration dissolved in DMSO. All the images of FM-stained BFA-compartment of PIN2-GFP were taken though a confocal laser microscopy (Fluoview FV1000, Olympus, Tokyo, Japan). FM 4-64 was excited by 515 nm and GFP were excited by 488 nm blue light emitted by Argon laser. Fluorescence emissions by FM 4-64 were taken between 630 and 700 nm. Fluorescence emissions by GFP were collected between 500 and 600 nm. Total areas of BFA-compartments were calculated by ImageJ software (ver. 1.43u for Mac OSX, http://imagej.nih.gov/ij/).

1.4 Results and Discussion

1.4.1 Light Promotes Endocytic Vesicle Recycling in Roots

In this study, I monitored the endocytic vesicle recycling process in root cells under different light conditions to elucidate the events reflecting the negative phototropic response in roots. A rate of endocytic vesicle recycling can be estimated with Brefeldin-A (BFA) treatment, which blocks a formation of vesicles from endoplasmic reticulum, thus reversibly preventing the transport of secretory proteins to the Golgi apparatus, resulting in the formation of round-shaped aggregated structure called BFA-compartment in cytosolic space. As Fig. 1-1(A) shows, BFA-compartments visualized with fluorescence probe, FM4-64 in red color, are present in epidermal cells in roots. Size and number of BFA-compartments indicate a speed of endocytic vesicle recycling reflecting root tropic response. If the size of observed compartments were big, it might indicate that roots are active in tropic movements. In Fig. 1-1(A)-a, big compartments was detected in the roots grown under light condition for 5 d, whereas roots grown under continuous dark condition showed smaller and less number of compartments (Fig. 1-1(A)-c). Although it was known that blue light-induced root negative phototropic curvature is BFA sensitive (Wan et al., 2012; Zhang et al., 2013), the result observed here suggests that roots always maintain high endocytic vesicle recycling activity for negative phototropism under continuous light-exposed condition.

1.4.2 Dark Treatment Attenuates the Endocytic Vesicle Recycling

As previously described, response of roots to incoming light are extremely quick (Yokawa et al., 2011). Light-exposed roots promote their elongation by seeking for darkness, which is known as negative phototropism (Yokawa et al., 2011). Silva-Navas et al. (2015) have recently proposed an improved root growth system ‘D-Root’ that allows growth of only the root portion kept in darkness. Interestingly, they reported that root illumination shortens root length and promotes early emergence of lateral roots. Xu et al. (2013) also reported another version of improved-Petri dish system for the proper root growth and showed different PIN2 localization between light and dark grown roots. It is important in these new experimental systems allowing restricted exposure of plants only in the aerial parts. However, there are concerns that light sources set in these sources could be too close, causing systems should not be close enough causing localized increase in temperature and unexpected reflection of light.

Zhang et al. (2013) demonstrated that 30 min of unilateral blue light illumination changes the

distribution of PIN3 (PIN-FORMED 3; auxin efflux carrier) on illuminated side versus shaded side in columella cells in. How can roots set a cellular situation back to normal growth condition, namely, under darkness (Fig. 1-1 (A)-c)? Laxmi et al. (2008) showed that accumulated fluorescence signal from enhanced GFP-fused PIN2 (PIN2-GFP) on plasma membrane in light grown roots was reduced to 62% of the initial level by 12 hours after dark treatment.

In the present study, the fate of BFA-compartments in root cells during the light-to-dark transition phase (dark adaptation) was also assessed. As shown in Fig. 1-1(A)-b, small BFA-compartments were observed in roots with light-to-dark history, in which plant root were grown under light condition for 4 days, followed by 24 hours of darkness. Interestingly, within 24 hours in darkness (Fig. 1-1 (A)-b), endocytic vesicle recycling almost went back to a steady state as shown by dark-grown roots (Fig. 1-1(A)-c).

1.4.3 Light-grown PIN2 Localization in Dark Treatment

It was shown that PIN2 is re-localized from plasma membrane to vacuole when roots were transferred from light to dark condition (Laxmi et al., 2008). In this study, the re-location of PIN2-GFP signal from plasma membrane to vacuoles in the dark-grown and of 4 d light/24 h dark-treated seedlings were also observed as showed in Fig. 1-1(C)-a, b and c. Intriguingly, the PIN2-GFP signal found in vacuoles and vacuole-like small compartments of 24-hours dark-treated roots was stronger than in dark-grown ones. Loss of fluorescence signal reflecting the presence of PIN2 in cytosolic region in 5 days dark-grown roots (Fig. 1-1(C)-c) might be attributed to the induced degradation of PIN2 proteins in the lytic vacuoles (Kleine-Vehn et al., 2008). However, what is interesting is that PIN2 proteins were still maintained in the vacuolar-like compartments for at least 24 hours after the shift to dark condition (Fig. 1-1(C)-b). As summarized in Fig. 1-2, these findings suggest the ability of roots to prepare for upcoming unfavorable light situation, in order to respond as quickly as possible with a minimum cost. Obviously, PIN2 proteins can be stored within multi-vesiculate body (MVB) or pre-vacuolar compartments (PVC) for short period (Wan et al., 2012). In fact, this unique behavior of PIN2 proteins can be regarded as a “buffering memory” of plant root cells. Once light comes again, endocytic vesicle recycling of PIN2 in root cells can be immediately activated (Fig. 1-1(A)-a), (as shown in Fig. 1-1(C)-a) to drive negative root phototropism.

In contrast, after experiencing several days under the dark condition, PIN2 will then be transported to vacuoles and degraded by lytic action (Fig. 1-1(C)-c). In a normal

laboratory-based growth condition, roots are exposed to repeated cycles of light and dark a day, due to standard lighting regime. Therefore, root of such seedlings continuously experience the "buffering memory" of illumination and they are in "alert" modus with respect of PIN2 behavior.

In laboratories, plants are normally propagated under 16 h light/8 h dark photoperiod, conferring a circadian rhythm to the plants. As I observed in this study, plant roots regulate a rate of endocytic vesicle recycling, possibly controlling other components signal transduction in response to external light stimuli. A direct or indirect association of such a circadian rhythm with endocytic vesicle recycling, which may play some roles in many tropic behaviors in plants, should be further studied in proper way of environmental setups. Also, it would be very intriguing to assess in further study if light treatment to only shoot parts modify endocytic vesicle recycling or other light-perceptive reactions in roots.

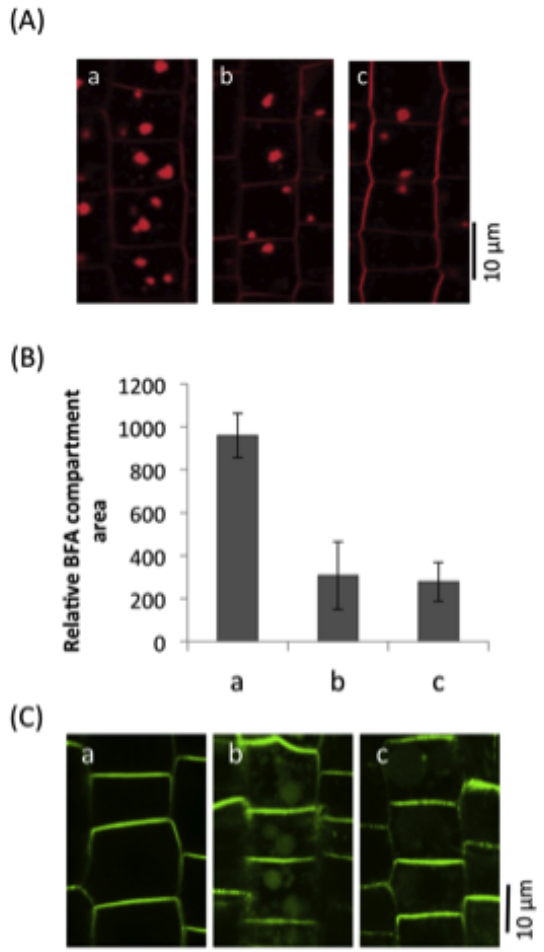

Fig. 1-1. Effect of light on endocytic activities in root transition zones. (A) Confocal images of roots which were incubated in 35 μM BFA. Seedlings were germinated and grown in (a) the light (16 h light/8 h dark) for 5 d (control), (b) 16 h light/8 h dark for 4 d followed by 24 h dark treatment, (c) Seedlings were germinated and grown in the dark for 5 d. (B) Relative BFA compartment area calculated from cells with 50 μm square (n = 4-7). (a), (b) and (c) indicates the identical growth conditions as mentioned above. Error bars indicate standard deviation of the mean. These sizes of BFA compartment were measured by ImageJ. (C) Confocal images of PIN2-GFP. (a), (b) and (c) indicates the identical growth conditions as mentioned.

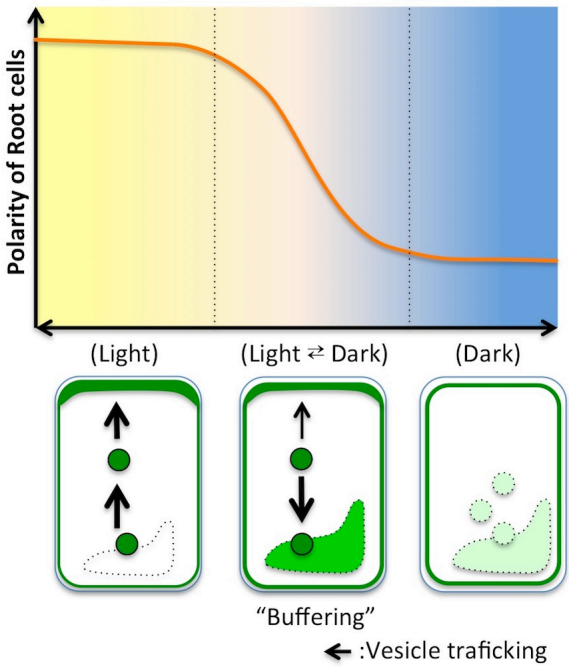

Fig. 1-2. PIN2 distributions in root apex cells under varying light regime as indicated in Fig 1-1. (Left) PIN2 proteins accumulate at the plasma membrane under cross walls. (Middle) If light is shaded, PIN2 proteins are gradually re-localized from recycling plasma membrane to late endosomes (also known as multi-vesicular bodies/pre-vacuolar compartments). (Right) After several days in darkness, retrieved PIN2 proteins are degraded within the late endosomes of dark-adapted roots.

Chapter 2

Ethanol and Dimethyl Sulfoxide Affect Plasma Membrane Rigidity, Alter Vesicle Recycling and F-actin Polarity in Root Apex Cells

2.1 Abstract

Dimethyl sulfoxide (DMSO) and ethanol (EtOH) are essential solvents. However, these compounds are reported to interact with the plasma membrane (PM) and alter its mechanical properties. Using intact roots of *Arabidopsis*, this study assessed the impact of these compounds on vesicle recycling and F-actin, both of which are closely related to membrane properties and signal transduction. While 1% DMSO disturbed vesicle recycling but not F-actin polarization, 0.1% EtOH affected both vesicle recycling and F-actin polarization. The PM was distorted by both of these chemicals in plasmolyzed root cells. DMSO and EtOH likely modify not only PM properties, but also endocytic vesicle recycling and associated signaling pathways.

2.2. Introduction

Dimethyl sulfide (DMSO) and ethanol (EtOH) are broadly used as solvents for preparing many reagents for biological experiments. DMSO was first synthesized and reported in 1867 (Saytzeff, 1867) and it has become a popular molecule for many biological applications over the last 60 years. DMSO is also used as a cryoprotectant and as a radical scavenger, and it is used in cosmetics and medical treatments to increase the permeability of chemicals or drugs through skin (Yu and Quinn, 1994). DMSO was reported to enhance and change permeability of ceramide membranes (Notman et al., 2007) and to disrupt networks of water molecules near the surface of

lipid membranes through dehydration of water molecules (Cheng et al., 2015). The previous work demonstrated that DMSO disturbs the polar distribution of membrane proteins in neurons (Winckler et al., 1999). Another study showed that endocytosis of glucose transporter GLUT4 was inhibited *via* DMSO (Berenguer et al., 2011). DMSO-dependent generation of reactive oxygen species was observed in yeast cells (Sadowska-Bartosz et al., 2013). Furthermore, DMSO induces the cold stress response gene by changing the membrane rigidity and F-actin reorganization *via* the increasing of cytoplasmic Ca^{2+} (Örvar et al., 2000).

In mammals, EtOH impairs N-methyl-D-aspartate (NMDA) receptor signaling on climbing fiber synapses in Purkinje cells in mice (He et al., 2013). Moreover, actin cytoskeleton is a key cellular component subjected to EtOH through NMDA receptor signaling in mice (Offenhäuser et al., 2006). In plants, NMDA-like receptors, called glutamate receptors (GLRs), are known (Chiu et al., 2002; Weiland et al., 2016). However, little is known about how EtOH affects plant cells. EtOH is also known to show general anesthetic properties, and the action of EtOH has been proposed to be accomplished at the membrane lipid level (Patra et al., 2006). Beer brewers sometimes suffer from the problem of brewing so-called “stuck fermentation”, which occurs when yeast becomes dormant and stops the fermentation process, thereby spoiling the product. The reason for this is not yet clear, but it is thought that at about the 10% content level, EtOH changes the membrane structure of yeast cells and brings them into dormancy (Patra et al., 2006). Interestingly, it has been reported that plants produce EtOH from pyruvate endogenously through the reaction of their alcohol dehydrogenase under anoxic stress conditions (Johnson et al., 1994; Chung and Ferl, 1999). It seems that plants use endogenous EtOH as a stress-induced signaling molecule. However, the biological function of EtOH as a signaling molecule is still largely unknown.

Cell membranes are a highly active and dynamic part of cells, functioning as an important interface between the outside and inside of those cells. The elaborate control of membrane homeostasis is crucial to many cellular events. It is known that even small disturbances of membrane structures have a big impact on membrane proteins, ion channels or endo/exocytic vesicle recycling (McNeil and Steinhardt, 2003). For biological experiments, many studies use these two compounds as solvents. Possible effects of DMSO and EtOH on endocytic vesicle recycling, which are directly affected by membrane rigidity, have not been well studied. The present study monitored the effects of these compounds on *Arabidopsis* root apex cells in terms of membrane-associated events. The results show that DMSO or EtOH inhibited *Arabidopsis* root growth, and endo/exocytic vesicle recycling was interrupted. F-actin polarization was also

disturbed by EtOH treatment but not by exposure to DMSO. Distortion of the plasma membrane (PM) structure was found in the presence of these chemicals. These results suggest that DMSO and EtOH affect cell membrane properties important to membrane recycling. These findings obtained from plant cells may also provide insight into the biological effect of these compounds in living cells.

2.3 Materials and Methods

2.3.1 Plant Growth Condition

Five-day-old seedlings of *Arabidopsis* grown in the light condition were stained with 4 μM FM4-64 membrane-staining fluorescence probe (Sigma, Germany) for 10 min. These seedlings were then treated in 0 or 0.1 or 1% (v/v) DMSO or 0 or 1% (v/v) EtOH dissolved in 0.5x MS for one hour, followed by a 35 μM Brefeldin A (BFA, Sigma, Germany) treatment for 30 min. Confocal images were taken to compare the effects of DMSO and EtOH on the rate of endocytosis. To monitor exocytosis, recovery from BFA-inhibited recycling was monitored. The seedlings were first stained with 4 μM FM4-64 and then soaked in 35 μM BFA for 30 min. After the BFA was washed out, the seedlings were incubated in each concentration of DMSO or EtOH for one hour. The confocal images were taken using confocal laser microscope (Fluoview FV1000, Olympus, Tokyo, Japan). FM4-64 dye was excited by 515 nm emitted by Argon laser and fluorescent emission of FM4-64 was collected between 630 and 700 nm. BFA-induced compartment size (area) was measured and averaged with ImageJ software.

2.3.2 Confocal Observations of Endo/Exocytic Vesicle Recycling

Seedlings of the transgenic *Arabidopsis* ABD2-GFP (actin-binding domain 2) line (Voigt et al., 2005) were grown in the light condition (16h light/8h dark) for five days. They were incubated in different concentrations of DMSO or EtOH prepared in 0.5x MS for two hours prior to confocal microscopy analysis. ABD2-GFP were excited by 488 nm emitted by Argon laser and emission were collected between 500 and 550 nm.

2.3.3 Visualization of F-actin

Seedlings of the transgenic *Arabidopsis* ABD2-GFP were grown in the light condition (16h light/8h dark) for five days. They were incubated in different concentrations of DMSO or EtOH

prepared in 0.5x MS for two hours prior to confocal microscopy analysis. ABD2-GFP were excited by 488 nm emitted by Argon laser and emission were collected between 500 and 550 nm.

2.3.4 Plasmolysis Experiments

Five-day-old seedlings grown in the light condition were stained with 4 μM FM4-64 for 10 min. Root cells were plasmolyzed by treating with 800 mM of mannitol for 10 min to visualize the properties of the PM surface. The seedlings were then incubated in different concentrations of DMSO or EtOH for one hour. An FM4-64 stained membrane was observed under confocal microscopy with the setting described above.

2.3.5 Statistical Analysis

All numerical data obtained here were analyzed and tested with appropriate statistical methods. Tukey's HSD (honestly significant difference) was applied to test a level of significance at $p < 0.05$ using R software (R for Mac OS X Cocoa, http://www.R-project.org).

2.4 Results and Discussion

2.4.1 Root Growth

First, the study assessed the effects of 0.5x MS media containing DMSO or EtOH on the root growth. As Fig. 2-1A and B show, 0.01% (v/v) DMSO and 0.01% (v/v) EtOH inhibited root growth, and significant inhibition of growth was observed when EtOH reached 1% of concentration. Interestingly, a low concentration of DMSO and EtOH (both at 0.01%) showed inhibitory effect to roots only when grown in the light condition (normal growth situation in plant laboratories). This finding is consistent with the author's previous report that *Arabidopsis* roots change their response to stress situations when roots are under light stress (Yokawa et al., 2014).

2.4.2 Endo/Exocytic Vesicle Recycling

Since DMSO and EtOH have been reported to affect membrane structure and rigidity, this study assessed endocytic vesicle recycling in the presence of DMSO or EtOH in root epidermal cells.

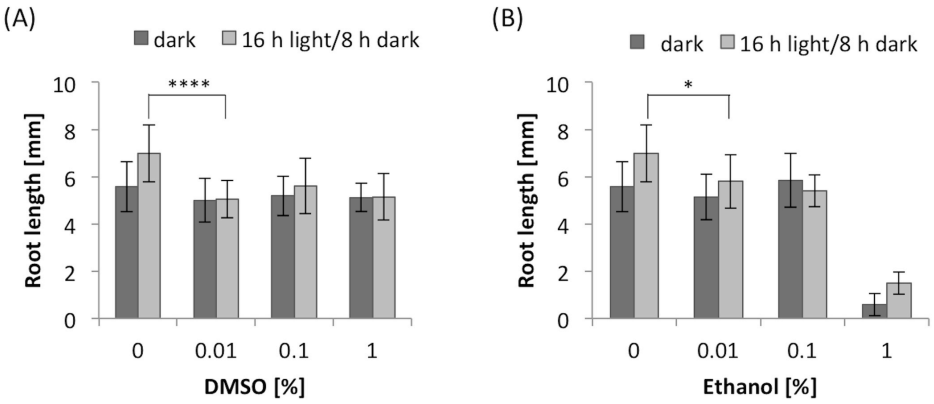

Fig. 2-1. Root lengths are affected by DMSO or EtOH. (A) Length of four-day-old roots on 0, 0.01, 0.1 or 1% concentration of (A) DMSO or (B) EtOH. Error bars indicate the standard deviation of the mean (SD) (n = 20-28). Statistical significance was determined by Student t-test: * $P < 0.05$, *** $P < 0.0001$.

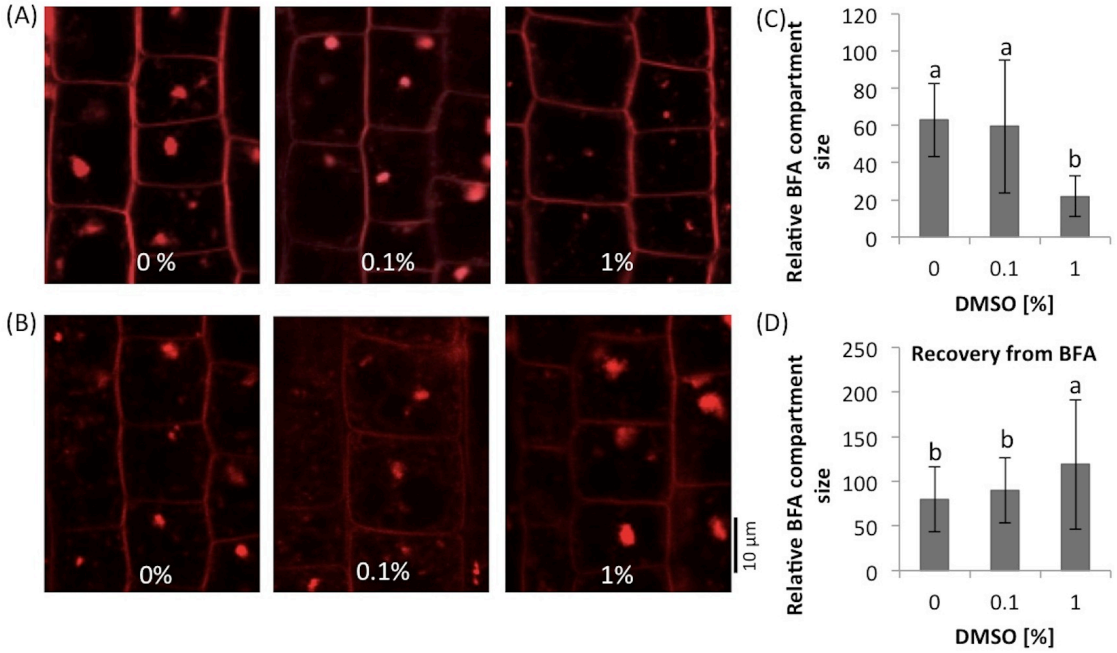

Fig. 2-2. BFA-induced compartments in cells of the transition zone of seedlings grown under light regime and exposed to DMSO. Confocal pictures of BFA compartments after DMSO treatments. (B) Recovery from BFA after DMSO treatment. After being incubated in 0 or 1% DMSO for one hour. (C) Relative Sizes of five BFA compartment were measured in seven different seedlings (n = 35). Error bars indicate SD. Different letters (a, b) in the graphs indicate significant difference (Tukey's HSD test, $P < 0.05$). (D) Relative BFA compartment size of recovery from BFA. Sizes of five BFA compartments were measured from five different seedlings (n = 25). Error bars indicate SD. Different letters in the graphs indicate a significant difference (Tukey's HSD test, $P < 0.05$).

Small BFA-induced compartments were observed when roots were treated with 1% DMSO (Fig. 2-2A and C). This indicates that DMSO slows endocytosis feeding into the endocytic vesicle recycling. Besides the rate of endocytosis, exocytosis or outward recycling (exocytosis *via* recycling endosomes) can also be monitored. The speed of the disappearance of BFA-induced compartments can be compared between control and DMSO-treated root cells. Roots first treated with BFA for 30 min were washed out with distilled water and then treated with a DMSO solution. Interestingly, the 1% DMSO treatment increased the speed of outward recycling *via* the exocytic pathway (Fig. 2-2B and D). Here 1% DMSO was shown to affect both endo- and exocytic pathways.

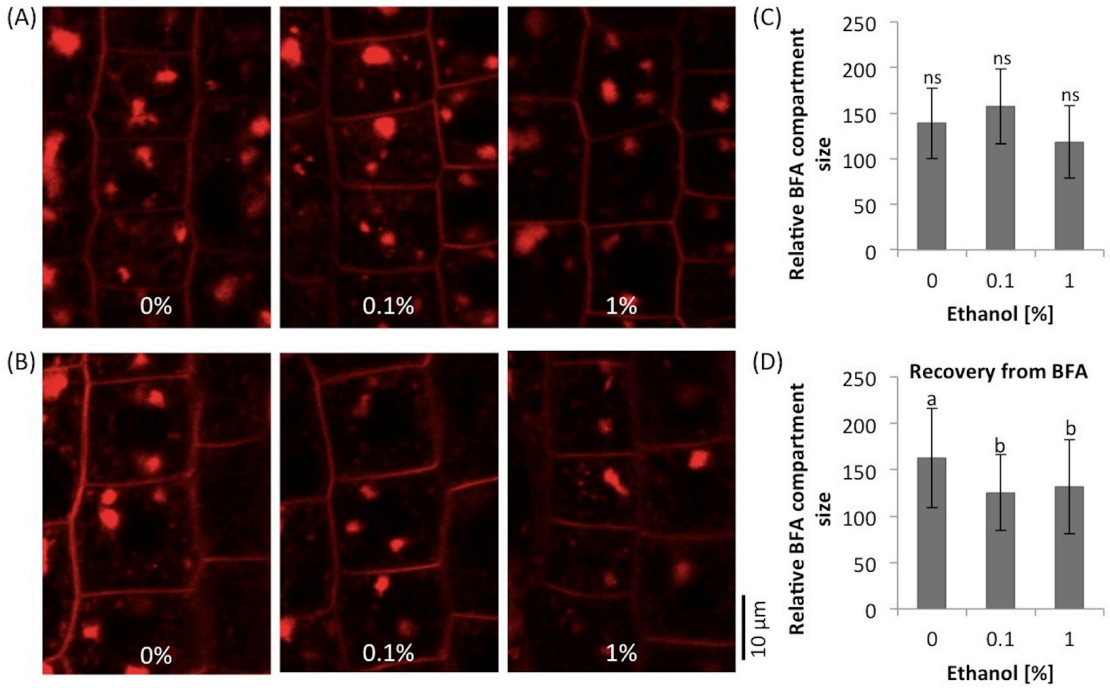

Fig. 2-3. BFA-induced compartments of root apex transition zone cells exposed to EtOH. (A) Confocal images of BFA-induced compartments after EtOH treatments. (B) Recovery from BFA exposures in light-grown seedlings. (C) The sizes of five BFA-induced compartment were measured in seven seedlings. Error bars indicate standard deviations from the mean (n = 35). (D) Reduction of BFA compartment sizes after recovery. Five BFA-induced compartment sizes were measured in six seedlings (n = 30). Error bars indicate SD. "ns" in the graphs indicates no significant difference and different letters (a, b) indicate a significant difference (Tukey's HSD test, $P < 0.05$).

Similarly, the impact of EtOH on vesicle recycling was assessed. As shown in Fig. 2-3A and C, both 0.1 and 1% of EtOH had no effect on endocytosis (Fig. 2-3B and D). In mammalian studies, 5 mM (*ca.* 0.29%) EtOH regulates endosomal recycling of dopamine transporters in

HEK-293 cells (Methner and Mayfield, 2010). This endosomal recycling is accomplished *via* BFA-insensitive pathways, because BFA does not modify dopamine receptor localization (Prou et al., 2001). However, EtOH slowed down the outward recycling *via* the exocytic pathway from the concentration of 0.1% (Fig. 2-3B and D). Taken together, in plants DMSO and EtOH likely modify BFA-sensitive signaling pathways. Moreover, it is intriguing that DMSO and EtOH affect recycling vesicle trafficking in different manners although both compounds are known to affect lipid membranes. Plants are known that produce EtOH endogenously through the catalytic reaction of their alcohol dehydrogenase, catalyzing a conversion from pyruvate to EtOH under anoxic stress condition (Johnson et al., 1994; Chung and Ferl, 1999). EtOH may be used as a stress-induced signaling molecule in plants, although further investigation of EtOH functions in plant cells is still necessary.

2.4.3 F-Actin Polarity

Since it has been reported that F-actin has an important role in endocytic vesicle recycling, the influence of DMSO and EtOH on F-actin localization in root epidermal cells was observed using an ABD2-GFP transgenic *Arabidopsis* line (Voigt et al., 2005). As the results in Fig. 2-4 show, the alignment of F-actin localized in the cross-wall (pointed with white arrow head in Fig. 2-4) disappeared in the presence of 0.1 and 1% EtOH, whereas DMSO showed no change. This result indicates that DMSO (1%) might inhibit endocytosis, as shown in Fig. 2-2, not *via* modulation of F-actin polarity, but possibly by interfering with membrane rigidity, as it has been shown that latrunclin B (inhibitor of actin polymerization) inhibits endocytosis in maize root cells (Baluška et al., 2002). It has been reported that F-actin distribution is affected by 2% DMSO in hepatocytes cells (Yamamoto, 1989). Örvar et al. demonstrated that 3% DMSO changes the membrane rigidification and F-actin organization *via* the increasing of $[Ca^{2+}]_{cyt}$ (Örvar et al., 2000). In the root apex transition zone, cross-walls (end-poles) connecting two adjacent cells longitudinally within cell files have been shown to play important roles for maintaining root polarity (Baluška et al., 2003; Lindsey, 2009). In the root apex transition region, the presence of abundant F-actin and active endocytic vesicle recycling was reported (Baluška et al., 2001; Ottenschläger et al., 2003; Baluška et al., 2003). In mice cells, actin cytoskeleton is both a target and effector of EtOH-mediated impacts on synapses through the disturbance of N-methyl-D-aspartate (NMDA) receptor signaling (Offenhäuser et al., 2006; Adler, 2006; Sordella, 2006). The plant-specific glutamate-like receptors (GLRs), expressed in plant cells (Weiland et al., 2016), may mediate the EtOH affecting vesicle recycling in a similar

way. However, further studies are required in this respect. The depletion of cross-wall localized F-actin found here in the EtOH-treated root apex cells might have an impact on disturbing exocytosis, endocytosis, root growth and polarity, as well as cellular physiological activities in general.

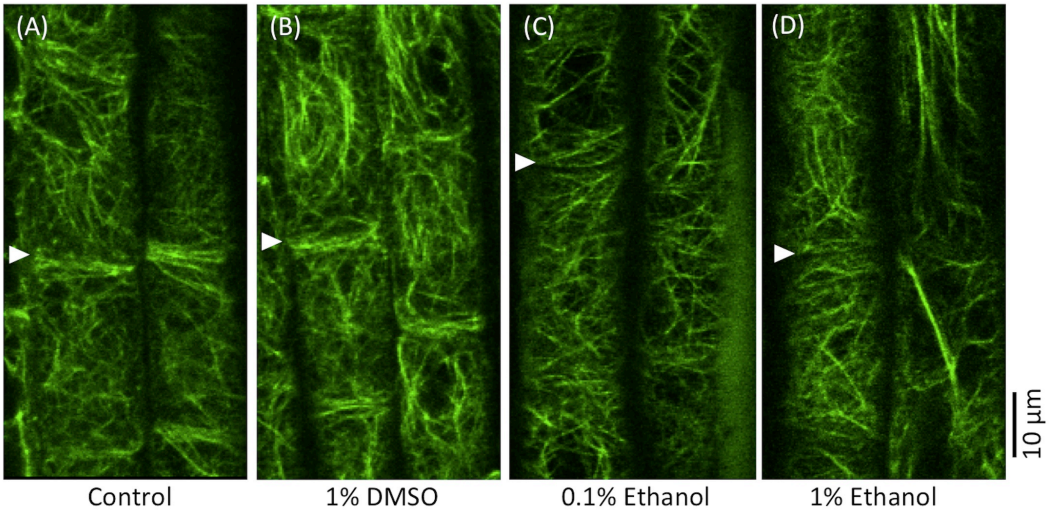

Fig. 2-4. Visualization of F-actin in epidermal cells in the root elongation zone. Five-day-old light-grown seedlings were incubated in (A) 0.5x MS, (B) 1% DMSO in 0.5x MS, (C) 0.1% EtOH in 0.5x MS, or (D) 1% EtOH for two hours. Arrowheads indicate the position of the cross-walls. Five replications produced similar results. A representative example was presented for each condition.

2.4.4 Membrane Properties

As shown, the endo/exocytic vesicle recycling is modulated by DMSO or EtOH; this is due to the alteration of membrane properties in root cells. As described in the introduction, it has been reported that both compounds directly change membrane mechanical conditions (Winckler et al., 1999; Notman et al., 2007; Cheng et al., 2015). Plasmolyzed plant cells are often used to visualize the membrane shape or membrane adhesion to the cell walls. Here, root epidermal cells were stained with an FM4-64 endocytic tracer, and subsequently plasmolyzed *via* 800 mM of mannitol treatment. As the results show, a distortion of the PM shape was observed in the presence of DMSO or EtOH. Fig. 2-5 shows the images taken with the confocal microscope. Interestingly, a shrunken shape of cells was found in DMSO-treated roots compared to in the control. On the other hand, EtOH-treated cells showed a swollen shape. These results suggest that these two compounds probably alter membrane conditions through different actions, and that the PM cannot maintain its structure and form in a hypertonic solution. The imbalance of

endo/exocytic vesicle recycling rate in the presence of either DMSO or EtOH (Figs. 2-2 and 2-3) might be caused *via* the alterations of membrane rigidity. It was reported that the endocytic recycling activity depends on membrane rigidity (Nakayama et al., 2012). Moreover, DMSO induces $[Ca^{2+}]_{cyt}$ elevation, which activates stress response genes, with 1% DMSO in alfalfa cells (Örvar et al., 2000), with 2% DMSO in *Brassica napus* cells (Sangwan et al., 2001), and with 2% DMSO in *Arabidopsis* (Whalley et al., 2011). $[Ca^{2+}]_{cyt}$ elevation with DMSO might also be involved in the activity of endocytic recycling because it has been indicated that $[Ca^{2+}]_{cyt}$ controls endocytosis (Steer, 1988). As the previous studies reported, DMSO perturbs lipid membrane structure, forming a water network on the surface (Gurtovenko and Anwar, 2007; Cheng et al, 2015). Moreover, DMSO also perforates membranes (Fernandez and Reigada, 2014). de Menorval et al. demonstrated in their *in silico* experiments that a low dose of DMSO merely undulated the lipid layer, and intermediate concentrations increased membrane permeability to water and calcium (de Menorval et al., 2012). In a yeast study, it was reported that a DMSO treatment facilitated phospholipid biosynthesis and membrane proliferation (Murata et al., 2003). The concentrations of DMSO and EtOH tested here are broadly accepted and essential in the preparation of many types of reagents as solvents for many biological experiments. Furthermore, for the regulation of EtOH-inducible genes, the optimal concentrations of EtOH are 1% (v/v) (Caddick et al., 1998) and 2% (Roslan et al., 2001) for *Arabidopsis*, or 0.5% to 2% for *Populus* (Filichkin et al., 2006). At these EtOH concentrations, membrane-associated cellular events and F-actin are affected as documented in the present study. It has been reported that *Arabidopsis* roots up-regulate the alcohol dehydrogenase gene (ADH) in anoxic or hypoxic situation (Johnson et al., 1994; Chung and Ferl, 1999). Since ADH in plants is known to catalyze the production of EtOH under hypoxic conditions, great caution must be taken when *Arabidopsis* roots are grown in hypoxic circumstances such as phytagel-solidified media. Otherwise, ADH-generated endogenous EtOH in root cells might disrupt normal cellular events. It has been demonstrated that DMSO and EtOH, at concentrations known to be experimentally effective, modify membrane properties affecting endocytic vesicle recycling and cell polarity in root apex cells. The detailed explanation of interactions between DMSO/EtOH-modified membranes, vesicle recycling and F-actin organization requires further experimental studies.

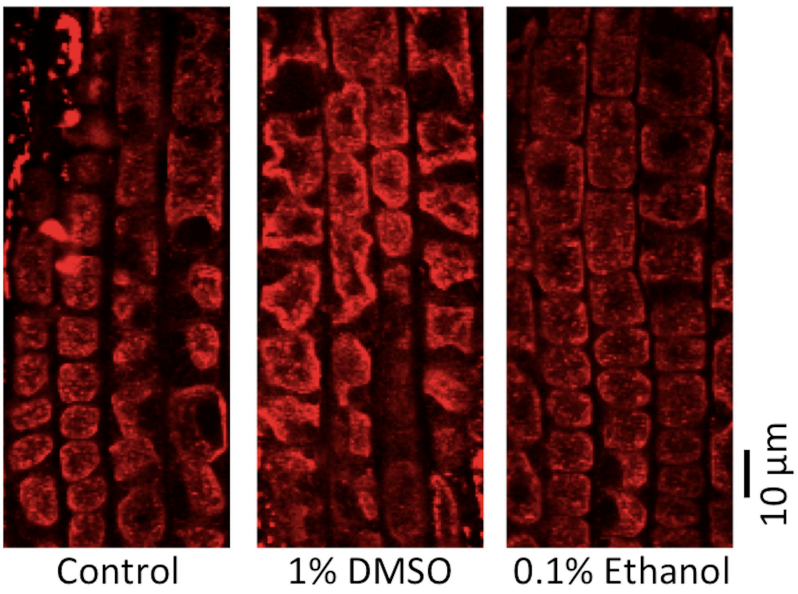

Fig. 2-5. Confocal pictures of plasmolysis with 800 mM mannitol in epidermal cells in the root transition zone. Five replications gave similar results. A representative example is presented for each condition.

Chapter 3

MES Buffer Affects *Arabidopsis* Root Apex Zonation and Root Growth by Suppressing Superoxide Generation in Root Apex

3.1 Abstract

In plants, growth of roots and root hairs is regulated by the fine cellular control of pH and reactive oxygen species. MES, 2-(N-morpholino)ethanesulfonic acid as one of the Good's buffers has broadly been used for buffering medium, because the buffer capacity of MES ranging pH 5.5-7.0 (for *Arabidopsis*, pH 5.8) and it is thought to suit for plant growth with the concentration at 0.1% (w/v). However, in nature, roots require different pH values on the surface of specific root apex zones, namely meristem, transition zone and elongation zone. Despite the fact that roots always grow on a media containing buffer molecules, little is known about impact of MES in culture on root growth. Here, I have checked the effects of different concentrations of MES buffer using roots of *Arabidopsis thaliana*. My results show that 1% of MES significantly inhibited root growth, the number of root hairs and length of meristem, whereas 0.1% promoted root growth and root apex area (region spanning from the root tip up to the transition zone). Furthermore, superoxide generation in root apex disappeared at 1% of MES. These results suggest that MES disturbs normal root morphogenesis by changing the reactive oxygen species (ROS) homeostasis in root apex.

3.2 Introduction

Good et al (1966) selected and reported buffers with less toxicity and less reactivity to biological compounds. Since then, these buffers were introduced to enormous amount of laboratory-based experiments. Since eighties of the last century, many studies using plant

hydroponic culture have been reporting the availability of MES molecules for buffering pH in liquid culture media. Imsande and Ralston (1981) demonstrated that 1-2 mM of MES solution has an excellent buffering capacity and it shows neither inhibition of nodulation nor lowering of nitrogen fixation in soybean hydroponic culture. Bugbee and Salisbury (1985) reported that MES did not significantly decrease growth as measured by seven growth parameters. 5 mM of MES did not leave impact on growth or uptake of most nutrients. Potassium uptake was even enhanced by the MES buffer in non-nodulated seedlings of soybean (Schuttler, 1987).

Meanwhile, some studies reported considerable problems caused by a chemical reaction between buffer molecules and other compounds in the media. In the presence of high concentrations of MES and PIPES, the polymerization of purified tubulin was observed using electron microscope (Waxman et al., 1981). For plant experiments, MES was reported to lower nitrogen fixation and plant growth in white clover (Rys and Phung, 1985). Medeiros et al. (1993) reported that 2 mM MES in hydroponic culture reduced shoot and root dry matter yields in maize (*Zea mays* L.) and increased accumulation of N, Ca, Mg, Mn and Zn in shoot. Miyasaka et al. (1988) reported that Mg, Mn and Zn uptake was decreased in winter wheat. MES was reported to perturb normal growth of cucumber in hydroponic condition, because MES oxidizes Mn^{2+} to Mn^{3+} and precipitates it from the nutrient solution, and thus nutrient uptake was inhibited due to this direct chemical reaction (Stahl et al., 1999). Importantly, 4 mM MES cannot maintain pH value for long period in hydroponic condition. The pH was decreased from 6.5 to 4.0 within 5 days (Nicholas and Harpera, 1993). It was also reported that MES inhibits the adventitious root formation from apple stem disks at 10 mM of concentration (de Klerk et al., 2008).

In addition to compounds prepared in media such as nutrients, there are numerous biomolecules in living organisms which interact with these buffers. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered as important primary signaling molecules playing a role in the propagation of cellular physiological information. As an interaction with RNS, Lomonosova et al. (1998) found out the production of hydrogen peroxide in the presence of peroxynitrite ($ONOO^-$; one of RNS) and HEPES buffer. HEPES was also shown to consume superoxide (one of ROS) and nitric oxide (NO) (Keynes et al., 2003). Very intriguingly, the combination of HEPES and riboflavin, often added as vitamin, in the culture medium drastically reduced NO content under the laboratory light environment (Keynes et al., 2003). Light-activated riboflavin accelerated the reaction mediated by HEPES (Keynes et al., 2003). In fact, piperazine ring-based buffers such as HEPES and PIPES have the ability to form radicals

(Grady et al., 1988). These findings suggest that such buffers potentially interfere with cellular ROS/RNS homeostasis. In this respect, a biochemical study demonstrated that a normal peroxidase activity for oxidizing phenolics strongly interfered with the presence of MES at concentrations of 5 mM in the solution, due to the replacement of target substrate by MES molecule (Baker et al., 2007).

Surprisingly, 2.5-10 mM (*ca*. 0.05-0.2% w/v) of MES has been used as a proper concentration for culture media since *Arabidopsis* has been introduced as model plant several decades ago. It implies that plant peroxidase, one of main functions is to compose cell wall, might be affected as roots always contact to buffer-containing media. Roots require precise control of pH value and ROS homeostasis for their normal morphogenesis in different specific zones (namely; root tip, meristematic zone, transition zone and elongation zone). Therefore, the effect of buffer, which potentially modifies the culture environments, must be assessed. Here, I therefore investigated the followings using roots of *Arabidopsis* seedlings, (1) MES enhanced the root length as well as the number of root hairs at 0.01% (w/v) and 0.1%, whereas 1% inhibited, (2) 0.1% MES enhanced the root waving phenotype, (3) 0.1% MES promoted the enlargement of meristematic zone, (4) 1% MES depleted apical root meristems. Less superoxide accumulation at the root apices was found in the MES-exposed roots when compared to the control roots.

3.3 Materials and Methods

3.3.1 Plant Growth Condition

Seeds of *Arabidopsis thaliana* were sterilized by 2% sodium hypochlorite (NaClO, ROTH, Karlsruhe, Germany) containing 0.1% Triton-X (ROTH, Karlsruhe, Germany) for 5 min. These seeds were washed in water for 4 times. Seeds were planted on 0.5x MS media (Duchefa, Haarlem, The Netherlands) containing 1% (w/v) sucrose (pH5.8 with KOH) solidified with 0.4% (w/v) phytagel (Sigma, Steinheim, Germany). Each concentration of MES (Duchefa, Haarlem, The Netherlands) was added to 0.5x MS media before autoclaved. The petri dishes were incubated at 4˚C for 1 day for imbibition and were put vertically at 23-25˚C in 16 h light / 8 h dark (light intensity: ~120 μmol/s/m2, humidity: ~50%). At the time point of day 3 and day 6, pictures of the seedlings were taken with EOS Kiss X7 (Canon, Tokyo). Root lengths were measured by ImageJ software (ver. 1.43u for Mac OSX, http://imagej.nih.gov/ij/). The number of waves were counted and compared in 6 day-old roots grown in different MES concentrations.

3.3.2 Microscopic Observation

Images of root hairs were taken through 0.8x objective lens of a stereomicroscope Leica MZFLIII (Solms, Germany). Images of root apices were taken through 10x objective lens of a light microscope Leica DM750 (Solms, Germany). Root hairs and distances from root tip to the root transition zone were measured by ImageJ software.

3.3.3 Histochemical NBT Staining for Superoxide Detection

To detect the presence of superoxide in root apex grown in different concentrations of MES, 6 day-old seedlings were incubated for 5 minutes in the staining solution of 300 μM nitroblue tetrazolium salt (NBT) (Fluka, Germany) dissolved in 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$ (pH = 9.5). Seedlings were then observed and imaged under a stereomicroscope.

3.3.4 Statistical Analysis

All numerical data obtained here were analyzed and tested in appropriate statistical methods. Tukey's HSD (honestly significant difference) was applied to test a level of significance at $P < 0.05$ using R software (R for Mac OS X Cocoa, http://www.R-project.org).

3.4 Results

3.4.1 Effect of MES Buffer on Root Growth

Arabidopsis Col-0 (wild type) seeds were germinated and grown on the media containing four different concentrations of MES (0, 0.01, 0.1, and 1%). The MES-containing media at this range of concentration showed no effect on seed germination (data not shown). The root was then measured at the time point of 3 and 6 days after germination.

Three days after germination, the average of root length showed significant differences between 0, 0.01, 0.1, and 1% MES treatments. As Fig. 3-1B shows, 0.01% and 0.1% of MES promoted the growth whereas 1% of concentration inhibited. Similarly, 6 days after germination, both 0.01% and 0.1% of MES still promoted the root growth (Fig. 3-1A and C). These data indicate low MES concentration (in the range from 0.01 to 0.1%) enhances root growth from early developmental stage of seedlings, however, 1% MES inhibited. In addition to the growth, I observed the effect of MES on root growth behavior, so-called waving phenotype, which is

known to reflect a root tropic growth affected by several physical factors such as gravity, light, touch etc. (Okada and Shimura, 1990; Simmons et al., 1995). Therefore, the frequency of root waving on each MES containing plate was also assessed. In the result, waving frequency was enhanced as MES concentration increased (Fig. 3-1A and D).

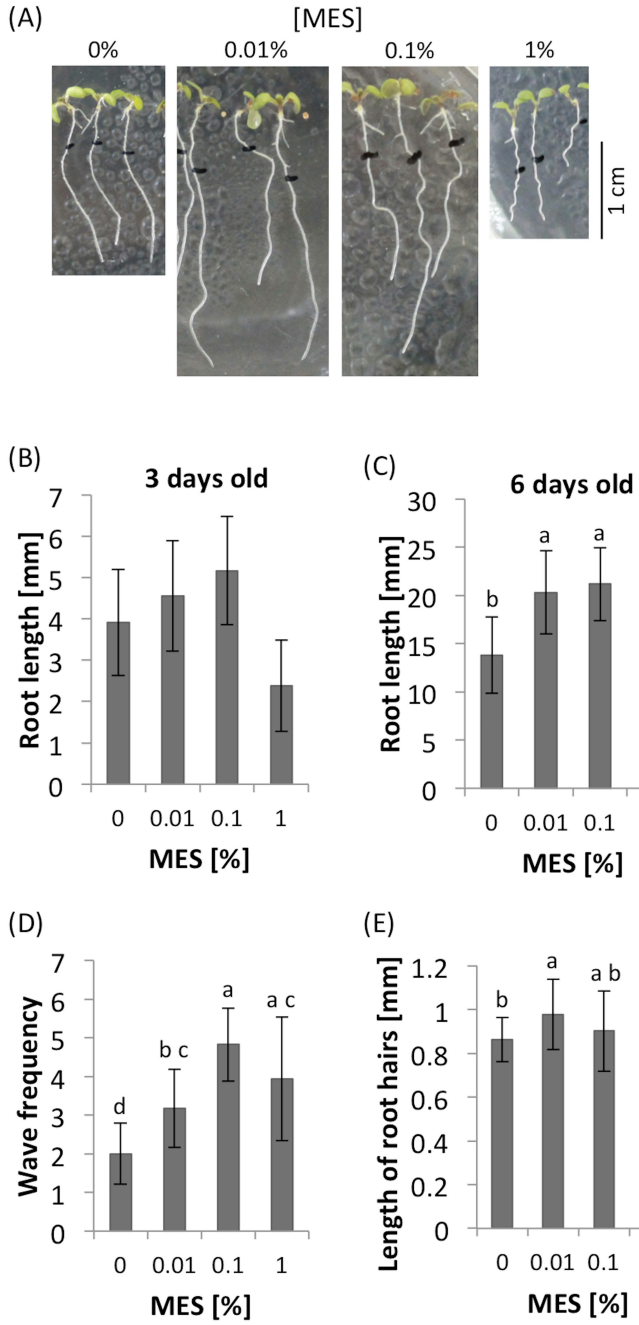

Fig. 3-1. Root growth and morphology of different concentrations of MES. (A) Appearances of seedlings at day 6. The seeds were germinated on 0.5x MS medium containing each concentration of MES. Black marks indicate positions of root tip at day 3. (B) Root length at day 3 after germination on each concentration of MES. Error bars indicate standard deviation of the mean (SD) (n = 62-72). (C) Root length at day 6 after germination on each concentration of MES. Error bars indicate SD (n = 34-65). Different letters (a-c) in the graphs indicate significant difference (Tukey's HSD test, P < 0.05). (D) The number of waves of the roots. Error bars indicate SD (n = 17). Different letters (a-d) in the graphs indicate significant difference (Tukey's HSD test, P < 0.05). (E) Root hair formation affected by MES. Lengths of root hairs were measured at position where longer root hairs emerge, because the distance from the tip varies under different MES treatments. Five root hairs were measured from 5 seedlings. Error bars indicate SD (n = 25). Different letters (a-d) indicate significant difference (Tukey's HSD test, P < 0.05).

As we described in the introduction part, pH and ROS have an important role for root growth. The formation of root hairs also requires fine control of those parameters. Here the impacts of MES on root hairs in 6 days after germination seedlings were compared. As Fig. 3-1E shows, 1% of MES drastically inhibited normal root hair formation. Lengths of five root hairs from five different roots (n = 25) in each treatment were measured and averaged. In the result, MES increased the length of root hairs when it is at 0.01% ($p < 0.05$) compared to control, but not at 0.1%. Interestingly, 1% MES strongly suppresses the formation (Fig. 3-1E). The total number of root hairs did not show significant differences among four MES concentrations (data not shown).

3.4.2 Root Morphology in Apex Region in the Presence of MES

As I observed, the presence of MES changed the root growth and its tropic behavior indicated by waving growth. Next, I have focused on root apex region in roots grown in different MES concentrations. This region is known to play an important role for polar auxin transport, which controls root tropic behavior, and it is most sensitive part of roots to external environment. Fig. 3-2A shows a scheme of a root apex. Cells with asterisks indicate the typical cells in the transition zone. In this study, I have defined the border between transition zone and elongation zone as following morphological category: the cell lengths obtain values which are two times longer as the cell widths (Baluška et al., 2010). Arrowheads in Fig. 3-2B indicate positions of the border between transition zone and elongation zone. A dashed horizontal line indicates the position of root tips. As depicted in Fig. 3-2B and C, the treatment of MES resulted in altering the size of root apex region including transition zone. MES at 0.1% concentration significantly enlarged the size of the area, whereas 1% decreased. I observed that only the number of cells in root apex region (from the root tip to the elongation zone) was affected by MES exposure, not cell size, namely 10-15 μm of cell length (growth direction) in the region was observed in all MES treatments. Interestingly, the abnormality in cell shape in root apex was observed in 1% MES growth condition (also shown Fig. 3-3). It suggests that high MES condition might interfere with the overall root morphogenesis similarly to root hair formation (Fig. 3-1E)

3.4.3 Superoxide Localization in Root Apex

As I have described in the introduction part, MES was reported to interact or interfere with biological redox machineries. Many studies have already revealed that proper control of redox homeostasis or ROS signaling are ultimately essential for root growth and root tropisms. Here I

tried to detect the distribution of superoxide in roots of *Arabidopsis* using a histochemical staining method, nitroblue tetrazolium (NBT) staining. When NBT compound reacts with superoxide, formazan visualized as blue precipitation in cells is immediately formed. Superoxide is known as one of ROS existing in apex region, which controls cell proliferation (Tsukagoshi et al., 2010). As shown in Fig. 3-3, NBT staining pattern in root apex region was observed in control, 0.01% and 0.1% MES condition. However, it completely disappeared in the 1% MES growth condition. It means that superoxide required for normal root growth in apex region is continuously diminished by 1% MES in growth media during culture on a plate for 6 days.

3.5 Discussion

3.5.1 MES Effect to pH and ROS Homeostasis

Because of the pKa value in acidic region, MES compound has been used for pH buffer by adding to plant culture media in terms of nutrient uptake. In *Arabidopsis* research, MES was also introduced to buffer pH value in agar-solidified plate culture. In this study, I have demonstrated the effects of MES on *Arabidopsis* root growth, morphogenesis and tropic behavior. Furthermore, staining superoxide in root apex indicated that MES strongly interferes with ROS homeostasis at 1% of MES, but not at 0.1%, which has an important role for root growth.

Foreman et al. (2003) demonstrated that superoxide produced by plasma membrane-associated NADPH oxidase is necessary for intracellular Ca^{2+} elevation leading to root hair formation and cell expansion. The treatment of diphenylene iodonium (DPI), inhibitor for flavo-proteins including NADPH oxidase, resulted in suppressing ROS accumulation and lacking of root hairs (phenocopy of *rhd2*-4 mutants; Foreman et al., 2003). The inhibitory effect of 1% of MES on root growth and root hair formation can be concluded as the result of disruption of superoxide production (Fig. 3-3).

Besides ROS homeostasis, pH is also important factor for cell expansion and root growth regulation. It was already reported that root hair formation requires acidification of cell wall (Bibikova et al., 1998), interplay between extracellular pH and ROS production (Monshausen et al., 2007). Therefore, the interruption of root hair initiation observed here might be interpreted as a high buffering capacity of MES at 1% concentration in the phytagel media. On the other

hand, 0.01% and 0.1% MES treatment significantly enhanced root growth (Fig. 3-1A-C), and 0.1% MES enhanced root hair length (Fig. 3-1E) compared to control conditions. This is probably because of modest buffering ability at this range of concentrations (0.01-0.1%) support continuous acidification required for root hair tip growth as well as for the root growth.

3.5.2 MES Impacts on Transition Zone and Tropism of Roots

In this study, I found that 0.1% MES increased the area of apex region including from root tip to the border between transition zone and elongation zone (Fig. 3-2). With stereomicroscope, the increase of cell numbers in this root apex region was observed. Transition zone of the root apex plays an important role for all root tropic behaviors based on polar auxin transport, which is accomplished *via* endocytic vesicle recycling as found in cells of this region (Baluška et al., 2010). As I have observed, the enlargement of the region in the presence of 0.1% MES (Fig. 3-2) enhances waving phenotype of growing roots (Fig. 3-1D). This root waving phenotype is known as a result of root tropic response of physical contact to agar surface (Okada and Shimura, 1990; Simmons et al., 1995). It was also reported that this phenotype became stronger if roots grow in light condition (Okada and Shimura, 1990). In addition, root growth pattern (waving and skewing) was also reported to change by the hardness of agar medium. 1.5% agar medium show stronger slanted-growth than 0.8% or 3% (Rutherford and Masson, 1996). Huang et al. (1995) showed that the rigidity of phytagel was altered by pH or the concentrations of nutrient components in media. Since root growth patterns depend on environmental factors (e.g., light and gravity) as well as physical contacts to media, high buffer contents are possibly changing physical properties of agar, affecting root growth on solidified plates. Very interestingly, the size of root apex correlates with the waving frequency (Fig. 3-1D), suggesting that the MES-induced enlargement of this root apex region results in changes of root tropic behavior.

Tsukagoshi et al. (2010) reported that UPBEAT1, a transcription factor, regulates the expression of peroxidase in root apex, determining the borderline between transition zone and elongation zone. There are two different ROS types present in these two root apex zones playing distinct roles, hydrogen peroxide in elongation zone and superoxide in transition zone is for cell differentiation and is for cell proliferation, respectively. In response to environmental conditions, roots regulate mode of growth by modulating the position of this borderline *via* UPBEAT1 regulation.

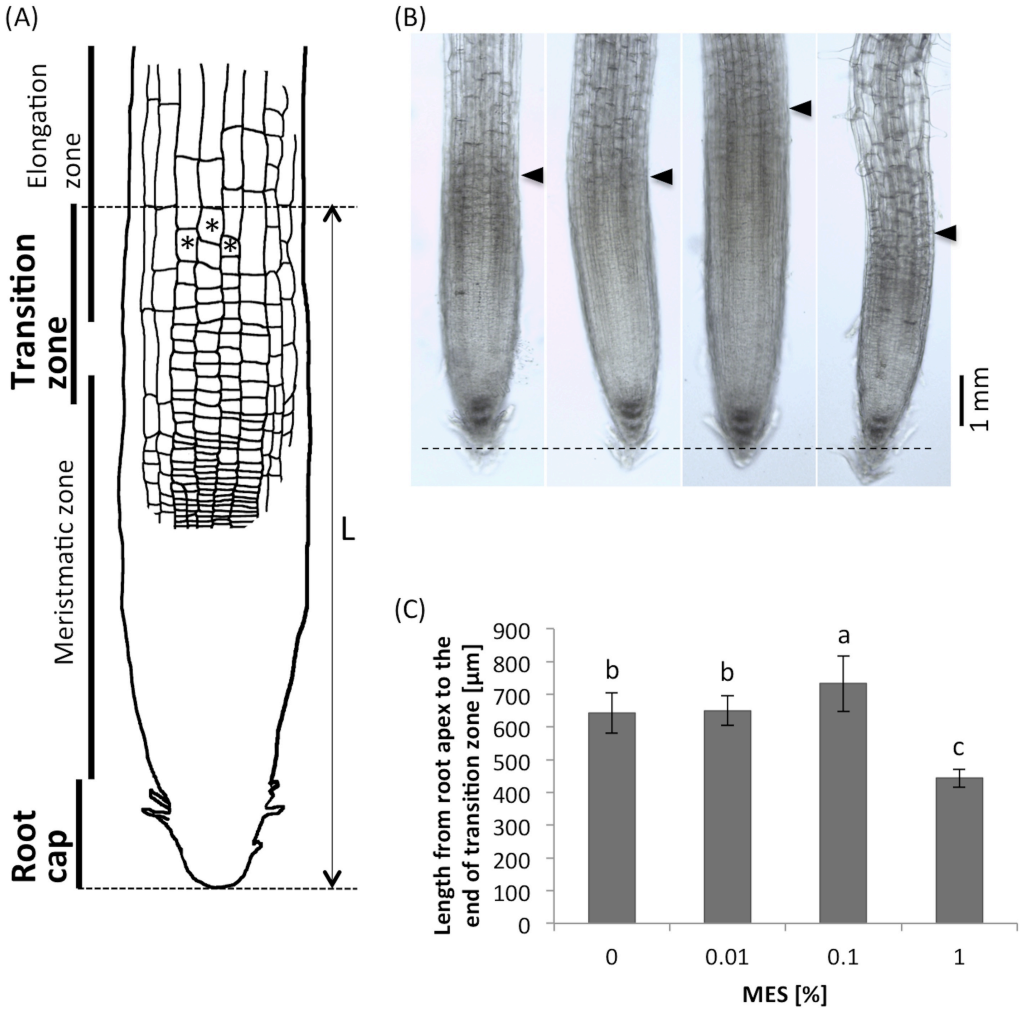

Fig. 3-2. Comparison of the root apex length. (A) A scheme of the root apex. Cells with asterisks indicate the typical cells in transition zone. In this study, we define the border between transition zone and elongation zone as following morphological category: the cell lengths obtain values which are two times longer as the cell widths (Baluška et al., 2010). (B) Microscope images of the root apices. Arrowheads indicate positions of the end of transition zone. A dashed horizontal line indicates the position of the root tip. (C) Lengths of the root apices (from the root tip to the end of the transition zone). Error bars indicate standard deviations of the mean (n = 9-11). Different letters (a-c) indicate significant difference (Tukey's HSD test, $P < 0.05$).

Possible reasons for MES-altered the area of transition zone can be considered as follows. (1) Indirect effect due to MES buffering ability. Similar to ROS in roots, pH must also be

controlled in different zones in roots (He et al., 2015). Therefore, roots changed the morphology as a result of an attempt to recover pH homeostasis or escape from such a situation. (2) Direct interaction of MES with peroxidases. It was already reported in a biochemical study as aforementioned. Because of the molecular structure of MES, it interferes with peroxidase activities oxidizing phenolic compounds (Baker et al., 2007). Peroxidases in root apex region might be affected in the presence of MES. It was reported that the appropriate control of ROS homeostasis by cell wall peroxidase is essential to regulate root cell elongation (Liszkay et al., 2004). As Fig. 3-3 shows, since superoxide in transition zone was completely disappeared at 1% concentration, it is likely that MES disturbs ROS-generating pathway, possibly *via* enzymes (peroxidases) or direct scavenging. A schematic summary is shown in Fig. 3-4.

3.5.3 MES for Laboratory-Based Experiments

In addition to extracellular interaction of MES and biological compounds, it is likely that MES compound is also taken up by roots and transported to other tissues in plant body such as leaves. This suggests that incorporated MES molecule in cellular space might cause lasting reactions interfering with many extracellular and intracellular signaling molecules. For example, HEPES buffer, structurally analogous to MES, drastically consumes endogenous NO in the presence of riboflavin under laboratory light condition (Keynes et al., 2003). Culturing plants under light in a growth chamber needs great caution if growth media contain such buffers. Impacts of light on growing *Arabidopsis* roots have been reported recently. Under illuminated condition, roots alter their physiological conditions, growth rate and tropisms (Yokawa et al., 2011; 2013; 2014; Wan et al., 2012; Mo et al., 2015; Novák et al., 2015; Kagenishi et al., 2016). Importantly, roots are evolutionarily optimized to grow in darkness in nature. Light activates root photoreceptors, or probably other light-absorbing compounds in root cells. In this regard, light may even bring unexpected results through a reaction with buffer compounds inside or outside of root cells as already discussed above (Keynes et al., 2003). Moreover, as I have noted in the introduction part, 4 mM of MES (*ca*. 0.1%) could not maintain pH value for 5 days in liquid media. The pH was gradually decreased from 6.5 to 4.0. Potential problems with MES were pointed out previous study (Nicholas and Harpera, 1993). All this suggests that the long-term experiments (longer than a day), that require fine pH control in liquid or solidified media, must be interpreted with a great care.

Growth conditions, buffers, other supplemental compounds such as riboflavin (vitamins) have been chosen to grow *Arabidopsis* as a model plant for laboratory-based experiments, we

must take into account such artificial environments for further root research.

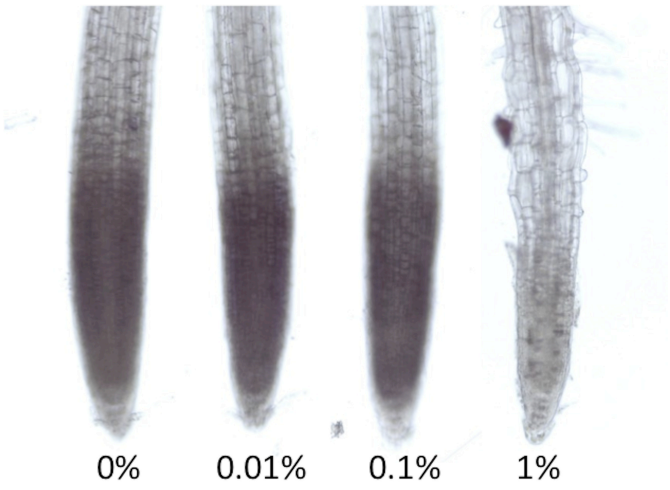

Fig. 3-3. Stereomicroscope pictures of NBT staining for superoxide detection. Seedlings were grown in different concentrations of MES for 6 days. These were incubated for 5 minutes in NBT. The representative picture is shown here (n = 5).

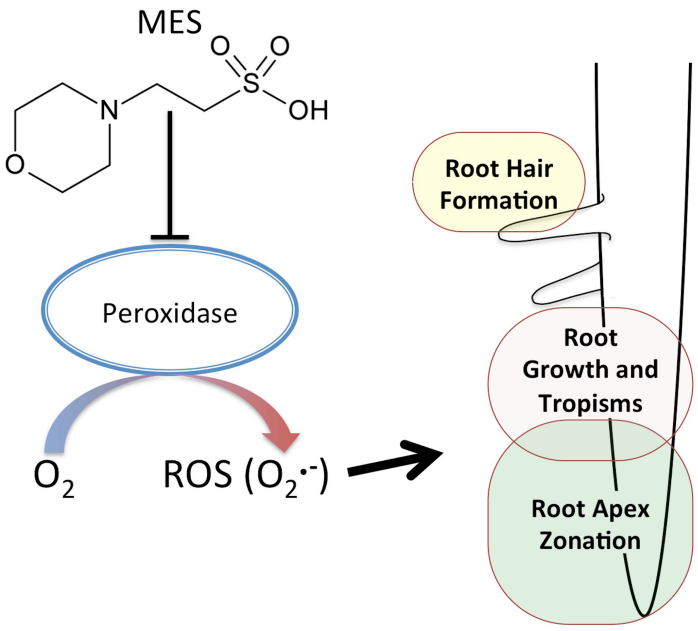

Fig. 3-4. A schematic diagram of MES effects on root growth. MES disturbs ROS-generating pathway in the root apex, possibly *via* enzymes (*e.g.* peroxidases), affecting the root apex zonation. The ROS ($O_2^{\bullet-}$) is involved in root hair formation, root growth and root tropisms.

Chapter 4

Extracellular ATP (eATP) Inhibits Endocytic Vesicle Recycling and Gravitropism of *Arabidopsis* Roots *via* NADPH oxidase-Mediated ROS Signaling

4.1 Abstract

Extracellular adenosine-5'-triphosphate (eATP) is known as a signaling molecule both in mammal and plant cells. In mammals, the study of eATP accelerated after a purinoceptor to eATP was cloned from the brain (Lustig et al., 1993). In contrast, the existence of the plant purinoceptor DORN was reported only very recently. Nevertheless, some studies have previously reported, even before the discovery of the eATP receptor, that eATP is involved in regulating plant growth and environmental adaptation. However, the processes behind the eATP signaling that modulates root growth and tropisms are largely unknown. The aim of the present study was to investigate the mechanisms of eATP signaling in the root apex of *Arabidopsis*. The results show that the endocytic vesicle recycling in root apex cells was modified by eATP. This study reports that the eATP-induced inhibition of root elongation and endocytic vesicle recycling requires reactive oxygen species (ROS) generated *via* NADPH oxidase C (AtRBOHC).

4.2 Introduction

Adenosine-5'-triphosphate (ATP) is known not only as an energy source for all living organisms, but also as an important signaling molecule both in mammal and plant cells (Choi et al., 2014). Burnstock (1972) reported the possibility that two types of receptors function to perceive ATP as a neurotransmitter in mammals, and two mammalian extracellular eATP

receptors, P2X (Valera et al., 1994) and P2Y (Webb et al., 1993), were later also identified. Afterwards, eATP signaling studies were accelerated. Based on the findings in mammalian cells, some pharmaceutical companies have developed a painkiller that targets purinoceptors or controls the metabolism of eATP.

Although it has been reported that plants do not have homologs of mammal purinoceptors (reviewed in Tanaka et al., 2010), the plant purinoceptor P2K (K for kinase) was recently found (Choi et al., 2014). P2K was identified by screening ethylmethane-sulfonate (EMS)-mutagenized *Arabidopsis* seedlings, and monitoring ATP-induced calcium responses. The identified P2K in the plant was named DORN1 (Does not Respond to Nucleotides1) (Choi et al., 2014). However, the effects of exogenous ATP on plants were often reported even before the discovery of the DORN1 receptor. For example, eATP enhanced curvatures of excised pea tendrils in the dark (Jaffe and Galston, 1966); Venus's flytrap closed faster when exposed to eATP than in control experiments (Jaffe, 1973); and eATP increased levels of nuclease and accelerated leaf senescence (Udvardy and Farkas, 1973). However, these researchers all assumed that the exogenously applied ATP acted as an energy resource of plant cells as it increased the speed of plant movement or growth. Meanwhile, Thomas et al. (1999) and Lew and Dearnaley (2000) suggested the existence of eATP produced by plants by reporting data from experiments using plant ectoapyrases (enzymes that can catalyze ATP or ADP). Later, apyrases were shown to play a key role in growth control (Wu et al., 2007), to regulate stomata apertures (Clark et al., 2011), and to control the polar auxin transport (Liu et al., 2012). Moreover, suppression of these apyrases raises levels of eATP and induces cell wall changes, implying the role of eATP in stress responses (Lim et al., 2014). Until now, a number of studies have demonstrated many roles of eATP in plant growth or tropisms. As is illustrated by Table 2, eATP has been shown to be involved in plant viability regulation, growth, development, stress responses, and adaptation to environmental challenges.

The release of eATP to extracellular space is generally detected based on the reaction of luciferin and luciferase, which require ATP to emit photons (chemiluminescence). Using this detection method, previous studies have shown that wounding, abiotic stress (Dark et al., 2011), biotic stress (Kim et al., 2006), and touch stress (Jeter et al., 2004; Weerasinghe et al., 2009) enhanced the release of eATP from plant cells. Thomas et al. (2000) proposed that an ATP-binding cassette (ABC) transporter might transport eATP into extracellular space. Interestingly, a high release of eATP was found in cells in the root apex transition zone caused by touch stimuli (Weerasinghe et al., 2009) or pathogen elicitors (Kim et al., 2006). The root

apex transition zone is recognized at root apices at approximately 200-520 μm from the root tip of *Arabidopsis* (Verbelen et al., 2006). This region acts as an important environmental sensor for light, gravity, and mineral nutrients. Cells in this root apex region have high activities of polar auxin transport across the cells, mediated through the high activities of the endocytic vesicle recycling (Baluška et al., 2010).

Table 2. eATP effect to plant.

ATP response in plant	Effective concentration	Plant	Reference
Abolishment of pollen germination	2 mM	*Arabidopsis thaliana*	Steinebrunner et al., (2003)
Decrease of pollen growth	150 μM (ATPγ)	*Arabidopsis thaliana*	Reichler et al., (2009)
Promotion of curvature of Excided tendril	1 mM	Excised pea (*Pisum sativum*)	Jaffe and Galston, (1966)
Enhancement of closing speed of trap	100 μM	Venus flytrap (Dionaea muscipula)	Jaffe, (1973)
Enhancement of stomata opening	5-20 mM Unknown 2 mM	*Commelina communis* *Arabidopsis thaliana* *Arabidopsis thaliana*	Nejidat et al., (1983) Clark et al., (2011) Hao et al., (2012)
Increase hypocotyle elongation	100-200 μM	*Arabidopsis thaliana*	Roux et al., (2006)
Cell viability of leaves	Unknown	*Pseudomonas syringae* pv. *tabaci*	Chivasa et al., (2005)
Reduction in hypocotyl elongation	0.4-1 mM	*Arabidopsis thaliana*	Roux et al., (2006)
Inhibition of Root growth	3 mM	*Arabidopsis thaliana*	Tang et al., (2003)
Enhancement of lateral root growth	3 mM	*Arabidopsis thaliana*	Tang et al., (2003)
Normal growth of Root hair	Unknown	*Medicago truncatula*	Kim et al., (2006)
Induce of cell death	500 μM	*Populus euphratica*	Sun et al., (2012)
Skewing of root	2 mM	*Arabidopsis thaliana*	Yang et al. (2015)

At the cellular level, it has been reported that eATP changes vesicular trafficking to repair the plasma membrane during cold stress (Deng et al., 2015). Moreover, several signaling pathways are necessary for the perception of eATP. It has been shown that reactive oxygen species (ROS), calcium (Ca^{2+}) and nitric oxide (NO) are important players for eATP signaling in plants. Both eATP and eADP have been reported to depolarize membrane potential in *Arabidopsis* root hairs (Lew and Dearnaley, 2000). Moreover, eATP induced the increase of cytosolic Ca^{2+} (Demidchik et al., 2003) through the generation of superoxide ($O_2^{\bullet-}$) *via* NADPH oxidase on the plasma membrane that activates the Ca^{2+} channel opening (Song et al., 2006; Jeter et al., 2004; Demidchik et al., 2009; Shang et al., 2009; Wang et al., 2014). The altered $[Ca^{2+}]_{cyt}$ by eATP promoted gene expression of mitogen-activated protein kinases (MAPK), which is known as a stress responsive factor (Jeter et al., 2004; Demidchik et al., 2009). eATP is also known as a regulator of the pathogenesis-related (*PR*) gene (Chivasa et al., 2009). $[Ca^{2+}]_{cyt}$ activated the generation of ROS controlled by calmodulin in response to environmental changes (Song et al., 2006). In addition, the eATP-dependent NO production was observed in *Arabidopsis* (Tonón et al., 2010), in tomato cell suspension (Foresi et al., 2007), and also in the hairy root of *Salvia miltiorrhiza* (Wu et al., 2008). It has also been suggested that eATP is involved in modulating extracellular pH control in neuronal cells, because of its nature as a phosphoric acid (Vroman et al, 2014).

The aim of the study presented in this chapter was to investigate how eATP affects endocytic vesicle recycling through ROS signaling in the root apex. Since eATP has a strong effect on root tropisms, it was postulated that primary signaling factor, ROS in response to eATP, might control vesicle recycling. In the study, the *Arabidopsis* loss-of-function mutant line, *rhd2-4* (*root hair defective 2-4*), which encodes NADPH oxidase C (*AtRBOHC*) and catalyzes the production of $O_2^{\bullet}$, was used, as RBOHC is known to be expressed in the root apex of *Arabidopsis*. In addition, the study analyzed the endocytic vesicle recycling of root apex cells in the *Arabidopsis* mutant lines of plant eATP receptor *dorn1-1* (pointed mutation of *DORN1*) and *oxDORN1* (ectopic expression line) roots.

The eATP-mediated inhibition of endocytosis was observed in eATP-treated Col-0 (wild type) root cells using brefeldin A (BFA; inhibitor of exocytosis) treatment. In contrast, the acceleration of exocytosis was observed with these eATP treatments. Damage of the cell membrane caused by eATP was observed by Evans blue staining. The results reveal that the transition zone cells showed the highest sensitivity to eATP in the Col-0 roots, while the *rhd2-4* roots were much less sensitive to eATP. In conclusion, eATP-induced inhibition of root

elongation and modulation of endo/exocytic vesicle recycling activity require ROS generated by NADPH oxidase C.

4.3 Materials and Methods

4.3.1 Plant Growth Condition

Seed of the *rhd2-4* mutant line were kindly provided by Professor Liam Dolan (University of Oxford, UK). Seeds of the *dorn1-1* and *oxDORN1* mutant lines were kindly provided by Professor Gary Stacey (University of Missouri, USA). Seeds of *Arabidopsis thaliana* were sterilized with 0.2% NaClO (ROTH, Karlsruhe) containing 0.1% Triton-X (ROTH, Karlsruhe) for five min. After washing them in water four times, the seeds were dried on filter papers. The seeds were then planted on 0.5x MS media (Duchefa, Haarlem) containing 0.1% MES, 1% (w/v) sucrose (pH 5.8 with KOH) solidified with 0.4% (w/v) phytagel (Sigma, Steinheim). The petri dishes were incubated at 4˚C for one day for seed imbibition. These were put vertically at 22˚C in the light (18h light/6h dark, light intensity: ~120 μmol/s/m^2, humidity: ~50%) or in the dark.

4.3.2 Long-Term Effects of eATP Exposure on Root Growth

Four-day-old dark-grown seedlings were transferred to a new medium containing different concentrations of ATP-disodium salt (Serva, Heidelberg), 0.5x MS, 1% (w/v) sucrose (pH 5.8 with KOH) solidified with 1% (w/v) agar. ATP was added to agar after autoclave to prevent it from degradation by heat. These petri dishes were incubated for four days in the light (16h light/8h dark) or in the dark. Images of root lengths were captured and measured by ImageJ software (ver. 1.43u for Mac OSX, http://imagej.nih.gov/ij/).

4.3.3 Short-Term Effects of eATP Exposure on Root Growth

ATP was solved in distilled water. Four-day-old dark-grown seedlings were immersed and treated with different concentrations of ATP solution for one min. After washing seedlings in distilled water, they were placed back onto the agar plate (0.5x MS, 0.1% MES, 1% (w/v) sucrose (pH5.8 with KOH) solidified with 0.8% (w/v) agar) and grown in the dark condition for five days. Lengths of roots were photographed and measured by ImageJ software.

4.3.4 BFA-Induced Compartments in Transition Zone Cells

Five-day-old seedlings were first treated with different concentrations of ATP in solutions. 4 μM FM4-64 (endocytic tracer, Sigma, Steinheim) stained roots for 10 min. FM4-64 was diluted and prepared with distilled water from 8 mM (2000x) stock solution dissolved in DMSO (Sigma, Steinheim). The seedlings were then incubated in 35 μM BFA for 30 min. BFA was prepared from 1000x stock solution in DMSO. Images were taken with a laser-scanning confocal microscope (Fluoview FV1000, Olympus, Tokyo).

FM 4-64 was excited by 515 nm and detected between 630 nm and 700 nm. The size of the BFA-induced compartments was manually measured on a computer screen with Image J software and calculations were made in Microsoft Excel.

4.3.5 Recovery from BFA-Induced Compartments After BFA Washout

Five-day-old seedlings were first incubated with 35 μM BFA for 30 min to generate a BFA-induced compartment, followed by a BFA washout and the treatment of 4 μM FM4-64 dye for 10 min. These BFA-pretreated seedlings were then soaked in different ATP solutions for one min. BFA compartment size in root epidermal cells in the transition zone was manually measured with the ImageJ software, and the speed at which the BFA-induced compartments diminished was analyzed using Microsoft Excel.

4.3.6 Influence of eATP on Root Cell Membranes

Etiolated five-day-old seedlings were soaked in different concentrations of ATP for 1min. These seedlings were stained with 0.0025% Evans blue reagent (dye for damaged plasma membrane) for 30 min. After washing them out with distilled water three times, the seedlings were incubated in distilled water for 15 min. Pictures of these roots were taken by microscopy and the relative areas of Evans blue stain were evaluated using ImageJ.

4.3.7 Effect of pH Values on BFA-Induced Compartments

Light-grown five-day-old seedlings were stained with FM4-64 for 15 min. The seedlings were incubated in 0.5x MS with different pH values (4.5, 5.8, 8.0 adjusted with HCl or KOH) for 30 min followed by the incubation of 35 μM BFA for 90 min. The size of the BFA-induced compartments was analyzed using ImageJ.

4.4 Results

4.4.1 Effects of eATP on Root Growth

The effects of eATP on root growth were observed by comparing lengths between control and ATP-treated roots. Etiolated four-day-old seedlings were transferred to new phytagel-solidified medium containing different concentrations of ATP and incubated for five days in the light (16h light/8h dark) or in the dark. In the light condition, root growth of Col-0 was inhibited with 1 or 3 mM eATP, whereas growth of *rhd2-4* root was enhanced with 0.3, 1 and 3 mM ATP (Fig. 4-1A and C). In the dark condition, eATP did not inhibit root growth of Col-0, while *rhd2-4* roots were longer with 0.3, 1 and 3 mM eATP (Fig. 4-1B and D).

To observe the effects of short-term exposure of ATP on root growth, four-day-old seedlings were soaked in an eATP solution only for one min and incubated on new plates (without ATP) for five days in the light condition. Root elongation of Col-0 was inhibited already at 1 μM concentration of eATP. On the other hand, *rhd2-4* roots were inhibited only at 1 mM in the short-term (one min) eATP treatment. These results show that the *rhd2-4* roots are less sensitive to eATP than Col-0 roots are (Fig. 4-1E and F).

4.4.2 Endocytosis in the Transition Zone in Cells of ATP-Exposed Seedlings

The effect of eATP on endocytic vesicle activity was observed by analyzing the sizes of BFA-induced compartments in epidermal cells of the transition zone. Etiolated seedlings were soaked in different concentrations of ATP (1-30 μM) for one min. These seedlings were then treated with a BFA solution for 30 min. In the results, both Col-0 and *rhd2-4* had smaller BFA-induced compartments with the treatment of a concentration of ATP higher than 10 μM. However, the BFA-induced compartments were larger in *rhd2-4* roots than in Col-0 roots (Fig. 4-2 A and B).

4.4.3 eATP Enhances Exocytosis

The effect of eATP on exocytic activity was observed during the BFA washout experiments. The diminishing speed of the disintegration of the BFA-induced compartments reflects a lower rate of exocytosis. In this study, etiolated seedlings were first exposed to a BFA solution for 30 min to induce BFA compartments in root cells. These seedlings were then exposed with 0 (control) or with different concentrations of an eATP (1-100 μM) solution for one min, followed by microscopic observation 10 min after incubation in distilled water. The obtained results reveal

that eATP enhanced the recovery of the BFA-induced compartments, especially when the concentration was higher than 10 µM ATP (Fig. 4-3 A and B).

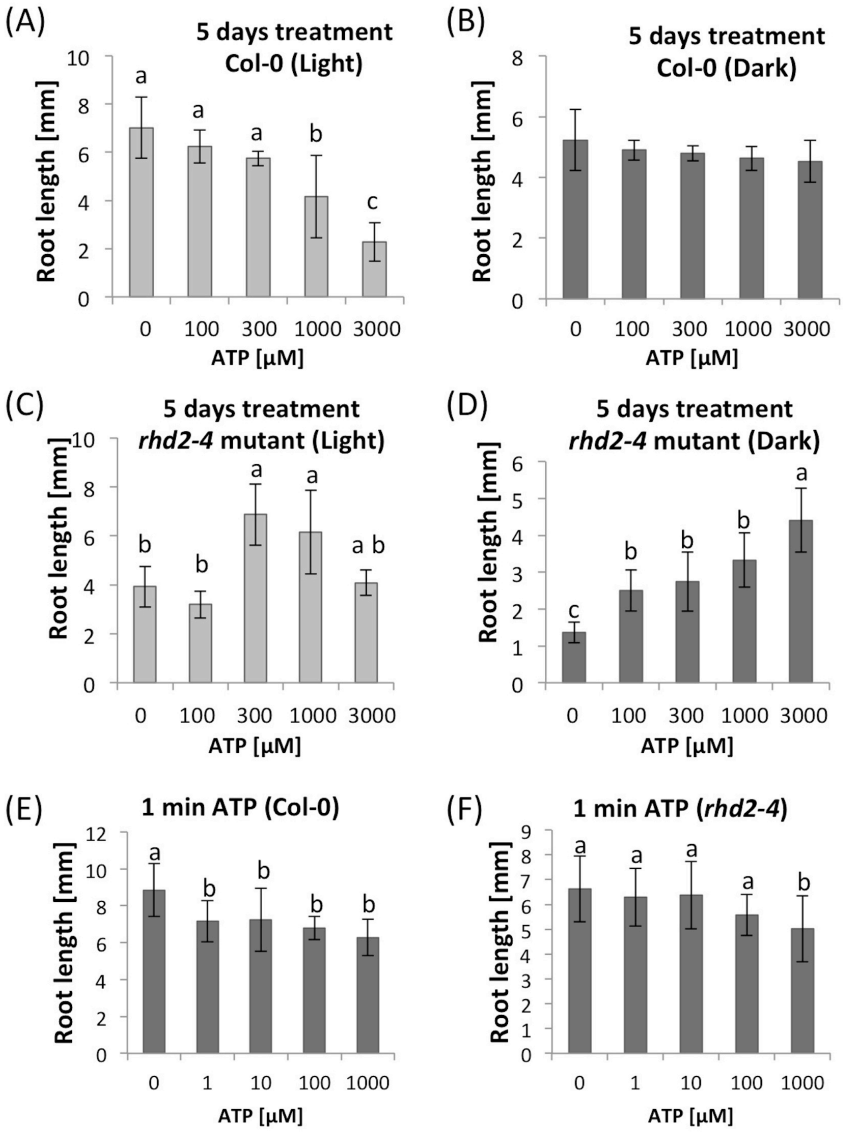

Fig. 4-1. eATP effects on root growth. (A, B) eATP effects on Col-0 root growth. Etiolated four-day-old seedlings were transferred to new media containing different concentrations of eATP. These petri dishes were incubated for five days in (A) the light (16h light/8h dark) or (B) the dark. (C, D) eATP affected *rhd2-4* root growth. After transfer into the new media, root lengths were measured using ImageJ software. Error bars indicate standard deviation of the mean (SD). Different letters (a-b) in the graphs indicate significant differences (Tukey's HSD test, $P < 0.05$, n = 8-13). (E, F) Root growth after eATP treatment for one min. Four-day-old (E) Col-0 or (F) *rhd2-4* dark-grown seedlings were exposed to different concentrations of eATP for one min. After washing out the eATP, seedlings were placed into new agar plates and grown in the dark condition for five days. Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: $P < 0.05$, n = 10-14.

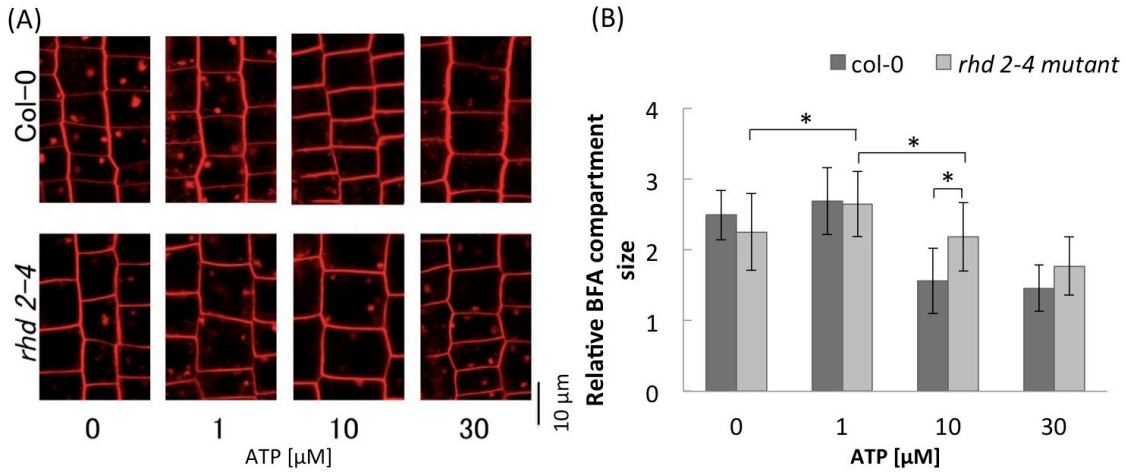

Fig. 4-2. BFA-induced compartments in the transition zone of ATP-exposed seedlings. (A) The representative images from confocal microscopy. Etiolated five-day-old seedlings were treated with different concentrations of ATP for one min. (B) The quantification of BFA-induced compartment sizes from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: * P <0.05.

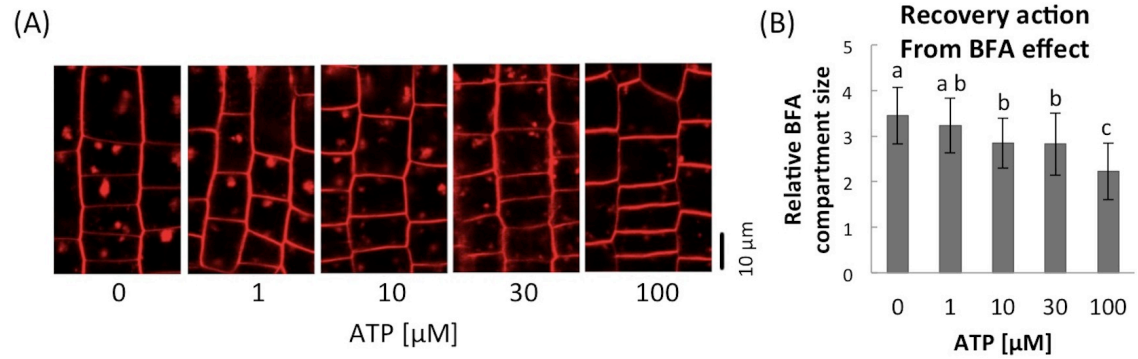

Fig. 4-3. The recovery of BFA-induced compartments in the presence of ATP. (A) The representative images from confocal microscopy. Etiolated five-day-old seedlings were first soaked in 35 μM BFA for 30 min after treatment of 4 μM FM4-64 dye for 10 min. These seedlings were then treated with different concentrations of ATP for one min and incubated in water for 10 min. (B) The quantification of BFA compartment size from five seedlings (n = 25). Error bars indicate SD. Different letters (a-c) in the graphs indicate significant difference (Tukey's HSD test, P < 0.05).

4.4.4 eATP-Induced Damage of Cell Membranes

Endocytosis and exocytosis are based on complex and dynamic machineries necessary for transport of vesicles and membrane-associated proteins or biomolecules. The results of the study shown above demonstrate that eATP affected both endocytosis and exocytosis in root apex cells, suggesting that eATP might control the endocytic vesicle recycling *via* direct impacts on membranes. Therefore, the influence of eATP on cell membrane was assessed and compared between Col-0 and *rhd2-4* using the Evans blue staining method (dye for damaged cell membrane). Etiolated seedlings were soaked in 0 or different concentrations of ATP (1-300 µM) for one min, and stained with Evans blue. After washing the seedlings in distilled water, blue-colored areas were compared. In the results, the staining intensity of Evans blue in the root transition zone of Col-0 with 10 µM ATP was higher than that of the *rhd2-4* roots. This finding suggests that the plasma membrane of *rhd2-4* mutant is less sensitive than the Col-0 is to the same concentration of eATP (Fig. 4-4 A and B).

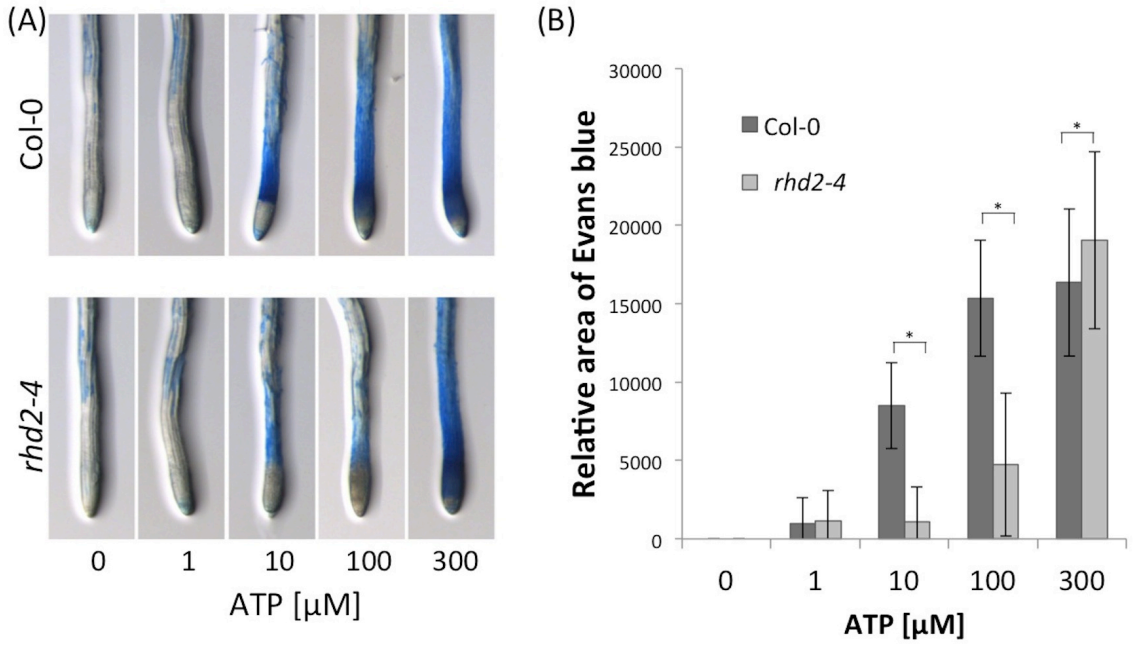

Fig. 4-4. The visualization of cell damage caused by eATP. (A) The microscopy images from seedlings stained with Evans blue. Etiolated five-day-old seedlings were soaked in different concentrations of ATP for one min. These seedlings were stained with Evans blue (dye for damaged cells) for 30 min. (B) ImageJ measured the relative area of Evans blue. Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: $*P < 0.05$, $n = 10$.

4.4.5 DORN1 is Involved in eATP Signaling in the Root Transition Zone

Endocytic recycling activity of the *DORN1* mutant lines (*dorn1-1*, point-mutated line; *oxDORN1*, ectopic expression line) was analyzed by comparing BFA-induced compartment size in cells of the transition zone. In this study, etiolated *dorn1-1* and *oxDORN1* seedlings were treated with 0 or 30 μM ATP for one min. These seedlings were then transferred and treated with BFA for 30 min. The results show that size of BFA-induced compartments in the *dorn1-1* roots was not altered by 30 μM eATP, whereas in the *oxDORN1* roots these sizes were reduced by the same concentration of eATP (Fig. 4-5 A and B).

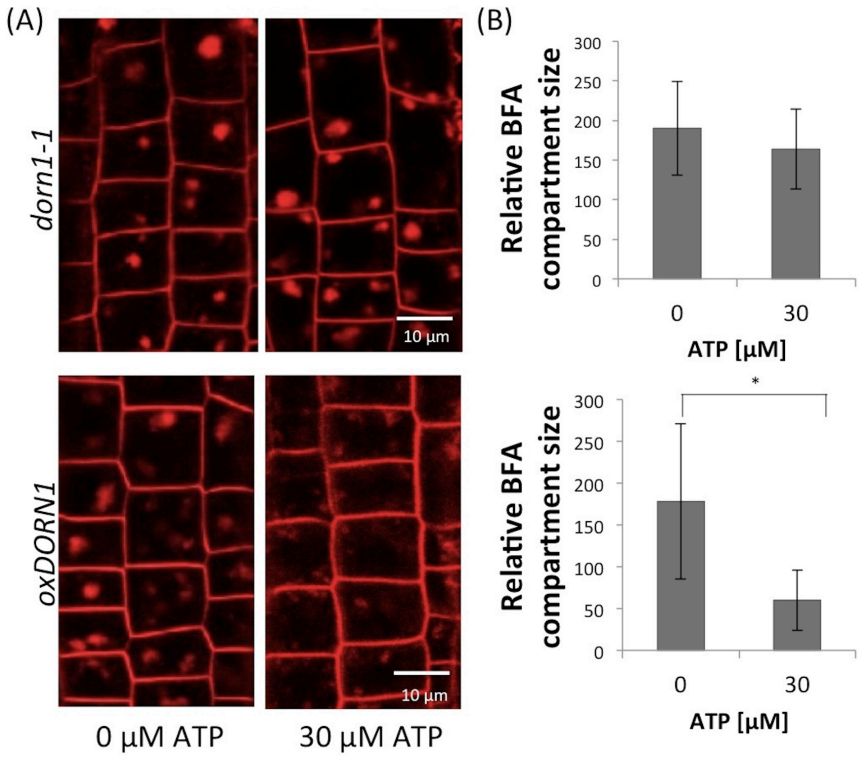

Fig. 4-5. BFA-induced compartments in cells of the transition zone of eATP receptor mutant lines, *dorn1-1* and *oxDORN1*. (A) The representative images from confocal microscopy. (B) The quantification of BFA-induced compartments from five seedlings (n=25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: $*P < 0.05$.

4.4.6 BFA-Induced Compartments in the Transition Zone of pH-Exposed Seedlings

Extracellular pH is known to affect root growth and tropisms. As described in the introduction, eATP is thought to be involved in modulating cellular pH values (Vroman et al., 2014). Although eATP-induced intracellular pH change is investigated in the next chapter, the effects of pH on endocytosis activity were observed in the present study as well. Seedlings were incubated in 0.5x MS with different pH values (4.5, 5.8, 8.0 adjusted with HCl or KOH) for 15 min with or without ATP. The seedlings were then treated with BFA solution to generate BFA-induced compartments. The obtained results show that a 15 min treatment with a pH 8 solution resulted in smaller BFA-induced compartments than at pH 4.5 and 5.8 (Fig. 4-6A and B).

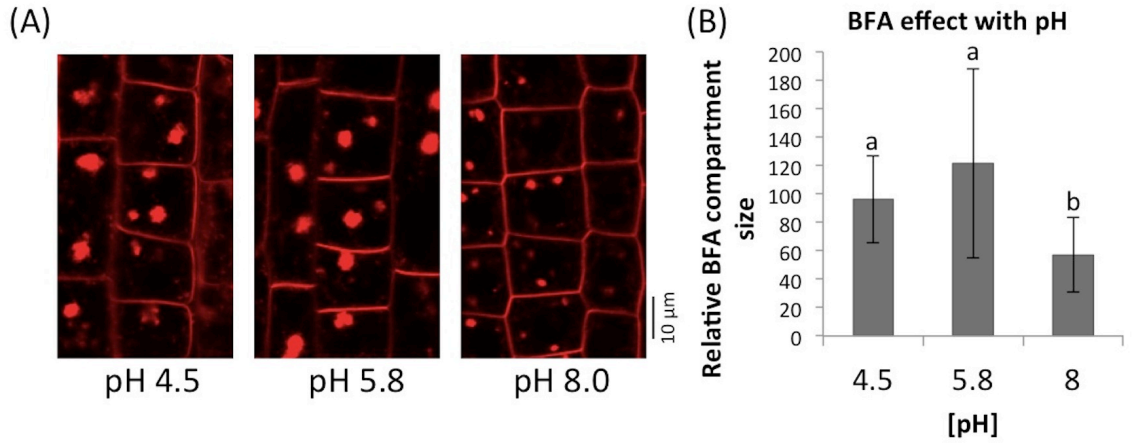

Fig. 4-6. BFA-induced compartments in the transition zone of pH-exposed seedlings. (A) Confocal images of the BFA-induced compartments. (B) Relative BFA compartment sizes were measured from six or seven seedlings (n = 30-35) using ImageJ. Error bars indicate SD. Different letters (a, b) in the graphs indicate significant differences (Tukey's HSD test, $P <$ 0.05).

4.5 Discussion

4.5.1 Root Sensitivity to eATP is Enhanced by the Light Conditions during Growth

As was discussed in Chapter 1, light exposure alters plant root development and growth. For example, it was previously reported that the illumination of roots alters physiological conditions,

growth rate, and root tropisms (Yokawa et al., 2011; 2013; 2014; Mo et al., 2015). This chapter first observed root responses to eATP under light- or dark-grown conditions by comparing root growth. The experimental procedures followed the published report that used eATP-containing agar plates (Tang et al., 2009). The present results show that eATP inhibited root growth of seedlings incubated in the 16h/8h light condition. However, this inhibition was not observed in the dark-grown roots. Previously, Tang et al. (2009) reported that only 3 mM eATP inhibited *Arabidopsis* root growth; however, the present data show that concentrations of eATP as low as 1 mM inhibited root growth. This difference between the eATP concentrations found in the present and previous study (Tang et al., 2009) might be due to degradation of ATP on the agar plates. ATP is known as an unstable molecule, and it can easily be hydrolyzed under high temperature circumstances or during long-term storage. To avoid heat-caused damage of the molecule, ATP was added to a melted agar (*ca*. 60˚C) after autoclaving. In addition to continuous exposure to the eATP treatment, seedlings were treated with eATP solutions for only one min to observe possible effects of this short-term exposure of eATP on root growth. Intriguingly, inhibited root growth was noted after only one min of 1 μM eATP treatment in Col-0 in the light-grown condition. Previous studies have reported that the touch-induced eATP release is completed within one min in *Arabidopsis* root apices (Weerasinghe et al., 2009). This suggests that eATP release followed by its perception from root cells might be a quick response, probably within a few minutes. In contrast to eATP inhibition of Col-0 root growth, root growth of *rhd2-4* was recovered by 0.3 and 1 mM eATP (Fig. 4-1 C and D). In the absence of eATP, the root length of *rhd2-4* line was shorter than that of Col-0. As has previously been shown, the generation of superoxide catalyzed by NADPH oxidase (*RHDC*) is important for root growth and root hair formation (Foreman et al., 2003). Therefore, superoxide generated *via* catalytic activity of NADPH oxidase C is important for root hair elongation. The present results show that eATP might phenocopy the *rhd2-4* roots, probably by the generation of ROS through some other mechanisms. This suggests that factors other than AtRBOHC may have a role in triggering further cellular signaling cascades based on ROS generated in response to eATP exposure. A chloromethyl derivative of 2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA fluorescence, ROS-staining probe) study also showed that ROS accumulation in *rhd2-4* mutant roots by 100 μM eATP treatment could be observed, although the ROS level was lower than in the wild type (Demidchik et al. 2009, Fig. 5B). Previous reports have also proposed that another eATP-driven ROS-producing pathway might exist that does not depend on only RBOHC. For example, it is known that ROS is also generated by the other RBOHs,

such as RBOHD and RBOHF, in roots (Kwak et al., 2003). Importantly, all RBOHs are expressed in the roots of *Arabidopsis*.

4.5.2 Endocytic Recycling is Disturbed by eATP

Since eATP has been reported to inhibit both root gravitropism and root growth, the present study examined the endocytic recycling activities after eATP and BFA treatments. As Fig. 4-2 shows, 10 μM eATP reduced BFA-induced compartment size. On the other hand, 10 μM eATP enhanced the recovery speed from the BFA-arrested state *via* vesicle recycling (Fig. 4-3). Together, these results suggest that eATP exerts opposite effects on these processes: it inhibits endocytosis, whereas exocytosis is enhanced. This imbalance of two vesicle recycling pathways, namely endo- and exocytosis, might consequently disrupt polar auxin transport mediated by PIN proteins. It has previously been reported that basipetal auxin transport is inhibited by 1 mM eATP (Tang et al., 2003) treatment. Moreover, the modulation of vesicle recycling by eATP has been observed in epidermal cells in the root transition zone (Fig. 4-2 and 3). These data suggest that has an important role for the perception of eATP molecules. In addition, it has been reported that 1 mM ATP generates an acidic pH situation (pH 4.8) in un-buffered solution (Tang et al., 2003). This study tested the effects of eATP solutions with no pH adjustment by following the previous report by Tang et al. (2003). Therefore, the study also investigated pH effects on BFA compartment size in the absence of eATP. In the results, pH 4.5 did not show any effect on the BFA-induced compartment size compared to a normal pH 5.8 level (Fig. 4-6A, B). Therefore, it can be concluded that the endocytic recycling was not affected by an acidic pH value. eATP is required to alter the recycling rate. However, as Tang et al. (2003) mentioned, eATP is more effective at inhibiting polar auxin transport at a low pH. Chapter 5 will show and discuss more results regarding the pH issue.

4.5.3 AtRBOHC Is Involved in eATP Signaling

A BFA treatment was used to observe the relationship between NADPH oxidase (AtRBOHC) and endocytic vesicle recycling. It has been reported that AtRBOHC is a major contributor of ROS generation by eATP in roots (Kim et al., 2006; Demidchik et al., 2009). In my study of *rhd2-4* mutant line, 1 μM eATP increased the size of BFA-induced compartments whereas a concentration of eATP higher than 10 μM reduced their size (Fig. 4-2). The effects relating to the 1 μM eATP partially support the finding that eATP recovers the root growth of the *rhd2-4* mutant line (Fig. 4-1C, D and F). However, when the concentration of eATP reached 10 μM, it

inhibited endocytic recycling activity similarly to in the Col-0 roots. In comparison with the size of BFA-induced compartment for concentrations of ATP under 10 μM in Col-0 roots, *rhd2-4* mutant roots showed smaller compartments for the same eATP concentration (Fig. 4-2). This finding suggests that *rhd2-4* roots are less sensitive to eATP in terms of the modulation of endocytosis. This notion is also supported by the fact that ROS produced *via* AtRBOHC is involved in eATP signaling pathways. However, exogenously applied ATP still reduced the sizes of BFA-induced compartments in the cells of *rhd2-4* roots, suggesting that other factors might be involved in eATP signaling that modulate endocytosis as well as root growth.

4.5.4 Cell Permeability was Altered by eATP

As discussed above, root growth of both Col-0 and *rhd2-4* was altered in the presence of eATP. Furthermore, eATP also affected the rate of vesicle recycling of both endo- and exocytic pathways in root cells, suggesting that eATP might affect membrane properties, possibly *via* ROS signaling or some more direct reaction. Therefore, this study compared the eATP-induced changes of cell membranes in Col-0 and *rhd2-4* roots using Evans blue. Evans blue solution is widely used to visualize the permeability of plasma membranes. Cell damage was observed in the transition zone with a concentration of eATP of 10 μM or higher. However, this damage caused by eATP treatment was not fatal to root growth, because roots kept growing even after a treatment of 1 mM eATP (Fig. 4-1). Therefore, it can be safely assumed that eATP exposure causes a transient change (or damage) of membrane properties. These results suggest that the root apex transition zone shows a prominent response to eATP treatment compared to other parts of root apices (Fig. 4-4). Interestingly, *rhd2-4* roots were less sensitive to the eATP treatment. This suggests that the cell damage observed here might be caused by the NADPH oxidase C-mediated accumulation of ROS after eATP perception. ROS is known to induce damage to plasma membranes (Blokhina et al., 2003). These results also support the previous work showing that eATP signaling is involved in ROS *via* NADPH oxidase C (Demidchik et al., 2009).

4.5.5 DORN1 Plays a Role in eATP Signaling to Endocytosis

In plants, the plant purinoceptor P2K was reported only recently (Choi et al., 2014). Endocytic activities in roots of the *DORN1* mutant lines, *dorn1-1* and *oxDORN1*, were observed in the root transition zone using a BFA treatment. In the results, no alteration of BFA compartment size by 30 μM eATP in *dorn1-1* was shown, whereas *oxDORN1* roots showed reduced BFA

compartment sizes after the eATP treatment (Fig. 4-5A and B). These results indicate that the eATP receptor DORN1 is also involved in transmitting eATP signaling to the endocytic recycling (Fig. 4-7). More detailed studies regarding DORN1 and endocytic vesicle recycling will be discussed in Chapter 5.

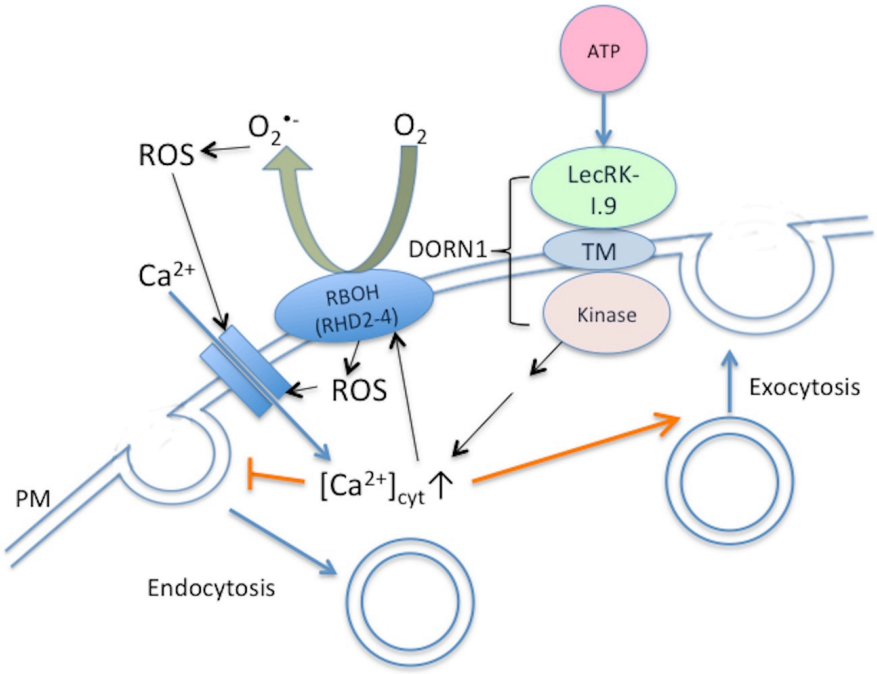

Fig. 4-7. A working model of eATP signaling mediated *via* endocytic vesicle recycling and ROS signaling. At the plasma membrane (PM), eATP stimulates DORN1, which consists of an extracellular legume-type lectin domain (LecRK-I.9), a single trans-membrane domain (TM), and an intracellular kinase domain. DORN1 signaling increases cytosolic free Ca^{2+}, $[Ca^{2+}]_{cyt}$ (Choi et al., 2014). The elevation of $[Ca^{2+}]_{cyt}$ stimulates AtRBOH (NADPH oxidase) activity on the PM *via* its EF hands (calcium binding motif) (Takeda et al., 2008). It is known that eATP generates ROS *via* AtRBOH (Song et al., 2006; Demidchik et al., 2009). The superoxide anion ($O2^{\bullet-}$) would then be generated in extracellular space, activating other ROS, such as H_2O_2 and $OH^{\bullet-}$. All these ROS species could then open the PM Ca^{2+}-permeable channel (Demidchik et al., 2009). Therefore, $[Ca^{2+}]_{cyt}$ levels would be further increased. The elevated $[Ca^{2+}]_{cyt}$ would induce the inhibition of endocytic activity, whereas exocytic activity would be enhanced (these pathways are indicated with orange bars). Based on the findings in the chapter, eATP-triggered ROS and/or Ca^{2+}-signaling regulate of endocytic vesicle recycling in cells of the root apex transition zone.

Chapter 5

Endocytic Vesicle Recycling in the Root Apex Is Regulated by eATP *via* the DORN1 Receptor

5.1 Abstract

Extracellular ATP (eATP) is known as a signal molecule both in mammal and plant cells. In plants, several studies have demonstrated that eATP is involved in the regulation of plant growth and in plants' adaptation to the environment. For example, eATP has been shown to inhibit root gravitropism and cell/organ growth in *Arabidopsis*. In mammals, eATP studies have accelerated since the purinoceptor (eATP receptor) was identified. In contrast, the plant purinoceptor DORN1 (Does not Respond to Nucleotides 1) was reported only recently. DORN1 has been shown to increase intracellular Ca^{2+} levels by eATP perception. However, it is still unclear how DORN1 is involved in the regulation of root growth or tropisms using eATP signaling pathways.

This study analyzed the expression pattern of the eATP receptor gene *DORN1* (At5G60300) using the microarray database of the Genevestigator (https://www.genevestigator.com/). DORN1 shows a high expression in *Arabidopsis* root tissues compared to other parts of the plant. Interestingly, in the *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), the highest expression level of the *DORN1* gene was found in the root apex transition zone located between the apical meristem and the elongation zone. As was discussed in Chapter 4, eATP signaling is involved in ROS signaling and controls endocytic vesicle recycling in cells of the root transition zone.

The aim of the present study is to investigate the mechanisms of eATP signaling in the root apex mediated by the eATP receptor DORN1. The eATP application inhibited the

graviresponse of wild type roots, whereas the *dorn1-1* roots (loss-of-function mutant of *DORN1*) showed no inhibition from the eATP treatment. The activity of endocytic vesicle recycling, which controls root tropic growth, was examined in *DORN1* mutants (*dorn1-1* and *oxDORN1*) roots. In addition, ATP-dependent pH changes in the root graviresponse were also assessed. The results indicate that the DORN1 receptor plays a key role in mediating eATP-dependent regulation of endocytic vesicle recycling in cells of the root apex transition zone.

5.2 Introduction

As was mentioned in the previous chapter, the plant purinoceptor P2K (K for kinase) was recently reported. P2K is identified by a number of ethylmethane-sulfonate (EMS)-mutagenized seedlings for the ATP-induced calcium response, and it was coined as DORN1 (Does not Respond to Nucleotides 1) (Choi et al. 2014). Two purinoceptors were found in a mammalian study, and a lot of studies have reported the function of the receptors as perceiving the eATP molecule. In contrast, the study of plant eATP receptors has just begun, although some older papers have previously demonstrated that eATP is involved in regulating plant growth and environmental adaptation. Interestingly, it has been reported that root parts utilize eATP signaling in their tropisms. For example, eATP has been shown to inhibit root gravitropism and growth in *Arabidopsis* (Tang et al., 2003). Moreover, it has been shown that a root apex shows high activity of eATP release induced by touch stress (Dark et al., 2011). Since the plant eATP receptor was identified only recently, further studies that investigate the function of DORN1 in root environmental responses are highly required.

The gene DORN1 (At5g60300) encodes a lectin receptor kinase-I.9 (LecRK-I.9) (Choi et al., 2014). It has been reported that it is an adhesive protein that connects the plasma membrane and cell wall in cells of *Arabidopsis* (Gouget et al., 2006). Therefore, in plants, a role of DORN1 is proposed to be maintaining the structure of the plasma membrane. Endocytic vesicle recycling is a well-known membrane-associated cellular event necessary for many root tropisms (Baluška et al., 2010). In order to keep the recycling system functional, structural homeostasis and maintenance of the plasma membrane are essential. It has been reported that the endocytic recycling activity maintain optimal fluidity and rigidity of the plasma membrane, controlling subsequent signaling events based on receptors and transporters (Nakayama et al., 2012).

Chapter 4 showed that eATP signaling was involved in modifying endocytic vesicle recycling in the root apex transition zone. The present study postulates that if DORN1 maintains the contact between the cell membrane and wall, it would also be an important player for the regulation of endocytic vesicle recycling. Although DORN1 has been identified as an eATP receptor, the true function of this protein is still unknown. DORN1 was first found in a large-scale screening by monitoring the elevation of cytosolic Ca^{2+} with eATP treatments (Choi et al., 2014). However, it is unknown how the increase of Ca^{2+} and the control of endocytic vesicle recycling interact with each other in eATP signaling during root tropic responses. Therefore, in this chapter activities of endocytic vesicle recycling, which controls root tropic growth, are examined using DORN1 mutant lines (*dorn1-1*, loss-of-function; and *oxDORN1*, ectopic expression line).

In plants, it has been reported that eATP inhibits root gravitropic response (Tang et al., 2003). On the other hand, for root gravitropic response, cells in the root cap region require a pH change (Fasano et al., 2001; Monshausen and Sievers, 2002). The pH value is an important physiological factor for all living systems. It is maintained by the system of some buffering functions of cells a change in pH might damage cells. However, living systems actively maintain optimal pH values to allow growth and physiological processes (Scott and Allen, 1999; Cosgrove, 2000). Although many studies have reported the importance of pH for different types of living organisms (Fozo et al., 2004), including plants (Scott and Allen, 1999; Monshausen, et al., 2007), based on biochemical methods, the number of papers has declined in recent decades. Interestingly, in mammals, it has been reported that secreted eATP has the function of lowering extracellular pH in animal retinal neuronal cells (Vroman et al., 2014). Furthermore, Tang et al. (2003) report that eATP is more effective in the inhibition of root gravitropism and auxin transport in low pH conditions. In the present study, the effect of eATP on pH change in root cells was monitored. Besides the perception of eATP by the receptor and related signaling, a possible role of secreted eATP will also be discussed.

5.3 Materials and Methods

5.3.1 Crawling of Maize Roots is Modified with eATP in Darkness

Maize seeds (*Zea mays* L., cv Clemente) were rolled and incubated with damp filter paper vertically in the dark for five days at 23˚C for germination. Seeds with straight roots were selected for the study. The maize roots were placed on a slope (about 30 degrees) made of a wet

firm sponge under the dim light. The entire experimental set-up was covered with a glass container to preserve the humidity. 26 hours after incubation, droplets of eATP solution 0 (Control), 100 µM or 10 mM were applied to the root apices. The roots were continuously incubated in the dark. The time-lapse pictures of root growths were taken with an EOS Kiss X7 (Canon, Tokyo) equipped with a controller (Etsumi E6315, Canon, Tokyo) every five min.

5.3.2 Growth Condition of Arabidopsis thaliana

Seeds of the *dorn1-1* and *oxDORN1* lines were kindly provided by Professor Gary Stacey (University of Missouri). Seeds of pHusion was kindly provided by Professor Alexander Schulz (University of Copenhagen). Seeds of *Arabidopsis thaliana* were sterilized with 0.2% $NaClO_2$ (ROTH, Karlsruhe) containing 0.1% Triton-X (ROTH, Karlsruhe) for five min. After washing them in water four times, the seeds were dried on filter papers. They were then planted on 0.5x MS media (Duchefa, Haarlem) containing 0.1% MES, 1% (w/v) sucrose (pH 5.8 with KOH) solidified with 0.4% (w/v) phytagel (Sigma, Steinheim). The petri dishes were incubated at 4˚C for one day for seed imbibition. These were put vertically at 22˚C in the light (16h light/8h dark, light intensity: ~120 µmol/s/m^2, humidity: ~50%) or in the dark.

5.3.3 Preparation of Nucleotide Solutions

Adenosine 5'-monophosphate sodium salt (AMP), Adenosine 5'-diphosphate potassium salt (ADP), and ATP were obtained from Sigma. These chemicals were solved in distilled water and the concentration was adjusted to 10 mM, pH 7.0, with NaOH as a stock solution.

5.3.4 Measurements of Root Growth

Five-day-old seedlings were treated with different concentrations of ATP, AMP or ADP diluted in 0.5x MS containing 20 mM KPB (pH 5.8) for 30 min. The treated seedlings were placed back on new plates containing 0.5x MS, 1% (w/v) sucrose (pH 5.8 with KOH) solidified with 0.8% Bacto agar (BD Difco). The pictures of roots were taken with an EOS Kiss X7 (Canon, Tokyo). The root growths after four days grown on new media were measured using ImageJ software (ver. 1.43u for Mac OSX, http://imagej.nih.gov/ij/).

5.3.5 Gravitropic Response of Roots

Seedlings (4 day-old) of similar root length were selected and incubated vertically on a new culture (0.5% MS, 1% (w/v) sucrose, 0.8% (w/v) agar, pH 5.8) for one day. 2 µl of test

solutions prepared in 20 mM KPB (pH 5.8) were applied to the root apex region by pipetting. The petri dishes were rotated 90˚ and incubated in the dark to avoid light-induced tropic responses. Images of the results of root curvature or root elongation were taken with an EOS Kiss X7 and measured using ImageJ software.

5.3.6 Cytosolic pH Responses to eATP Treatments with pHusion

Five-day-old pHusion seedlings (transgenic *Arabidopsis thaliana* line of the pH sensor) were soaked in the control or ATP-containing solution prepared in 0.5x MS and 20 mM KPB (pH 5.8) for 30 min. EGFP was excited by 488 nm and detected between 500 and 600 nm. mRFP was excited by 543 nm and detected between 600 and 630 nm. pH-dependent relative fluorescence was calculated and compared based on 40 μm diameters of circle areas in the microscope images of the root tips. These calculations were processed using ImageJ software.

5.3.7 BFA-Induced Compartments in the Root Apex Transition Zone

Five-day-old seedlings were incubated in 4 μM FM4-64 for 10 min (endocytic vesicle tracer) and then incubated in test solutions in 0.5x MS and 20 mM KPB (pH 5.8). These seedlings were incubated in 35 μM BFA in 0.5x MS and 20 mM KPB. FM 4-64 was excited by 515 nm and detected between 630 nm and 700 nm.

5.3.8 EGTA-Induced Effects on BFA-Induced Compartments

Five-day-old seedlings were stained by 4 μM FM4-64 for 10 min. The seedlings were incubated in 0.5x MS and 20 mM KPB (pH 5.8) with or without 5 mM EGTA for 30 min before being incubated in 35 μM BFA in 0.5x MS for 90 min. 5 mM EGTA was adjusted from 10000x high stock solution (pH 7.0).

5.3.9 Recovery from BFA-Induced Compartments

Five-day-old seedlings were treated with 35 μM BFA in 0.5x MS for 90 min after 4 μM FM4-64 dye staining for 10 min. The seedlings were then soaked in a test solution for 30 min.

5.3.10 Plasmolysis with 800 mM Mannitol in the Root Apex

Five-day-old seedlings were stained by 4 μM FM 4-64 for 10 min. These seedlings were incubated in 800 mM mannitol for 10 min to induce plasmolysis of root cells. Shapes of root cells were observed under a confocal microscope.

5.3.11 Cytosolic pH Response to Gravity Stimulation of the Root Tip

Five-day-old pHusion seedlings were mounted on slide glass and incubated vertically with 0.5x MS containing 20 mM KPB (pH 5.8). After two hours, these prepared slides were rotated and incubated at a 0˚ or 90˚ angle for 30 min, followed by confocal observation in the same manner as the one described above in the pHusion section.

5.4 Results

5.4.1 eATP Attenuated Maize Root Crawling Behavior

When maize seedlings are placed horizontally on a slope in dim light, their root apices will try to grow downward and penetrate the ground because of gravitropism. If the ground surface is too hard for roots to penetrate, then root apices will lift up by bending in the elongation zone and will make the next penetration attempt after some growth. This series of root movements is called “crawling” (Hahn et al., 2006; Burbach et al., 20012). This movement consists of alternated and integrated root sensory responses: gravitropism and thigmotropism. This study investigated the crawling movement of maize roots with eATP. The data show that eATP-treated roots attenuated their crawling movement. The control treatment showed two crawling cycles (touching and creeping) on the sponge surface (at 175 min and 375 min) within 450 min. However, 10 mM eATP-treated roots did not show any crawling behavior, and their root apices were kept lifted up against the gravity vector from 100 min to 375 min. Finally, the eATP-treated root apices recovered their response to gravity after 450 min (Fig 5-1).

5.4.2 The Expression Pattern of the eATP Receptor DORN1 in the Root Transition Zone

First, the study analyzed the accumulation of DORN1 (At5G60300) in plant roots using the *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Brady et al., 2007; Winter et al., 2007). Interestingly, DORN1 seems to accumulate in the root apex transition zone (Fig. 5-2).

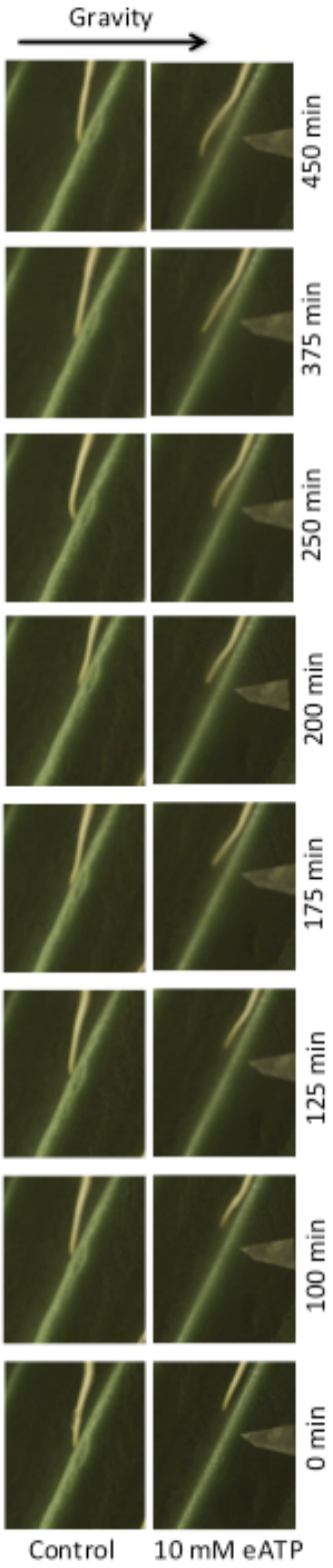

Fig. 5-1. Crawling test of maize root with or without eATP. Five-day-old maize roots were incubated on a slope made of a wet firm sponge for 26 hours in the dark. Root apices were then treated with 0 μM (control) or 10 mM eATP, respectively. The numbers next to the pictures indicate the time elapsed since the treatment of eATP. At 0 min, both the control and the eATP-treated root tips were on the sponge surface. The control showed two crawling cycles (touch and creeping) on the sponge surface (first: 175 min; second: 375 min) until 450 min. However, the 10 mM eATP-treated roots did not show any crawling behavior, and the root apices were kept lifted up against gravity from 100 min to 375 min. Finally, the eATP-treated root apices recovered their response to gravity after 450 min.

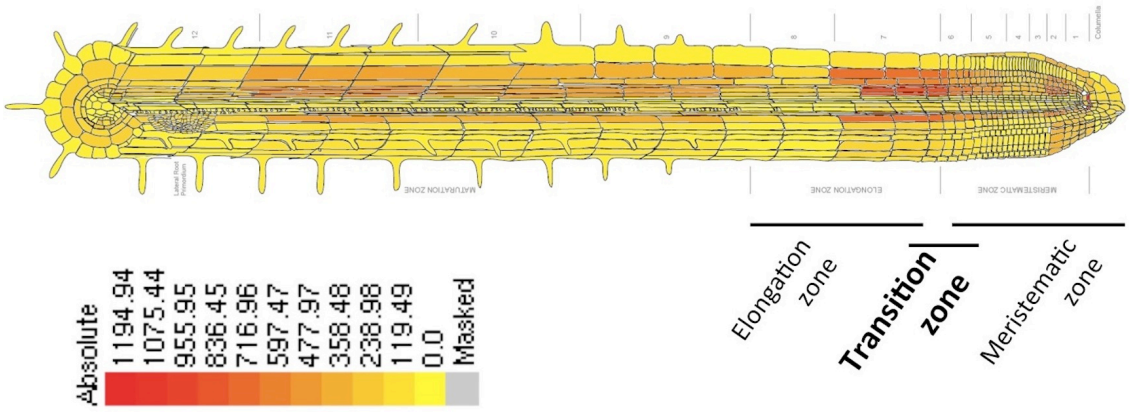

Fig. 5-2. Accumulation of DORN1 (At5G60300) in plant root, analyzed using the *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb. cgi;Brady et al., 2007; Winter et al., 2007). Absolute value indicates the expression level for the gene, At5G60300 compared to the highest signal based on the microarray data. High and low levels are shown in a red and a yellow color, respectively.

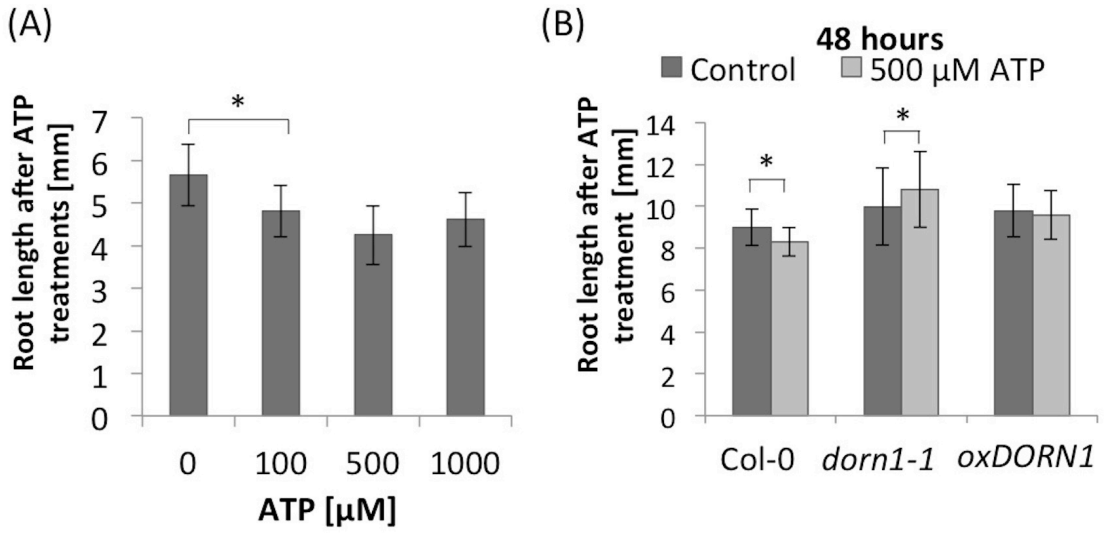

Fig. 5-3. Root length after eATP treatment for 30 min. (A) eATP effects on root growth. Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: **P* < 0.05, n=14, 15. (B) ATP effects on root elongation of mutant lines. The statistical significances of mean differences were analyzed using a Student *t*-test: **P* < 0.05, *n* = 11-13.

5.4.3 Root Growth is Inhibited by eATP (pH 5.8) Treatment

In this study, pH values of all ATP treatment solutions were adjusted to rule out the side effects of pH. The eATP (pH was adjusted to 5.8) impacts were observed by comparing root growth. eATP concentrations higher than 100 μM inhibited root growth 24 hours after the treatment in the light condition (Fig. 5-3A). The root growth of DORN1 mutant lines, *dorn1-1* and *oxDORN1*, were also measured with 500 μM eATP treatment. The results show that the roots of *dorn1-1* and *oxDORN1* lines were not inhibited even after 48 hours of incubation with 500 μM eATP (Fig. 5-3B).

5.4.4 eATP Inhibition of Root Gravitropic Responses

eATP-mediated inhibition of root gravitropism was also observed. Two μl of different concentrations of eATP were applied to root tips with droplet from pipets. The petri dishes with treated seedlings were rotated 90˚ and incubated for eight hours in the dark condition to prevent any light-induced tropic responses. Root curvatures were measured between the directions of root tips and the horizon (Fig. 5-4A). eATP concentrations of 100 μM or higher inhibited root gravitropic responses within five hours. However, the inhibition of gravitropism by eATP was recovered after eight hours (Fig. 5-4B).

Next, gravitropic responses of *dorn1-1* and *oxDORN1* roots were observed with a 500 μM eATP treatment. Root gravitropism of *dorn1-1* line was not inhibited, whereas gravitropism of *oxDORN1* roots was inhibited (Fig. 5-4C). The root elongation was also measured in the gravity-stimulated roots (Fig. 5-4D, E and F). The results show that 500 μM eATP did not inhibit the root elongation of Col-0 (wild type) and *dorn1-1* within five hours, whereas the growth of *oxDORN1* roots was inhibited.

5.4.5 Root Gravitropic Responses Are Modulated by Exogenous ATP, AMP and ADP

The inhibition of root gravitropism *via* AMP and ADP were also assessed in this study. Root bendings of the Col-0 and *oxDORN1* lines were inhibited by a five-hour exposure to 500 μM ADP (Fig. 5-5). Interestingly, *oxDORN1* roots were also inhibited by AMP (Fig. 5-5). Roots of the *dorn1-1* mutant line showed no effects of ATP, AMP or ADP on root gravitropism (Fig. 5-5A, B and C).

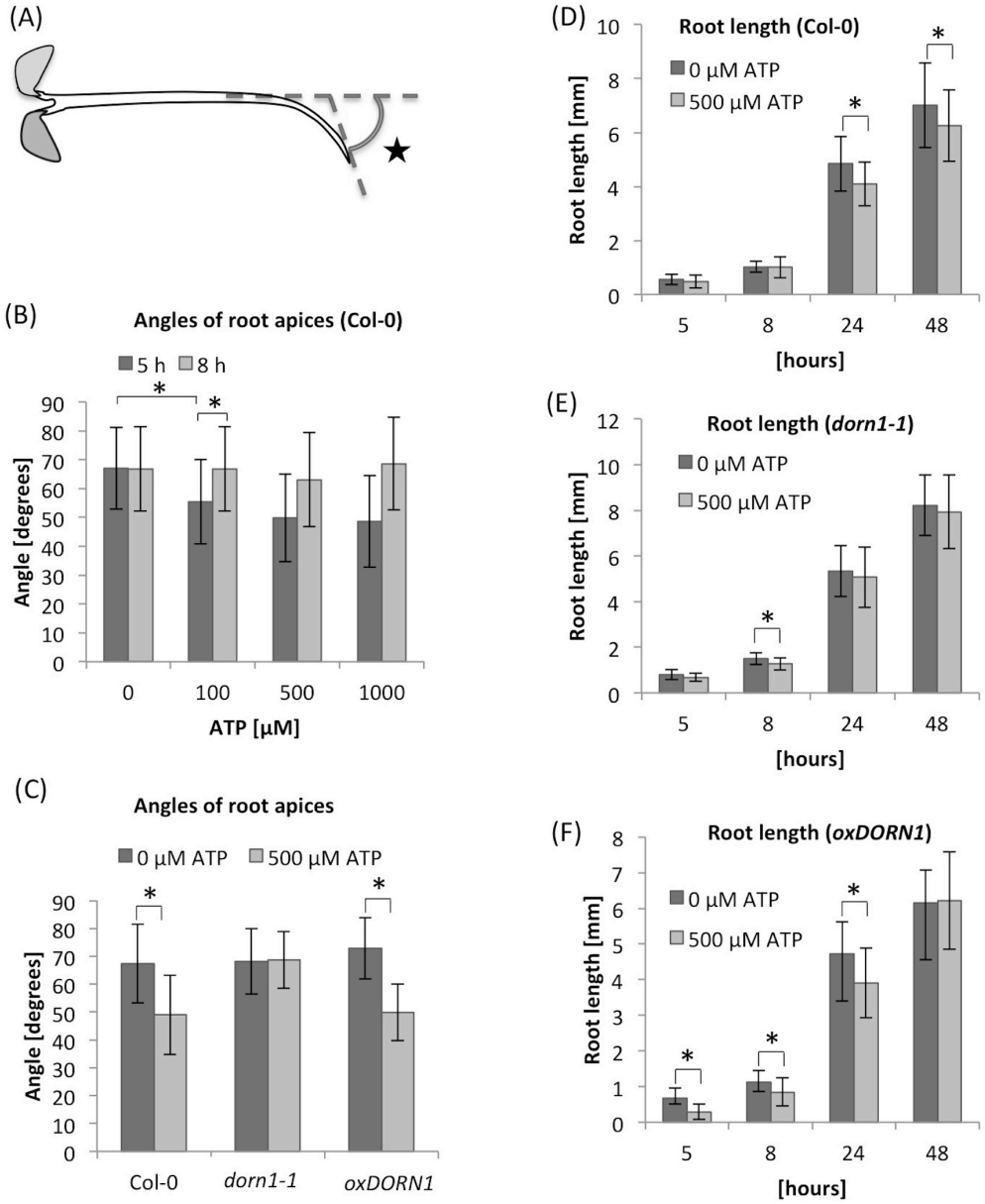

Fig. 5-4. Effects of eATP on root elongation and gravitropic responses. (A) Diagram root bending. Root apex angles were measured between the directions of root tips and the horizon (*). (B) Effects of eATP on root gravitropic responses. Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: $*P < 0.05$, n = 18-20. (C) Root gravitropic responses of DORN1 (eATP receptor) mutant lines. The statistical significances of mean differences were analyzed using a Student *t*-test: $*P < 0.05$, n = 20-38. (D-F) Root elongation in the dark after applying eATP to the root tip of (D) Col-0, (E) *dorn1-1* and (F) *oxDORN1*. Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student *t*-test: $*P < 0.05$, n = 19, 20.

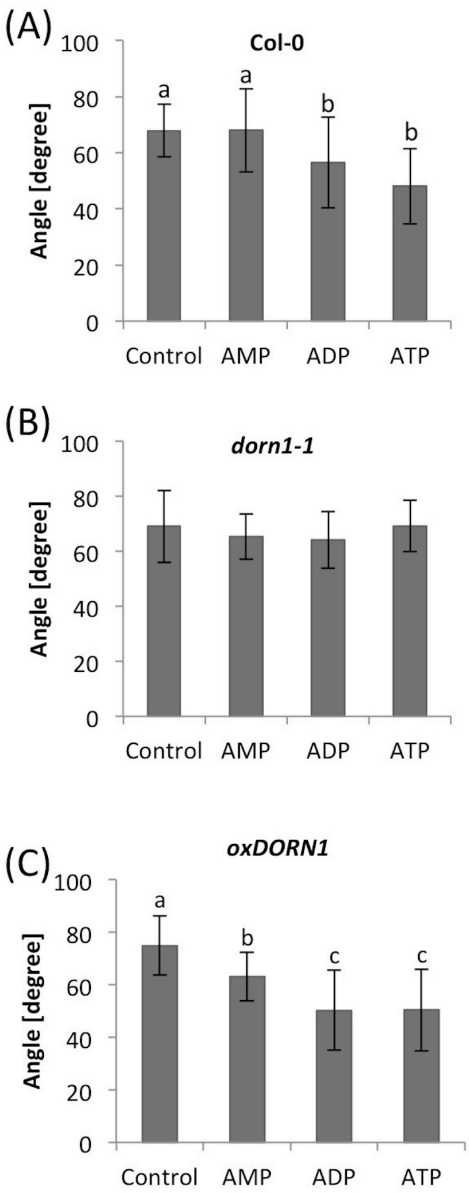

Fig. 5-5. Effects of eATP, eADP and eAMP on root gravitropic responses. Similar lengths of four-day-old seedlings were selected and transferred to new petri dishes. Two µl of each concentration of ATP, AMP or ADP in 20 mM KPB (pH 5.8) was applied to the root tips of Col-0 (A), *dorn1-1* (B) and *oxDORN1* (C). These petri dishes were rotated by 90˚ and incubated in the dark for 5 hours. Error bars indicate SD. Different letters (a-c) in the graphs indicate significant differences (Tukey's HSD test, $P < 0.05$).

5.4.6 eATP Affects Endocytosis in Epidermal Cells in the Transition Zone

Endocytic recycling activity was observed by comparing BFA-induced compartment sizes in cells of the root apex transition zone of Col-0 (wild type). First, the time-dependence of the eATP treatment was assessed. Five-day-old seedlings were first incubated in 0 or 500 µM eATP for five, 15, 30, 60 or 180 min (Fig. 5-6 and 7). The seedlings were then soaked in BFA for 90 min. The obtained results show that the five min eATP treatment increased the BFA-induced compartment sizes compared to the control treatment. In contrast, eATP treatments longer than 15 min reduced the BFA-induced compartment sizes compared to the respective control treatments.

There was no significant difference in the BFA-induced compartment sizes among eATP-treated plants, regardless of the treatment time of eATP.

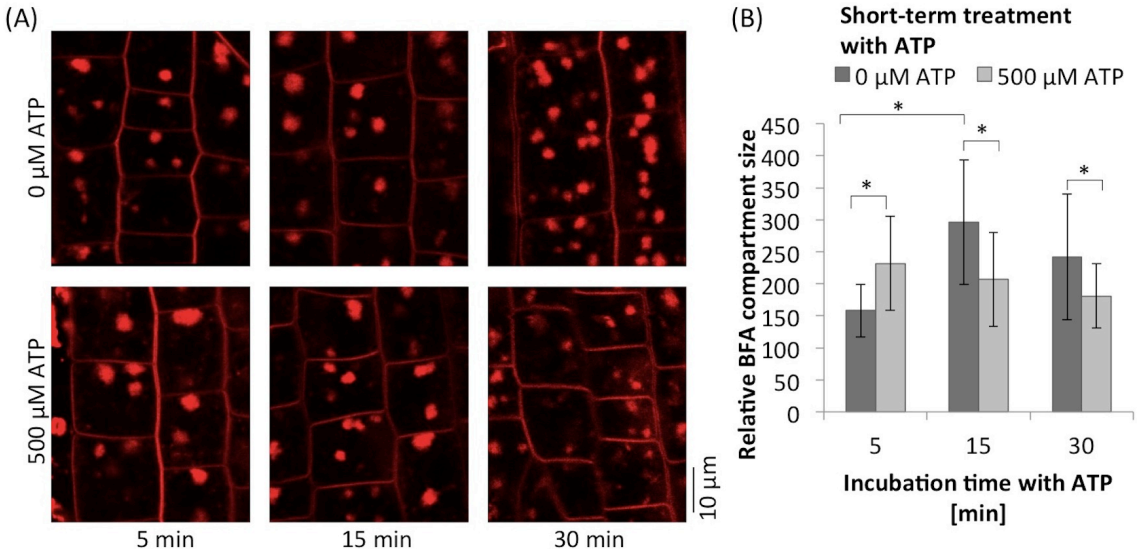

Fig. 5-6. BFA-induced compartments after short-term eATP treatments in epidermal cells of Col-0. (A) Confocal microscopy pictures of BFA-induced compartments with 0 or 500 µM ATP. (B) Relative BFA compartment size. BFA compartment sizes were measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: *P < 0.05.

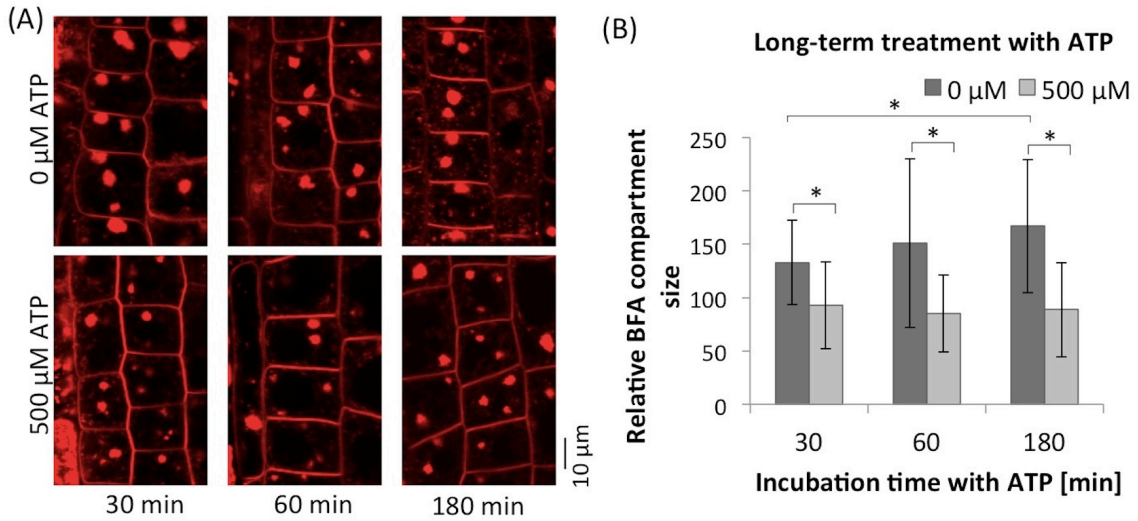

Fig. 5-7. BFA-induced compartments after long-term eATP treatments in epidermal cells of Col-0. (A) Confocal microscopy pictures of BFA-induced compartments with 0 or 500 µM ATP. (B) Relative BFA compartment size. BFA compartment sizes were measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: *P < 0.05.

5.4.7 eATP Affects Endocytosis in Roots of the eATP Receptor DORN1 Mutant Lines

Endocytic recycling activity in both the eATP receptor mutant *dorn1-1* and *oxDORN1* lines was analyzed using BFA-induced compartments in cells in the root apex transition zone, using the same method discussed above. In this study, seedlings were first treated with 500 μM of ATP solution for 30 min, followed by the treatment of the BFA solution for either 30 min or 90 min to compare the rate of the formation of BFA-induced compartments at different time points. Interestingly, *dorn1-1* roots showed larger BFA-induced compartment sizes than Col-0 roots (Fig. 5-8). 500 μM eATP reduced the sizes of the BFA-induced compartments in Col-0 and *oxDORN1* roots with 30 min BFA treatment, while they were not reduced in *dorn1-1* roots (Fig. 5-8). In conclusion, only the *oxDORN1* roots showed no significant difference in sizes of BFA-induced compartments after 90 min of BFA treatment (Fig. 5-9).

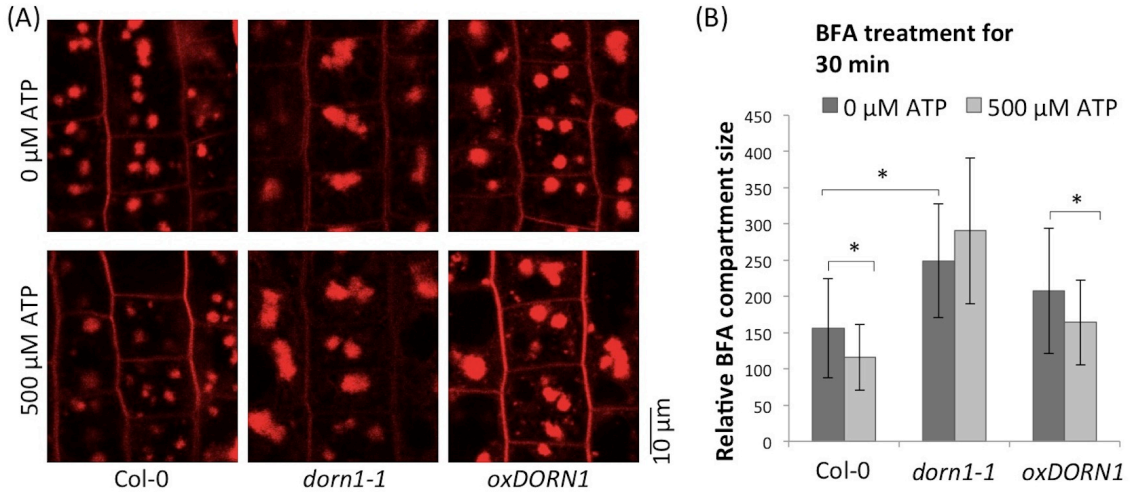

Fig. 5-8. The Effects of eATP on BFA-induced compartments in epidermal cells of Col-0 and mutants: 30 min BFA. (A) Confocal microscopy pictures of BFA-induced compartments with 0 or 500 μM ATP. (B) Relative BFA-induced compartment size. BFA compartments of Col-0, *dorn1-1* and *oxDORN1* were respectively measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: *$P < 0.05$.

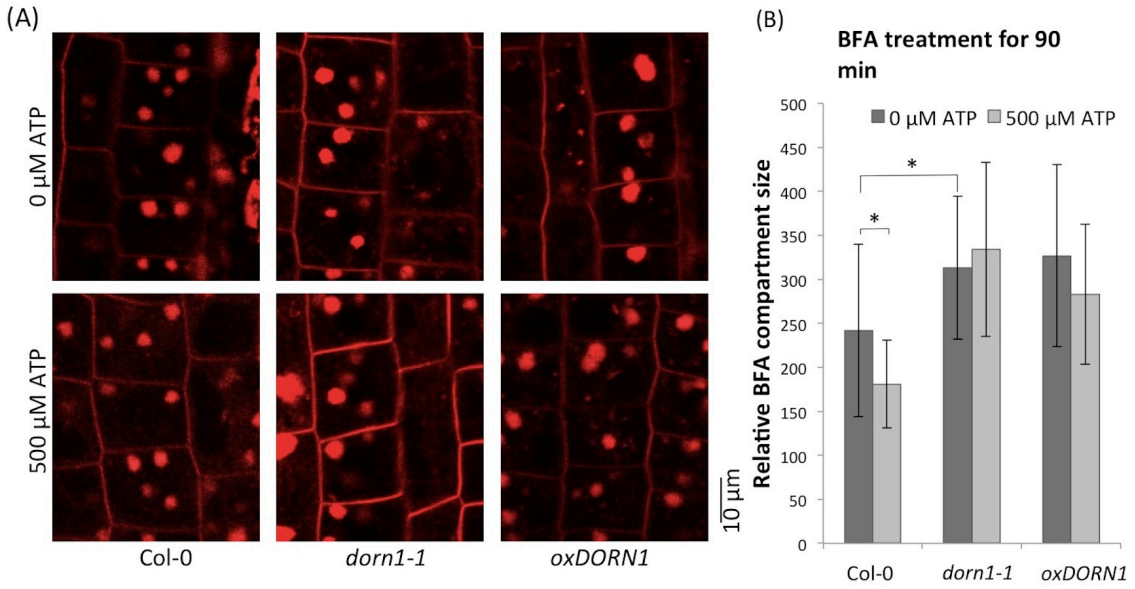

Fig. 5-9. Effects of eATP on BFA-induced compartments in epidermal cells of Col-0 and mutants: 90 min BFA. (A) Confocal microscopy pictures of BFA-induced compartments with 0 or 500 µM ATP. (B) Relative BFA compartment size. BFA compartments of Col-0, *dorn1-1* and *oxDORN1* were measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: *$P < 0.05$.

5.4.8 eATP Enhances Exocytosis in Col-0 and dorn1-1, but not in oxDORN1 Roots

Exocytic activity was assessed by BFA compartment size by washing out the BFA reagent from roots. Seedlings were incubated in BFA for 90 min. The seedlings were treated in 0 or 500 mM ATP. In the results, ATP enhanced the rate of recovery from BFA (meaning that compartments become smaller quickly) in Col-0 and *dorn1-1*, but not in *oxDORN1* (Fig. 5-10).

5.4.9 AMP and ADP Affect Endocytic, but not Exocytic, Pathways in Endocytic Vesicle Recycling

Besides ATP, the effects of AMP and ADP on endocytic vesicle recycling were also assessed by comparing BFA-induced compartment sizes. Seedlings were incubated in a control, AMP or ADP test solution for 30 min, followed by the treatment of BFA for 90 min. The obtained results show that the ADP treatment reduced BFA compartment size compared to the control treatment in epidermal cells, while AMP showed no effect on BFA compartment size. Next, exocytic activity was also observed by washout of BFA, as described above. The results show

that neither AMP nor ADP altered the speed of recovery from BFA compartments, reflecting the rate of exocytosis (Fig. 5-11).

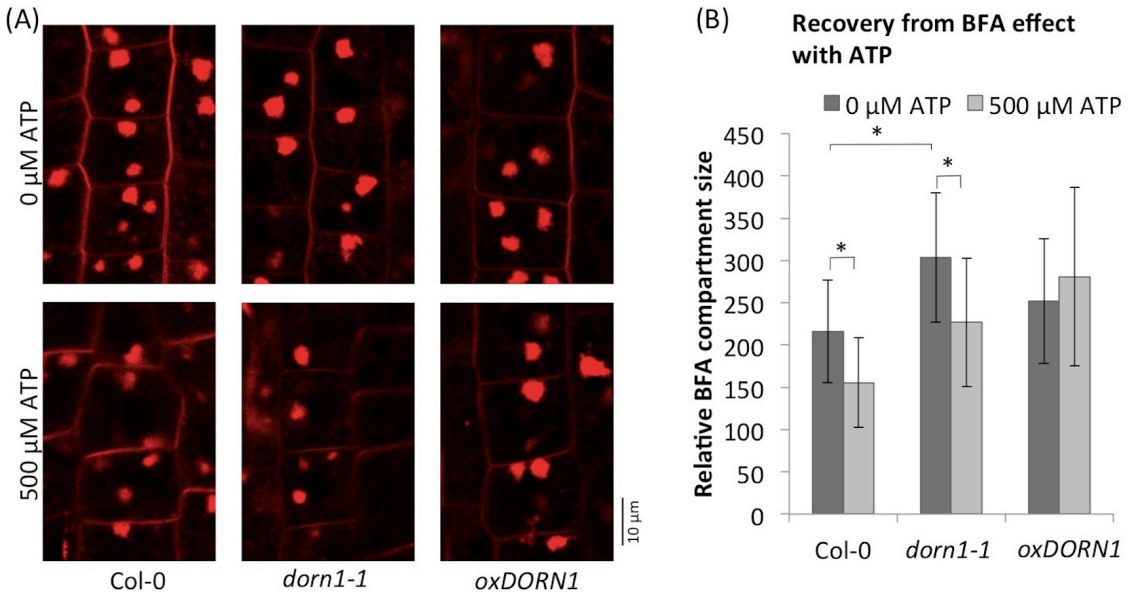

Fig. 5-10. Recovery of BFA-induced compartments in root epidermal cells of Col-0 and mutants. (A) Confocal microscopy pictures of recovery of BFA-induced compartments with 0 or 500 μM ATP. (B) Relative BFA compartment size. BFA compartments of Col-0, *dorn1-1* and *oxDORN1* were respectively measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: *$P < 0.05$.

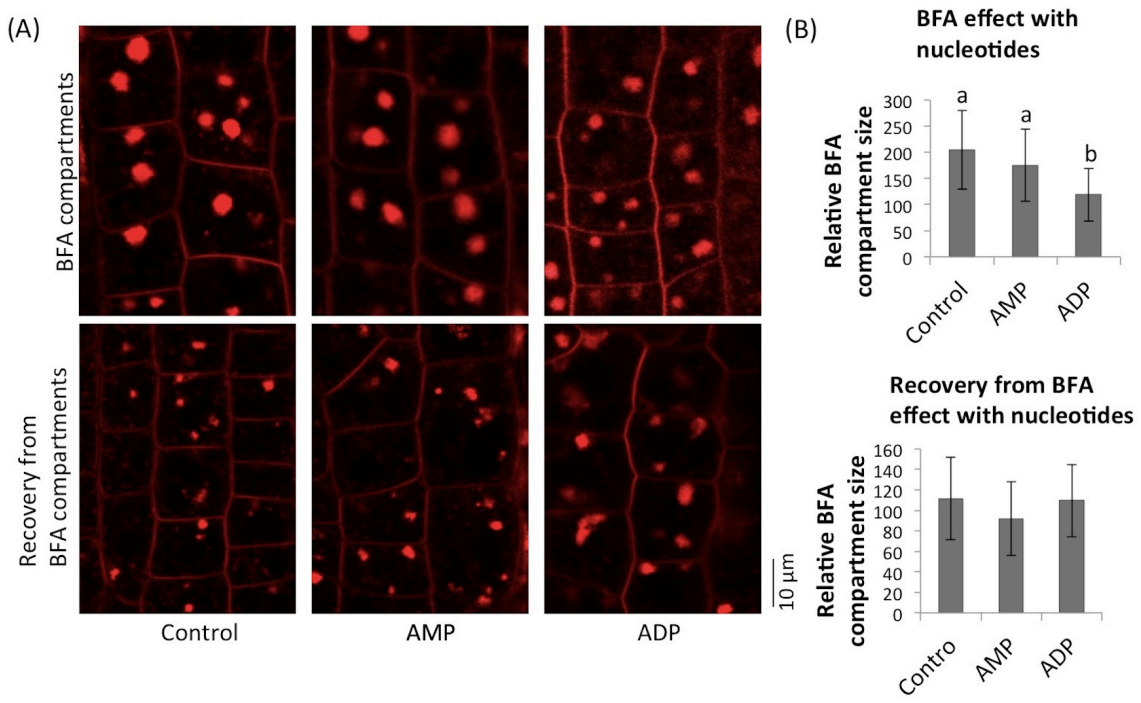

Fig. 5-11. BFA-induced compartments after AMP and ADP pre-treatments in epidermal cells of Col-0. (A) Confocal pictures of BFA compartment with AMP or ADP. (B) Confocal pictures of recovery from BFA. (C) Relative BFA compartment sizes were measured from five seedlings (n = 25). Error bars indicate SD. Different letters in the graphs indicate significant differences (Tukey's HSD test, $P < 0.05$).

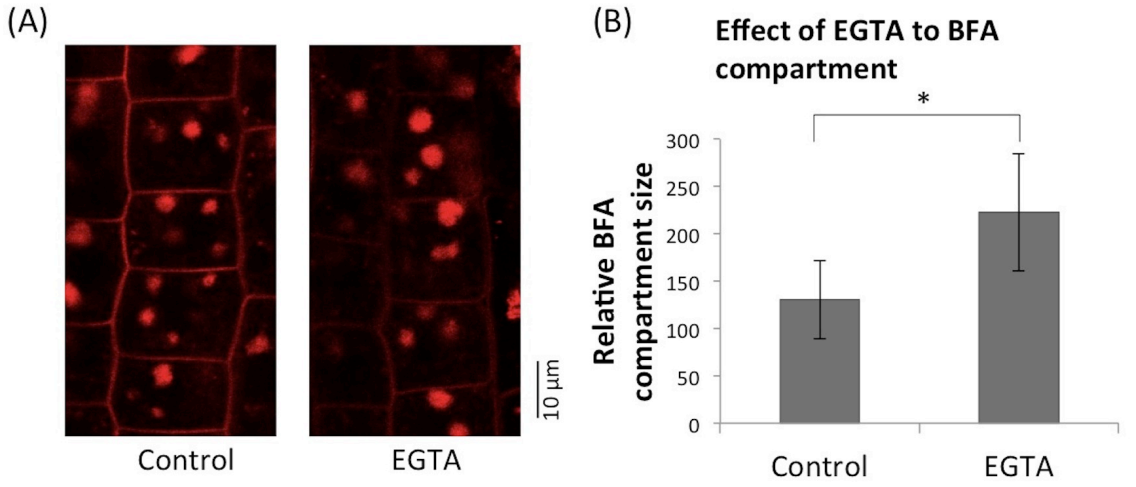

Fig. 5-12. BFA-induced compartments after EGTA pre-treatment in epidermal cells of Col-0. (A) Confocal pictures of BFA-induced compartments. (B) Relative BFA compartment sizes were measured from five seedlings (n = 25). Error bars indicate SD. The statistical significances of mean differences were analyzed using a Student t-test: $^*P < 0.05$.

5.4.10 Effect of Ca^{2+} on Endocytic Vesicle Recycling

The effect of Ca^{2+} on the formation of BFA-induced compartments, which correspond to the endocytic pathways, was observed with the treatment of an apoplastic calcium chelator, EGTA. Seedlings were incubated with or without 5 mM EGTA for 30 min before the BFA treatment of 90 min. The obtained results show that the pre-treatment with EGTA induced the formation of larger BFA compartments in cells of the transition zone than those in control cells (Fig. 5-12).

5.4.11 DORN1 Might Be Involved in Cell Wall-Membrane Adhesion and Rigidity of Plasma Membrane

eATP receptor DORN1 consists of a kinase, transmembrane domain, and a lectin receptor kinase-I.9 (Choi et al., 2010). It has been reported that the lectin receptor kinase is involved in the adhesion between the plasma membrane and cell wall in *Arabidopsis* (Gouget et al. 2006). In order to study the participation of DORN1 in maintaining rigidity of the plasma membrane, the plasmolyzed root cells in eATP receptor mutants (*dorn1-1* and *oxDORN1*) were observed using a treatment of a hypertonic solution of mannitol. In this study, seedlings were exposed to 800 mM mannitol for 10 min and the plasma membrane was visualized with the FM 4-64 dye. The results indicate that transition zone cells of the *dorn1-1* roots showed a shrunken shape, while cells of the *oxDORN1* roots showed a round-quadrate (swollen) shape. On the other hand, the Col-0 root showed oval-shaped cells (Fig. 5-13).

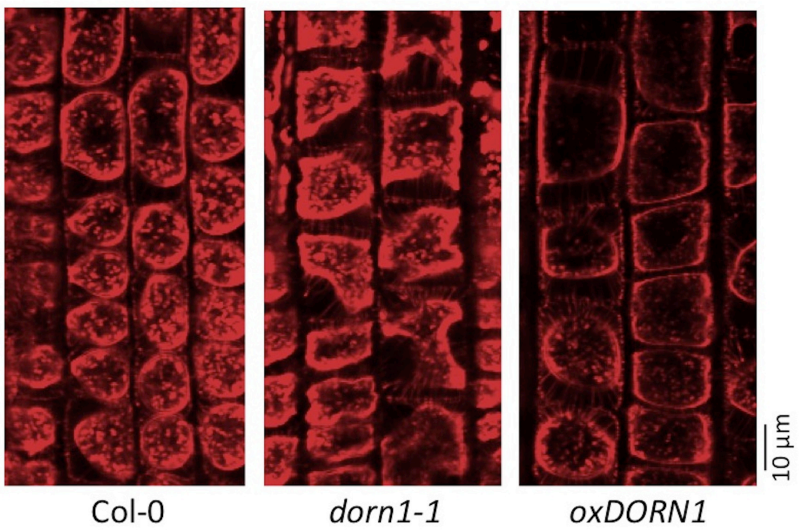

Fig. 5-13. Confocal pictures of mannitol-induced plasmolysis in root apex cells of Col-0 and eATP receptor mutants. Five replications yielded similar results. A representative example is presented.

5.4.12 Change of Cytosolic pH by eATP Treatment or Root Gravistimulation

It has been reported that changes in pH value are important for root graviresponse (Scott and Allen, 1999; Monshausen and Sievers, 2002). These reports show that eATP inhibits gravitropic response, but the mechanism is unknown. To assess these two factors, the effect of eATP on cytosolic pH was observed using a pHusion (pH sensor) transgenic *Arabidopsis* line. pHusion seedlings were soaked in an ATP solution (pH 5.8) for 30 min. The pH values were calculated using the ImageJ software and confocal microscopy images. The obtained results show that 500 μM eATP treatment shifted the cytosolic pH value to acidic conditions in cells of the root tip region (including the root cap cells). Next, pH changes during root gravitropic responses were observed. Gravitropic stimulation of roots for 30 min resulted in a shift of pH to acidic values in the apex (Fig. 5-14).

5.4.13 pH Effects on eATP-Mediated Endocytic Vesicle Recycling

BFA-induced compartment sizes were monitored after treatment with 500 μM ATP at three different pH values in cells of the transition zone (Fig. 5-14). As Fig. 5-14 shows, smaller BFA-induced compartments were observed when roots were exposed to eATP at acidic (low) pH values (at 4.5 and 5.8). This important result suggests that the eATP-dependent inhibition of endocytosis requires acidic conditions.

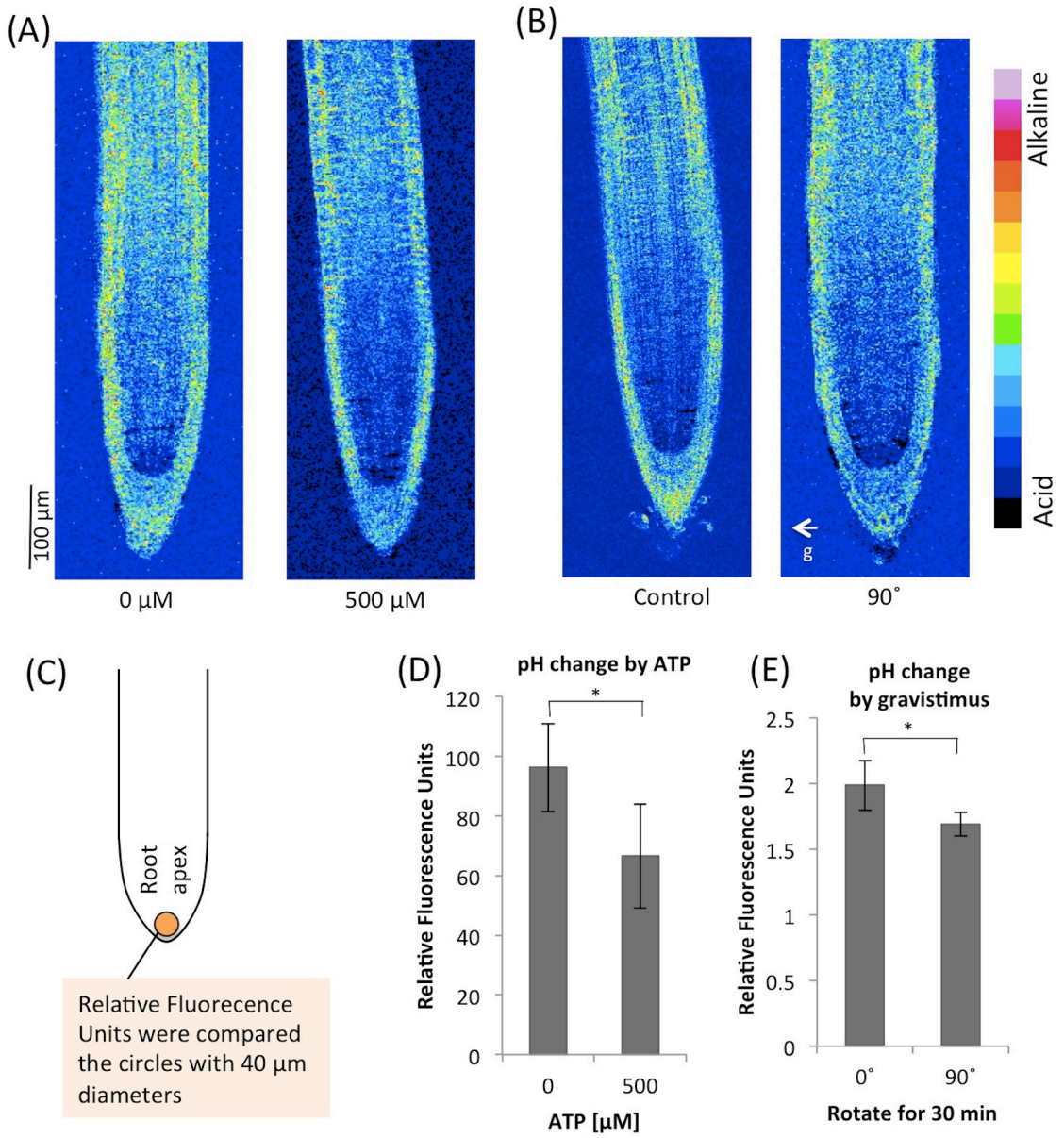

Fig. 5-14. pH responses to eATP or gravitropism. (A) Confocal microscopy pictures of the pHusion line (transgenic *Arabidopsis* line of the pH sensor) exposed to eATP. (B) Confocal microscopy images of the pHusion line responding to gravitropism. (D) Relative fluorescence unit by ATP. (E) Relative fluorescence unit by gravitropism. Error bars indicate SD. Statistical significances were determined using a Student *t*-test: (*$P < 0.05$, n = 6-7).

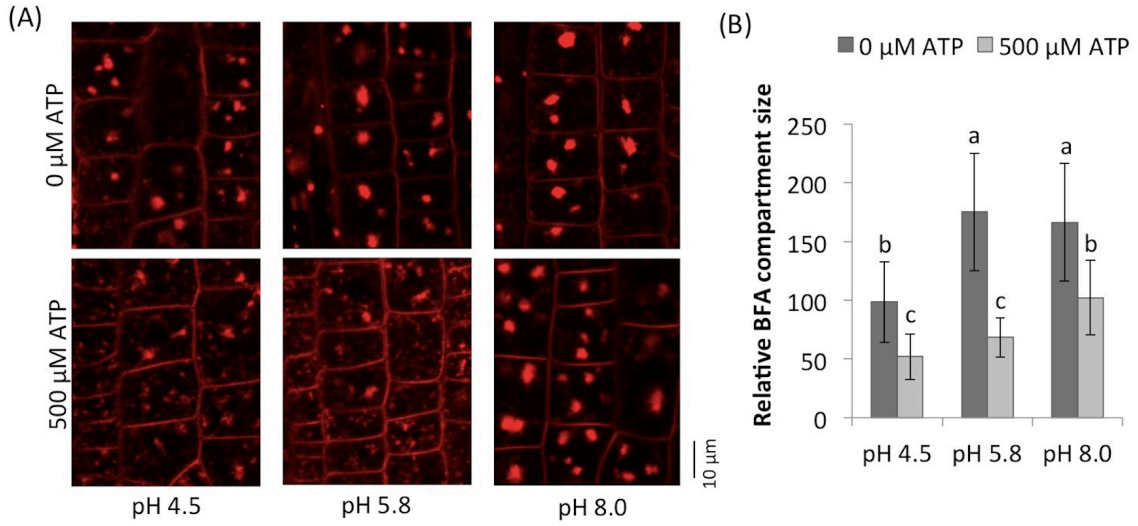

Fig. 5-15. BFA-induced compartments with each pH in epidermal cells at the transition zone of Col-0. (A) Confocal images of BFA-induced compartments. (B) Relative BFA compartment sizes were measured from four or five seedlings (n = 40-50) by ImageJ. Error bars indicate SD. Different letters in the graphs indicate significant differences (Tukey's HSD test, $P < 0.05$).

5.5 Discussion

5.5.1 eATP (pH 5.8) Effects on Root Elongation

eATP (adjusted with K-Phosphate buffer to pH 5.8) effects were observed by comparing root growth under different eATP levels. At levels higher than 100 µM, eATP (pH 5.8) inhibited root elongation within 24 hours (Fig. 5-3A). The effects of eATP (pH 5.8) become weaker with no pH adjustment as the ATP molecule lowers the pH of solutions. As was shown in Chapter 4, an un-buffered eATP solution inhibited root elongation at a low concentration (1 µM) compared to the buffered eATP solution (100 µM). This result might support the previously published report showing that eATP in low pH conditions is more effective at inhibiting polar auxin transport and gravitropism of roots (Tang et al., 2003). However, although Tang et al. (2003) reported that 3 mM eATP (pH was adjusted by MES buffer) inhibited root growth, in the present study eATP inhibited the root growth already at 100 µM. This difference between the eATP concentrations' effectivity might be caused by hydrolyzation of ATP on the agar plates, as ATP is an unstable molecule and is sensitive to temperature and long-term preservation.

Therefore, in the present study ATP was added to a melted agar (*ca*. 60˚C) after autoclaving. To avoid the effects of ATP degradation, seedlings were treated with eATP solutions only a short time (30 min) before starting the growth experiment.

5.5.2 The Impact of eATP on Crawling Movement and Root Gravitropic Responses

eATP attenuates the crawling movement of maize root apices (Fig. 5-1). This crawling root apex movement is a complex response of integrating root gravitropism and thigmotropism (Hahn et al., 2006). Next, the root gravitropic response was inhibited within five hours observed after the application of eATP (pH 5.8) in the root apices of *Arabidopsis* (Fig. 5-4B). However, the inhibition of root apex bending by eATP was recovered eight hours after the application of eATP in the range of concentration tested from 100 to 1000 μM. This result indicates eATP likely give reversible effect on root. Tang et al. (2003) reported that 3 mM of an eATP-containing medium inhibited the root apex gravitropic response, and this inhibition lasted for two days. If roots are kept growing under a high eATP concentration for so long time, the roots might not be able to recover from the inhibition of root gravitropism. eATP applied to root surfaces might be hydrolyzed due to a long duration of incubation, or degraded due to the catalytic activity of ecto-apyrase, known as nucleotidase, located on the cell surface. eATP is reported that might be an important signaling molecule involved in roots' ability to grow over the barrier surface requires temporary suspension of gravitropic responses (Weerasinghe et al., 2009; Tanaka et al., 2010; Yang et al., 2015). Here, my results suggest an additional mechanism of the root apex obstacle avoidance using eATP signaling (Fig. 5-17).

5.5.3 The Effects of AMP and ADP on Root Gravitropic Responses

The data obtained from this study show that 500 μM ADP inhibited root curvature within five hours in Col-0 and *oxDORN1* roots. However, the *dorn1-1* roots were completely insensitive to ATP, AMP and ADP (Fig. 5-5A, B and C). In mammalian studies, it has been reported that not only eATP but also other nucleotides, such as eADP and eAMP, act as signaling molecules (Burnstock et al., 2011). Similarly, according to the report by Demidchik et al. (2011), eATP and eADP molecules show distinct signaling roles in plant cells even though their structures are highly similar (ADP lacks only one phosphate moiety). DORN1 has also been reported to be responsive to eADP, but not to eAMP (Choi et al., 2014). Intriguingly, in the present study the AMP-dependent inhibition of root gravitropic response was only found in the *DORN1* ectopic

expression line, *oxDORN1*. Although it is still unclear how eAMP interacts with DORN1, it is important to be aware that it is the purine moiety, and not phosphate, that is critical for ATP signaling.

5.5.4 eATP Alters Endocytic Activity in Root Apex Cells

Endocytic recycling is essential for root apex gravitropic responses and growth (Abas et al., 2006). The inhibition of endocytic recycling in the Col-0 root apices by eATP supports the finding that eATP also inhibits root gravitropism and growth in Col-0 (Fig. 5-3B and C). Different treatment times of eATP exposures have distinct impacts on root endocytic activity. A five min eATP treatment enhanced the endocytic vesicle recycling, whereas exposures longer than 15 min showed inhibitory effects. This two-phased action of endocytic modulation by eATP suggests a possibility of different modes of eATP signaling, depending on time in roots. This finding also supports the previous report showing that a low concentration of eATP (20 or 50 μM) enhances vesicle trafficking, whereas high eATP concentrations (500 or 1000 μM) inhibit vesicle trafficking (Deng et al., 2015). Importantly, the BFA washout experiments also indicated that eATP enhances the rate of exocytosis (Fig. 5-10), suggesting that eATP disturbs the balance of the endo- and exocytic pathways in root apex cells.

Endocytic recycling activity in *dorn1-1* and *oxDORN1* root apices is also relevant. Endocytic recycling was not inhibited (it even increased) in the *dorn1-1* roots, while the activity in the Col-0 roots was inhibited in the presence of 500 μM ATP (Fig. 5-8, 9 and 10). As shown in Fig. 5-3B and 5-4C, root gravitropism of the *dorn1-1* roots was not inhibited with eATP either. These results show for the first time that eATP is perceived via the DORN1 receptor, and that still-elusive eATP-DORN1 mediated processes modulate endocytic vesicle recycling during root apex graviresponse. However, the *oxDORN1* roots showed no differences in the BFA washout experiments, whereas Col-0 and *dorn1-1* roots showed faster recovery (which means an enhanced rate of exocytosis). Taken together, these findings based on BFA experiments suggest that eATP signaling controls endocytic vesicle recycling activity *via* DORN1.

5.5.5 DORN1 Plays a Role in Cell Wall Adhesion and Rigidity of the Plasma Membrane

DORN1 might have a role in maintaining the structure of the plasma membrane, because the gene *DORN1* (At5g60300) encodes a lectin receptor kinase-I.9 (LecRK-I.9) (Choi et al., 2014), which has been reported to participate in adhesion of the plasma membrane and cell wall in

Arabidopsis (Gouget et al., 2006). In order to observe the condition of the plasma membrane among three *Arabidopsis* lines used here, this study observed plasmolyzed root cells exposed to 800 mM mannitol in the root transition zone. The results show that *oxDORN1* roots are more tolerant to plasmolysis induced by mannitol, as their cells maintained a round-quadrate shape. In contrast, the plasma membrane observed in the *dorn1-1* roots was shrunken considerably (Fig. 5-12). This result supports the finding that different endocytic activities are found in different eATP receptor mutant lines (Fig. 5-8, 9 and 10). It has been reported that the cell expansion of the plasma membrane controls endocytic vesicle recycling activity and subsequent plant signaling (Nakayama et al., 2012). The present results suggest that eATP receptor DORN1 might have a role in maintaining the plasma membrane structure with possible impacts on the cell wall adhesion domains as well as the endocytic vesicle recycling.

5.5.6 Ca^{2+} is Necessary to Induce BFA Compartments

DORN1 has been reported to induce an increase of $[Ca^{2+}]_{cyt}$ (intracellular calcium ion) with an eATP treatment (Choi et al., 2014). In order to find a link between $[Ca^{2+}]_{cyt}$ and endocytosis, seedlings were soaked in EGTA solution as a chelator of Ca^{2+} from apoplastic space before BFA treatment. According to the present results, EGTA exposure resulted in larger BFA-induced compartments than in the transition zone cells of control roots (Fig. 5-12). Based on these results, this chapter proposes a working model of eATP signaling involving Ca^{2+} (Fig. 5-16). This model suggests that DORN1 allows eATP-mediated $[Ca^{2+}]_{cyt}$ elevation by opening Ca^{2+}-channels on the plasma membrane. This high Ca^{2+} then causes both the inhibition of endocytic pathways and the promotion of exocytic pathways. This unbalanced vesicle recycling activity then influences localization of proton pumps and PIN proteins involved in the root apex tropisms and behavior.

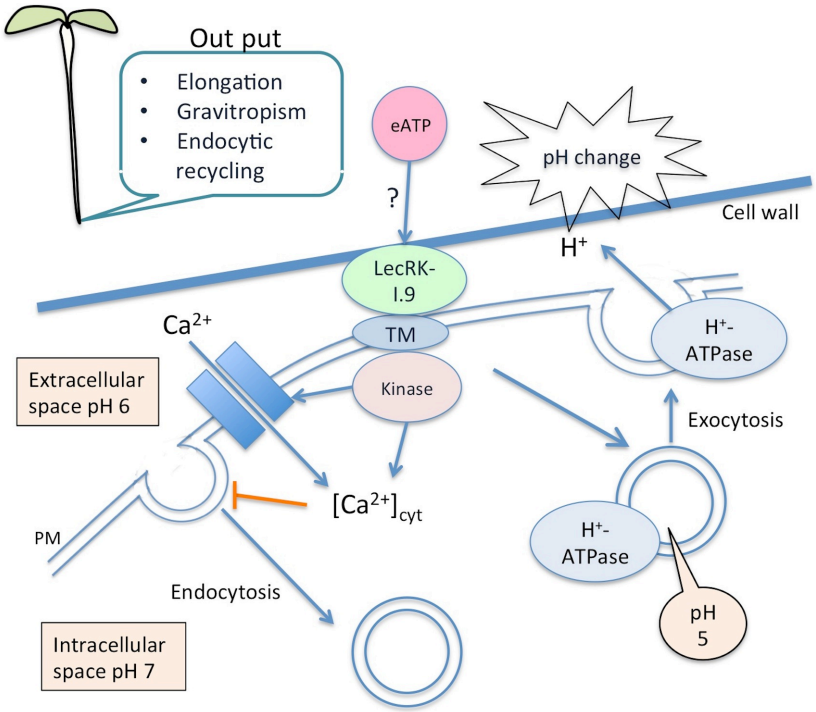

Fig. 5-16. Emerging model of eATP signaling in the root apex. At the plasma membrane, eATP stimulates DORN1, which consists of an extracellular legume-type lectin domain (LecRK-I.9), a single trans membrane domain (TM), and an intracellular kinase domain, which DORN1 induces elevation of $[Ca^{2+}]_{cyt}$ level (Choi et al., 2014). The elevated $[Ca^{2+}]_{cyt}$ is involved in the inhibition of endocytic activity, whereas exocytic activity is enhanced (orange arrows). The amount of DORN1 at the plasma membrane is also involved in the control of endocytic recycling activity, because DORN1 is involved in adhesion between the cell wall and plasma membrane (Gouget et al., 2006). It has been reported that endocytic acidity is involved in the control of the plasma membrane rigidity (Nakayama et al., 2012). The pH value within vesicles is lower than in extracellular space because of the H^+-ATPase activity (Hager et al., 1991). Therefore, when eATP signaling enhances exocytosis, extracellular pH could be lowered. In due course, root growth and gravitropism are inhibited by eATP signaling.

5.5.7 eATP Changes pH Conditions in Root Tip Cells

The pH in the root cap has been shown to have an important role in gravity responses (Fasano et al. 2001; Monshausen and Sievers, 2002). Here, it is the for first time reported that *Arabidopsis* roots exposed to eATP lower the cytosolic pH values in cells of the root tip region (Fig. 5-14). This finding of eATP-mediated pH change probably explains why eATP inhibited root gravitropic responses. In mammals, it has been reported that secreted eATP lowers extracellular

pH in retinal cells (Vroman et al., 2014). It is known that ATP is released *via* exocytic pathways into extracellular space (Tanaka et al., 2010). If plant cells secrete eATP that can locally lower pH in extracellular space, eATP could become even more effective in local regions. Tang et al. (2003) previously reported that at low pH values, eATP is more effective at inhibiting root gravitropism and auxin transport.

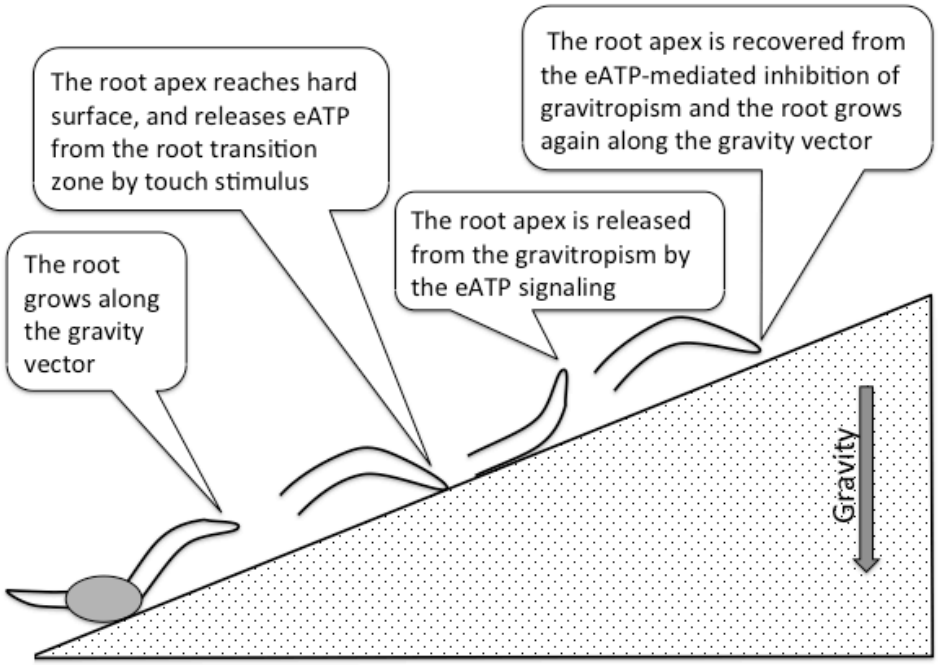

Fig. 5-17. Hypothetical model of root apex crawling controlled by eATP. First, the root grows directed to the gravity vector. Second, the root apex reaches a hard surface and releases eATP from the root transition zone by touch stimulus (Weerasinghe et al., 2009). Third, the root apex is released from the gravitropism by eATP signaling (Tang et al., 2003). Fourth, the root apex is recovered from the eATP-mediated inhibition of gravitropism and the root grows again directed to the gravity vector.

5.5.8 The Influence of pH on Endocytic Vesicle Recycling

It is known that extracellular pH affects root hair growth (Monshausen et al., 2007) and tropisms (Scott and Allen, 1999). It has been reported that impacts of exogenously applied ATP are dependent on the pH values, which influence the uptake of sucrose (van Bel and Reinhold, 1975) and the inhibition of polar auxin transport (Tang et al., 2003). Moreover, it has been reported that the presence of 1 mM ATP drops the pH value of un-buffered MS medium down to pH 4.8 (Tang et al., 2003). Of course, many eATP studies in plants have used a buffered eATP solution adjusted around pH 5.5 to 7.2 by MES (Song et al., 2006; Wang et al., 2014;

Jeter et al., 2004; Hao et al., 2012; Choi et al., 2014), NaOH (Zhang and Mou, 2009), or HEPES (Deng et al., 2015). Nevertheless, some other studies have reported that un-buffered eATP (with no pH adjustment) still affects plant growth or tropisms (Wu and Wu, 2008; Tang et al., 2003). The present study observed the effects of pH on sizes of BFA-induced compartments with an eATP treatment. The pH values were adjusted with HCl or NaOH. The results show that at a low pH (pH 4.5 and 5.8), eATP inhibited the formation of BFA-induced compartments (Fig. 5-15), whereas eATP showed no effect in alkaline environments. This suggests that the eATP-mediated modulation of endocytic vesicle recycling requires acidic conditions, which affect the conformation of ATP molecule.

5.5.9 eATP Functions as a pH Modulator?

This chapter has discussed the impacts of exogenously applied ATP on root growth and gravitropism mediated *via* endocytic vesicle recycling. However, ATP must first be released from the inside of cells in order to be a functional molecule extracellularly. As Vroman et al. (2014) demonstrated, eATP might be secreted out of cells to lower extracellular pH, which is required for changing physiological properties of cells. In fact, ATP itself is a phosphoric acid and potentially lowers pH value if it is released into the micro region between the plasma membrane and cell wall *via* vesicle-based exocytosis. Therefore, this chapter proposes that the lowering of the pH could be one of the roles of secreted eATP in root tropism, besides eATP's role as a signaling molecule perceived by the DORN1 receptor. However, the potential influence of pH values on endocytic vesicle recycling is still unclear.

It has been demonstrated that ATP binds divalent cations (Mg^{2+}, Ca^{2+}) at middle to alkaline conditions and forms a stable structure (Carvalho and Leo, 1967; Ramirez et al., 1980). It has also been reported that ATP is easily hydrolyzed under acidic conditions (Zhang et al., 2015), suggesting that although eATP is unstable in acidic regions, it can still behave as a reactive signaling molecule. However, further studies are necessary to explain how eATP release is controlled, or how pH is modulated by eATP locally.

General Discussion

Since plants are sessile organisms, plant roots interfere with a number of chemical compounds in the rhizosphere. The root apex transition zone shows high sensitivity to environmental stimuli based on the high activity of endocytic vesicle recycling. Therefore, investigations of the cellular events in the transition zone in response to environmental stimuli may provide a comprehensive understanding of plants' environmental adaptations in their sessile life.

The aim of this thesis was to investigate certain aspects of root adaptation to the environment by comparing the activity of endocytic vesicle recycling in the root apex transition zone after certain environmental stimuli such as light, MES buffer, solvents (DMSO and ethanol), and eATP as an extracellular signaling molecule in *Arabidopsis*. The thesis compared responses of endocytic recycling, root growth, and gravitropic responses after applying these stimuli. The obtained results indicate that the root apex transition zone shows dynamic changes of the endocytic recycling in response to light stimuli, solvents, eATP, and changes in pH values, resulting in changed root growth and root tropisms. Furthermore, endocytic vesicle recycling activity is likely to be involved in maintaining mechanical and structural homeostasis of the plasma membrane.

6.1 Impacts of Light, MES, DMSO and EtOH on Endocytic Vesicle Recycling

First, this thesis compared the BFA compartment size and PIN2-GFP localization in the root apex transition zone of *Arabidopsis* seedlings grown in different light conditions. The results show that light promoted the activity of endocytic vesicle recycling, whereas the darkness treatment attenuated the endocytic vesicle recycling. Responses of roots to incoming light are likely extremely quick. Yokawa et al. (2011) report that the root apex generated ROS in response to 10 seconds of illumination with blue light. Zhang et al. (2013) report that 30 min of unilateral blue light illumination changed the distribution of PIN3 on the illuminated side versus the shaded side in the root cap of columella cells in *Arabidopsis*. Laxmi et al. (2008) show that PIN2-GFP on the plasma membrane in light-grown roots was reduced to 62% of the initial level 12 hours after a dark treatment. The results presented in this thesis show that the PIN2-GFP

signal found in vacuoles and vacuole-like small compartments of 24-hour dark treatment roots was stronger than in the dark-grown roots. Therefore, the roots of such seedlings experience the "buffering memory" of illumination and they are in "alert" modus with respect to their PIN2 behavior.

6.2 DMSO and EtOH

Both DMSO and EtOH are essential as solvents for chemicals in many biological experiments. In the present studies, these chemicals altered endocytic recycling activity and root growth. Interestingly, a low concentration of DMSO and EtOH (both at 0.01%) inhibited root growth only in the light condition (normal growth situation in plant laboratories). This is consistent with the author's previous report that *Arabidopsis* roots change their response to stress situations when roots are under light stress (Yokawa et al., 2014).

1% DMSO changed both the end/exocytic pathways, whereas both 0.1 and 1% of EtOH had no effect on endocytosis. However, EtOH slowed down the rate of exocytosis with a concentration of 0.1%. Together, DMSO and EtOH changed vesicle trafficking in different ways. DMSO (1%) might inhibit endocytosis *via* modulation of F-actin assembly. The alignment of F-actin arrays localized at the cross-wall (designated with the white arrow head in Fig. 2-4) disappeared in the presence of 0.1 and 1% EtOH, whereas DMSO caused no change. End-poles play an important role for maintaining polarity of root growth (Lindsey, 2009; Baluška et al., 2003). In this region, the presence of abundant F-actin and active vesicle-based trafficking has been reported (Baluška et al., 1987, 2000, 2001, 2002). Root epidermal cells were plasmolyzed by osmotic pressure with 800 mM mannitol. It has been reported that both EtOH and DMSO molecule directly react with and change membrane conditions (Winckler et al., 1999; Notman et al., 2007; Cheng et al., 2015). In the present results, distortion of the plasma membrane shape was observed in the presence of DMSO or EtOH. Interestingly, a shrunken shape of cells was found in the DMSO-treated roots compared to the control roots. On the other hand, EtOH-treated cells showed swollen shapes. As mentioned above, DMSO and EtOH might change the rigidity of the plasma membrane in different ways, resulting in the inhibition of root growth.

6.3 MES

Next, this study investigated the effects of different concentrations of MES buffer using growing roots of *Arabidopsis thaliana*. MES is broadly used as a buffering medium in plant experiments. In the obtained results, MES changed root growth, morphogenesis and root tropic behavior. Furthermore, histochemical staining for superoxide (ROS) generation in the root apex revealed that MES strongly interferes with ROS homeostasis at 1%, but not at 0.1%. MES molecules were reported to have peroxidase reactivity. Peroxidase is abundant in plant cell walls, being active in the maintenance of normal ROS homeostasis (Baker et al., 2007). Since ROS has been shown to have an important role for root growth and root apex zonation (Tsukagoshi et al., 2010), all root growth studies must be taken into account for using the MES-containing buffer.

Importantly, in studies investigating root growth and tropisms, attention must be paid to the light conditions of the incubation room, as well as concentrations of solvents and components of media.

6.4 Extracellular ATP (eATP)

6.4.1 eATP Inhibits Root Growth

eATP inhibited the root growth of seedlings incubated in the 16h/8h light condition. However, this effect was not observed in the dark-grown roots. This suggests that roots become sensitive to eATP when they are illuminated, as has previously been reported (Yokawa et al., 2014). The experimental procedures followed the previous report that used eATP-containing agar plates (Tang et al., 2003). Although Tang et al. (2003) reported that 3 mM eATP inhibits root growth, in the present study as little as 1 mM eATP inhibited root growth. Next, pH-adjusted eATP (pH 5.8 with KOH) was applied to the root tips. The results of this thesis show that eATP inhibited the root growth already at 100 μM of concentration. These differences in eATP concentrations might be caused by the hydrolyzation of ATP on the agar plates. ATP is an unstable molecule that is very sensitive to temperature and other environmental factors in long-term experiments.

6.4.2 eATP Reversibly Inhibited the Crawling Movement and Gravitropic Responses in Root Apices

The root gravitropic response was observed after the application of eATP (pH 5.8 with KOH) in the root apices. In the results, concentrations of the eATP treatment higher than 100 μM of inhibited root bending within five hours. The inhibition of root bending by eATP was recovered eight hours after the application of eATP to the roots (Fig. 5-4B). This supports the hypothesis that eATP is important for signaling, allowing the root to grow over barrier surfaces. eATP-exposed roots are free from gravitropic responses due to the eATP-mediated loss of root gravitropism (Tanaka et al., 2010; Yang et al., 2015). The present results indicate that eATP inhibits root gravitropism in a reversible way. The recovery phase is needed to grow roots down along the gravity vector again after the avoidance of obstacles.

6.4.3 Other Extracellular Nucleotides

In mammalian studies, it has been reported that extracellular nucleotides act as signaling molecules; this has been found not only for ATP but also for adenine, AMP and ADP (Burnstock et al., 2011). The present results show that 500 μM ADP inhibited root gravibending within five hours in both the Col-0 and *oxDORN1* roots. However, *dorn1-1* roots are completely insensitive to ATP, AMP and ADP (Fig. 5-5A, B and C). Demidchik et al. (2011) reported that eATP and eADP are distinct in terms of their signaling roles in plant cells, even though their molecular structures are highly similar (ADP lacks only one phosphate moiety). DORN1 was reported to also be responsive to ADP but not to AMP (Choi et al., 2014). Intriguingly, AMP-dependent inhibition of root gravibending was found only in the *oxDORN1*. Although the relationship between AMP and *oxDORN1* is unclear, this finding might provides an important clue to the understanding of the purine moiety function of ATP signaling.

6.4.4 eATP Changes Endocytic Recycling via *DORN1 Activity*

Endocytic vesicle recycling is essential for root growth and gravitropic responses (Abas et al., 2006). This thesis has shown that five min of eATP (at pH 5.8) treatment enhanced the endocytic vesicle recycling, whereas treatments longer than 15 min inhibited recycling. This two-phased action of eATP suggests two different modes of eATP signaling, depending on the time factor. This finding also supports the previous report showing that low concentrations of eATP (20 or 50 μM) enhance vesicle trafficking, whereas high concentrations of eATP (500 or 1000 μM) inhibit it (Deng et al., 2015). The present BFA washout experiments (Fig. 5-9)

revealed that eATP enhanced the rate of exocytosis, suggesting that eATP disturbs the balance of endo- and exocytic pathways in root cells. Meanwhile, the endocytic recycling in the *dorn1-1* roots was not inhibited but rather increased. These results suggest that eATP is perceived in root apices *via* the function of DORN1, and that DORN1 mediates the modulation of endocytic vesicle recycling during root graviresponse. However, while eATP enhanced endocytic vesicle recycling in the Col-0 and *dorn1-1* roots, the *oxDORN1* roots showed no changes in the BFA washout experiments. Taken together, the BFA experiments suggest that eATP changes the endocytic vesicle recycling process *via* DORN1 activity.

6.4.5 DORN1 Plays a Role in Cell Wall Adhesion and Rigidity of Plasma Membrane

This study observed plasmolyzed root cells with 800 mM mannitol in the root apex transition zone. Importantly, the *oxDORN1* roots were more tolerant to mannitol-induced plasmolysis, as cells maintained a round-quadrate shape. In contrast, the dorn1-1 roots were more sensitive to mannitol and their cells were strongly plasmolyzed (Fig. 5-12). Interestingly in this respect, DORN1 (At5g60300) encodes a lectin receptor kinase-I.9 (LecRK-I.9) (Choi et al., 2014), which has been reported to participate in the adhesion of the plasma membrane and cell wall in *Arabidopsis* (Gouget et al., 2006). The present results show different endocytic and recycling activities found in different eATP receptor mutant lines (Fig. 5-8, 9 and 10). These findings are relevant, as it has been reported that the cell expansion of the plasma membrane controls endocytic vesicle recycling activity and subsequent plant signaling (Nakayama et al., 2012).

6.4.6 Ca^{2+} Mediates eATP-DORN1 Effects on Vesicle Recycling

EGTA was used as a chelator of Ca^{2+} from apoplastic space. EGTA-mediated Ca^{2+} chelating resulted in larger BFA-mediated compartments than those found in cells of the transition zone of control roots (Fig. 5-12). DORN1 has been reported to induce an increase in $[Ca^{2+}]_{cyt}$ (intracellular calcium ion) with eATP treatment (Choi et al., 2014). This indicates that the DORN1 response to eATP includes an increase in the $[Ca^{2+}]_{cyt}$ concentration by opening Ca^{2+} channels in the plasma membrane. This might result in both the inhibition of endocytic recycling and the promotion of exocytic pathways (Fig. 5-16). This unbalanced vesicle recycling activity then influences the localization of proton pumps and PIN proteins involved in root tropisms.

6.4.7 eATP Changes pH Values in Cells of Root Apex Regions

This is the first report on *Arabidopsis* roots showing that the treatment of eATP decreases the cytosolic pH value in cells of the root tip region (Fig. 5-14). The pH in the root cap has been shown to have an important role in gravity responses (Fasano et al. 2001; Monshausen and Sievers 2002). The finding shown in the Chapter 5 probably explains that ATP-mediated pH change inhibited root gravitropic response. In mammals, it has been reported that secreted eATP lowers pH in extracellular space in retinal cells (Vroman et al., 2014). It is known that ATP is released *via* exocytic pathways into extracellular space (Tanaka et al., 2010). If plant cells secrete eATP that can decrease pH in extracellular space locally, eATP could become more effective at a local region. Tang et al. (2003) previously reported that eATP was more effective in inhibiting root gravitropism and auxin transport at low pH values.

6.4.8 The Influence of pH Values on Endocytic Vesicle Recycling

This study observed the effects of pH on BFA-induced compartment size manipulated with an eATP treatment. At acidic pH values (pH 4.5 and 5.8), eATP inhibited the formation of BFA-induced compartments (Fig. 5-15), whereas no effect was found in an alkaline environment. It is known that extracellular pH affects root hair tip growth (Monshausen et al., 2007) and root apex tropisms (Scott and Allen, 1999). Moreover, it has been reported that an impact of exogenously applied ATP is dependent on pH value, which influences the uptake of sucrose (van Bel and Reinhold, 1975) and the inhibition of auxin transport (Tang et al., 2003).

Thus, this thesis proposes that the lowering of pH could be one of the roles of secreted eATP in root tropism, besides its role as a signaling molecule perceived by DORN1. However, the influence of pH values on endocytic vesicle recycling is still unclear.

It has been demonstrated that ATP binds divalent cations (Mg^{2+}, Ca^{2+}) at middle to alkaline conditions and forms a stable structure (Carvalho and Leo, 1967; Ramirez et al., 1980). It is also known that ATP is easily hydrolyzed under acidic conditions (Zhang et al., 2015), suggesting that although the ATP molecule is unstable in acidic regions, it can still behave as a reactive signaling molecule. However, further studies are necessary to explain how eATP release is controlled, and how pH is modulated by eATP locally.

6.4.9 NADPH oxidase C (AtRBOHC) is Required for eATP Signaling

This study also discovered the relationship between NADPH oxidase C (AtRBOHC) and eATP

signaling with *rhd2-4* by root growth, the ratio of cell damage, and endocytic recycling activity. It has been reported that eATP induced the increase of cytosolic Ca^{2+} (Demidchik et al., 2003) through the generation of superoxide ($O_2^{\bullet-}$) *via* NADPH oxidase on the plasma membrane that activates the Ca^{2+} channel opening (Song et al., 2006; Jeter et al., 2004; Demidchik et al., 2009; Shang et al., 2009; Wang et al., 2014). In the present results, *rhd2-4* roots, mutated in NADPH oxidase C, responded more weakly than Col-0 roots to eATP exposures. This result supports the notion that the eATP signaling in root apices requires ROS generation by NADPH oxidase C.

This thesis showed the responses used by roots to adapt to their extracellular environment by comparing the activity of endocytic vesicle recycling in the root transition zone, root growth, and root gravitropism after some environmental stimuli, such as light, MES buffer, solvents (DMSO and ethanol), and eATP in *Arabidopsis*. Further, endocytic recycling activity was revealed to have the function of maintaining plasma membrane rigidity. As was already mentioned, the root apex transition zone may have an important role in controlling the rhizosphere. The present data show that the root apex zonation was changed by ROS in the presence of an MES buffer. Moreover, ROS was also shown to be an important second messenger in eATP signaling, controlling endocytic vesicle recycling *via* NADPH oxidase. This might suggest that plant root changes its cellular activity and root apex zonation by using common signaling molecules such as ROS. This might provide a very important clue to understand how the root transition zone functions as an excellent sensor of its environment, and flexibly adapts to it. The findings based on the present study also bring new knowledge to develop new technology for agriculture, because light and pH factors can easily be controlled and ATP can be an eco-friendly fertilizing compound to improve crop plant growth.

Conclusions

The results of this study reveal that eATP functions as a signaling molecule in the root apex. The roles of two membrane proteins in eATP signaling, NADPH oxidase and the eATP receptor DORN1 (At5G60300) in the transition zone, were elucidated. Interestingly, the highest expression level of DORN1 was shown in the root apex transition zone. This thesis also reported that eATP

was involved in ROS generation by NADPH oxidase C (AtRBOHC). In the future, the interplay of NADPH oxidase and eATP receptors on the plasma membrane of the root apex transition zone will provide a clue to determine the detailed mechanisms that regulate the endocytic vesicle recycling activity after the DORN1-mediated eATP perception in plant root apices.

It is suggested that eATP may be released from the root apex transition zone by touch stress when the root tip touches obstacles. Released eATP, acting as signaling molecule, allows the root apex to grow away from the obstacle's surface, as the root apex is freed from gravitropic response by eATP signaling (Tanaka et al., 2010). The results obtained in my studies reveal that eATP inhibited gravitropic root response after five hours, and the inhibition was recovered within eight hours (Fig. 5-4). This finding suggests that the recovery from eATP-inhibited graviresponse might allow roots to grow towards gravity again after avoiding obstacles.

eATP may inhibit root gravitropism not only with the inhibition of PIN2-dependent auxin transport (Tang et al., 2003), but also *via* pH changes at the root tip. As described by Monshausen and Sievers, (2002), the pH in the root cap has an important role in gravity responses. I have shown that pH values were lowered with eATP application to pHusion transgenic plants in the root tip (Fig. 5-14). This was the first attempt to observe pH changes in plants with eATP.

Next, this thesis demonstrated that five min of eATP (pH 5.8) treatment enhanced endocytic vesicle recycling in the root apex transition zone, whereas a longer 15 min treatment inhibited this process (Fig. 5-7). This two-phased action suggests the possibility of different modes of eATP signaling depending on the time factor. Plant root apices emerge to sensitively perceive diverse environmental changes *via* controlled releases of eATP into their rhizosphere.

Finally, the results of this thesis suggest that eATP controls the root apex transition zone responses to environmental stimuli by alteration of the activities of endocytic recycling, ROS generation, membrane rigidity, and root apex zonation. These studies provide the first insights not only into the neglected roles of eATP in plant cell physiology, but also for other research fields, such as agriculture and potentially pharmaceutical or medical studies in the future.

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Erklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit in allen Teilen selbst und ohne jede unerlaubte Hilfe angefertigt habe. Diese oder eine ähnliche Arbeit ist noch keiner anderen Stelle als Dissertation eingereicht worden. Die Arbeit ist an nachstehend aufgeführten Stellen auszugsweise veröffentlicht worden:

Kagenishi, T., Yokawa, K., and Baluška, F. (2016). MES buffer affects *Arabidopsis* root apex zonation and root growth by suppressing superoxide generation in root apex. *Front Plant Sci* 7, 79.

Kagenishi, T., Yokawa, K., and Baluška, F. (2016). Dynamic regulation of endocytic vesicle recycling and PIN2 localization in Arabidopsis roots under varying light qualities. *Environ Control Biol* 54, 51-55.

Ich habe früher noch keinen Promotionsversuch unternommen.

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