博士論文（要約）

Molecular mechanisms of adaptation to low calcium conditions in *Arabidopsis thaliana*

(シロイヌナズナにおける低カルシウム条件に対する適応の分子機構)

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Contents

Abbreviations

bp	base pair
CalS	callose synthase
CAPS	cleaved amplified polymorphic sequence
cDNA	complementary DNA
Col-0	Columbia-0
dCAPS	derived cleaved amplified polymorphic sequence
DDG	2-deoxy-D-glucose
DNA	deoxyribonucleic acid
EMS	ethyl methanesulfonate
FKS	FK506 sensitive
GFP	green fluorecent protein
GSL	glucan synthase like
GUS	β-glucuronidase
ICP-MS	inductive coupled plasma-mass spectrometry
JA	jasmonic acid
L*er*	Landsberg erecta
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SA	salicylic acid
SSLP	simple sequence length polymorphism
T-DNA	transferred DNA

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Abstract of thesis

Molecular mechanisms of adaptation to low calcium conditions in *Arabidopsis thaliana*

Plants are basis of existence of humanity. In a historical view, improvement of agriculture has preceded economic growth. Understanding of plant physiology and genetics is essential to make the agriculture further efficient in the future, thus, I believe that accumulation of knowledge on plant contribute to promotion of human welfare.

Plant growth depends on absorption of essential elements from soils and photosynthesis in leaves. Insufficient supply of essential elements causes deficiency symptoms negatively affecting agriculture. Seventeen essential elements including calcium (Ca) are described in plants. In plants, Ca is mainly transported from roots to shoots through xylem along transpiration stream. Transpiration rate per leaf is higher in old leaves and lower in new and small leaves, and also in organs where no or little stomata is, such as fruits. In addition, Ca is difficult to be translocated through phloem because of strong binding to pectin in cell wall. Due to these characteristics, even when Ca in soil is sufficient, Ca deficient symptoms often appear in new tissues accompanying necrosis: blossom-end rot in tomato; tip burn in Chinese cabbage. These deficiency symptoms reduce their commercial values. Breeding of low-Ca tolerant crops is a possible and promising solution for this problem, however, knowledge on low-Ca adaptation mechanisms in plants required for efficient molecular breeding is still limited.

In my thesis, I clarified a molecular mechanism of low-Ca adaptation in plants through genetical and physiological analyses of low-Ca sensitive mutants in *Arabidopsis thaliana*. In Chapter 1, I revealed that synthesis of callose, one of the cell wall polysaccharide, prevented cell death under low-Ca in *A. thaliana*. I found that the wild-type plants accumulated callose in response to low-Ca condition, depending on *Glucan synthase like* (*GSL*) *10*. I also demonstrated that the inhibition of callose synthesis enhanced cell death lesion. From these results, I established that callose synthesis is indispensable process

to prevent cell death for low-Ca adaptation. In Chapter 2, I clarified that redundant contributions of several *GSL* genes were required for the suppression of cell death and adaptation to low-Ca. I found that *gsl1-5* and *gsl8-11* mutants also exhibited the reduced callose accumulation under low-Ca. Further, I demonstrated that the *GSL1* and *GSL10* additively contributed to suppression of cell death under low-Ca. These results established that several *GSL* genes contribute to the ectopic callose accumulation, suppression of cell death, and low-Ca adaptation in *A. thaliana*. In Chapter 3, I demonstrated a potential involvement of defense responses in the development of cell death under low Ca.

Chapter 1: Callose synthesis suppresses cell death induced by low calcium condition in *Arabidopsis thaliana*

Necrosis in new leaves is one of the characteristics of Ca deficiency symptoms in plants. Mechanisms of necrosis induced by low-Ca condition or adaptation remains unclear.

A low-Ca sensitive mutant, *lcs3*, cannot develop true leaf under low-Ca. Before my study in Ph.D., it had been demonstrated in the laboratory of Plant Nutrition and Fertilizers at the University of Tokyo that the causal gene of *lcs3* (hereafter referred to as *gsl10-5*) is *GSL10*. In my master thesis, I have demonstrated that *A. thaliana GSL10* complemented a yeast β-1,3 glucan (callose) synthase mutant, establishing that *GSL10* encodes callose synthase.

In this Ph.D. study, I performed detailed physiological characterization and transcriptome analysis of *gsl10-5*. Observation of cell death by trypan blue staining revealed that *gsl10-5* developed cell death in new leaves under a low-Ca condition and the introduction of *GSL10* alleviated cell death phenotype in *gsl10-5*, showing that *GSL10* has a function of prevention of cell death under low-Ca conditions. Callose staining with aniline blue revealed that the wild-type plants accumulated ectopic callose in response to low Ca and that *gsl10-5* accumulated less callose than the wild-type plants. Callose accumulation in *gsl10-5* was recovered by introduction of the wild-type gene, showing that *GSL10* is a responsible gene of the ectopic callose accumulation induced by low Ca. These results suggest that callose synthesis is required for the adaptation to low Ca and prevention of cell death. To test this, the wild-type plants were grown under low-Ca condition in the presence of callose synthase inhibitors, 2-deoxy-D-glucose (DDG) or caspofungin. Shoot growth of the wild-type plants under low Ca with DDG or caspofungin were inhibited comparable to that of *gsl10-5* under low-Ca condition. Application of DDG enhanced the cell death and reduced callose accumulation in the wild type under low-Ca condition. These results showed that callose synthesis is required for the prevention of cell death under low-Ca. To get insight into the transcriptome change(s) in GSL10-mediated low-Ca adaptation, RNA-sequencing analysis was performed. The results showed similar expression profiles between wild-type plants treated with low Ca and *gsl10-5* grown under normal condition. Especially, the expressions of genes related to cell wall and defense responses are commonly altered, suggesting the requirement of *GSL10* for the

alleviation of both cell wall damage and defense responses caused by low Ca. These results demonstrate that callose synthesis is essential for the prevention of cell death and is a key part of plant strategy to survive under low-Ca condition.

Chapter 2: Contributions of *GSL* family to low-Ca adaptation in *A. thaliana*

Low-Ca sensitive mutants, *lcs4*, *lcs5* and *lcs6* cannot develop true leaves under low Ca as observed in *gsl10-5*. Before my study in Ph.D., in the laboratory, it had been revealed that the causal gene of *lcs4* and *lcs5* is *GSL1* (*lcs4* and *lcs5* are hereafter referred to as *gsl1-5* and *gsl1-6*, respectively) and that of *lcs6* is *GSL8* (*lcs6* is hereafter referred to as *gsl8-11*). It had also been demonstrated that *GSL1* and *GSL8* complemented a yeast β-1,3 glucan synthase mutant, suggesting *GSL1* and *GSL8* encodes a functional callose synthase.

In my Ph.D. study, I performed detailed physiological characterization of the *gsl* mutants. I analyzed the contributions of *GSL* genes to the low-Ca adaptation, the ectopic callose accumulation, and the suppression of cell death under low-Ca condition. To test whether *GSL1* and *GSL8* contribute to the callose accumulation under low Ca, I performed callose staining. *gsl1-5* and *gsl8-11* mutants showed the reduced ectopic callose accumulation compared to the wild-type plants, showing that callose accumulation is positively correlated with the adaptation to low-Ca condition. Under the low-Ca condition, double mutant of *gsl1-5* and *gsl10-5* showed enhanced growth inhibition and cell death compared to its parental single mutant lines. In the *A. thaliana* genome, 12 genes are annotated as putative callose synthase. I checked the low-Ca sensitivity of T-DNA insertion lines of other 9 members of *GSL* genes and *GSL11* T-DNA line showed sensitivity to low Ca. These results suggest that the callose synthesized by GSL proteins additively contributes to the tolerance and the prevention of cell death under low-Ca in *A. thaliana*.

Chapter 3: Analysis of the involvement of callose in a low-Ca sensitive mutant *lcs7*

Before my study in Ph.D., in the laboratory, it had been revealed that a low-Ca sensitive mutant *lcs7* exhibits growth inhibition under low-Ca condition similar to that of *gsl* mutants, and the low-Ca sensitivity of *lcs7* is caused by a gain-of-function mutation in a transcription regulation factor. From microarray analysis, it had been shown that callose degrading enzyme genes were up-regulated in *lcs7*, implying the possibility that the reduction of callose is the cause of low-Ca sensitivity of *lcs7*. In addition, it had been shown that double mutants between *lcs7* and *gsl10-5* or *gsl8-11* showed severe growth inhibition compared to single mutants.

In this Ph.D. study, I performed callose staining to test whether less callose accumulation is related with low-Ca tolerance in *lcs7* as is observed in *gsl* mutants. There was no significant difference

between the wild-type plants and *lcs7*, suggesting that the low-Ca sensitivity of *lcs7* is not caused by the reduction of callose and that the enhanced low-Ca sensitivity of double mutant between *lcs7* and *gsl* mutants is caused by additive effects of different low-Ca adaptation mechanisms. To speculate the reason for low-Ca sensitivity of *lcs7*, I re-analyzed the microarray data and performed gene ontology analysis. The results showed that the genes related to defense response and cell wall are enriched in differentially expressed genes in *lcs7*. These results suggest that the proper transcription regulation of defense responses and cell wall is crucial for the prevention of cell death under low Ca.

In this Ph.D. thesis, I concluded that *A. thaliana* adapts to low-Ca conditions by synthesizing callose, and that redundant contributions of *GSL* family are required for the suppression of the cell death. In addition, I suggested the involvement of defense response in cell death under low Ca. I believe this knowledge will contribute to the breeding of Ca deficiency tolerant crops and the improvement of agricultural productivity in the future.

Preface

Significance of plant research for humankind

I believe it is necessary for the improvement of agricultural productivity to take in depth considerations about the physiologies and properties of crops. Reinforcement of knowledge on the physiologies of crops/plants have contributed to the efficient cultivation of crops, the improvement of agricultural productivity and economic growth, and the pursuit of happiness of each person. In addition, it is my view that the reinforcement of knowledge itself, independent of its contents, is essential to deepen self-recognition of humanity. In other words, the reinforcement of knowledge is essential to understand how human lives and/or what human is. Thus, the significance of plant research is supported by both agricultural and scientific contexts.

Target of the study of plant nutrition-my view

Plants directly support humanity as a source of food. Thus, it is an important issue to cultivate plants appropriately, which directly influences the survival of human. In human history, cultivation methods have long been improved through experience and in 19^{th} century such knowledge is systemized and form several scientific fields (Liebig, 1855). Plant nutrition is among them.

Seventeen essential elements have been recognized for plants (Marschner, 2011). In other words, if any one of these essential elements was absent from the earth, plant would not continue to live on the earth and consequently we human could not live. Human body is composed of many different elements, most of which are originally from plants absorbed from soil. Plants is an interface enabling human and other lives to be connected to the earth and the universe. Plant nutrition is a pursuit for revealing how plants live on the earth by absorbing and utilizing the elements - fragments of this planet and this universe.

The purpose and features of this thesis and difference from previous studies

The title of my thesis is “Molecular mechanisms of adaptation to low calcium conditions in *Arabidopsis thaliana*”. However, as described in the following sections, several genes have been already identified to be essential for low-Ca tolerance. Thus, it is required to clarify the difference between present studies and previous studies.

First, my thesis focuses on the mechanisms of prevention of cell death under low-Ca conditions in Arabidopsis by using mutant which cannot expand true leaves under low-Ca. My thesis pays attention to the mechanisms to low-Ca tolerance originally equipped in Arabidopsis. A lot of previous studies employed reverse genetic approach to identify the genes in Ca homeostasis, however, these research are not sufficient due to its technique fault: only Ca related genes, such as Ca transporter and pectin related genes, have been tested. On the other hand, my thesis is using forward genetics and explore the hidden side of Ca homeostasis without *a priori* knowledge.

Second, my study focuses on one of the essential cell wall polymer, callose. Callose is consisted of β-1,3 glucan and has many biological functions. However, no report had been shown its relationship to the low-Ca tolerance in plants.

These two points differentiate my thesis from previous studies. To facilitate the understanding of the contents of the thesis, I described Ca in plants and plant cell wall in the following sections.

General introduction

Calcium in plant nutrition

There are 17 essential elements in plants (H, B, C, N, O, Mg, P, S, Cl, K, Ca, Mn, Fe, Cu, Zn, Mo and Ni) (Marschner, 2011). Ca is one of the macronutrients in plants, and a pile of knowledge on Ca have been accumulated so far. In the following sections, the functions, current knowledge on transportation and deficiency symptom of Ca in plants are discussed.

Functions of Ca in plants

In plants, Ca is required for many biological processes including the stabilization of cell wall, second messenger in signal transduction, and the stabilization of plasma membrane. In the following subsections, the involvement of Ca in each function is described.

1) Stabilization of cell wall

Ca contribute to the mechanical strength in plant cell wall by crosslinking pectin. Pectin is one of the cell wall matrix, a polysaccharide composed of homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Mohnen, 2008, for review). HG is a polymer of galacturonic acid linked with α-1,4 linkage. Galacturonic acid in HG has carboxyl group with both methylated and de-methylated forms. Ca^{2+} binds to de-methylated carboxyl groups of HG, and stabilize and gelate pectin (Powell et al., 1982). The gelation of pectin is important for the plant structural strength and developmental morphology. For example, the flower stem of *pectin methyl esterase 35* mutants cannot stand straightly (Hongo et al., 2012). This phenotype is correlated with the reduction of fluorescence from antibody against de-methylesterified pectin. In addition, it has been known that foliar application of Ca contributes to the prevention of infections by pathogens, seemingly by reinforcing cell wall with pectin-Ca^{2+} crosslink (Toivonen and Bowen, 1999).

2) Second messenger in signal transduction

It has been known that Ca^{2+} concentration in cytosol is maintained at a submicromolar level. On the other hand, Ca^{2+} concentration in apoplast is estimated to be about 1-10 mM. In other words, Ca^{2+} concentration in apoplast is approximately 10,000-fold higher than that in cytosol (White and Broadley, 2003, Conns et al., 2011). The difference of Ca^{2+} concentration between inside and outside of cell enables plant cells to use Ca^{2+} as a signal. This function of Ca^{2+} is often called as a second messenger. For example, when a physical stimulus is applied to the plasma membrane, Ca^{2+} channels on the plasma membrane open in response to stretching stress and allow apoplastic Ca^{2+} to flow into cytosol (Nakagawa et al., 2007). Elevation of cytosolic Ca^{2+} concentrations lead to the activation of downstream responses such as mitogen associated protein kinase (MAPK) cascade

and/or the activation of a number of enzymes (Lecourieux et al., 2006, for review).

Ca^{2+} is also a second messenger to induce cell death (Kurusu et al., 2005). For example, in the process of pathogen infection, recognition of pathogen-associated molecular patterns (PAMPs) causes influx of Ca^{2+} into cytosol through cytosolic-nucleotide gated channel (CNGC). It has been demonstrated that this Ca^{2+} influx leads to the activation of the MAPK cascade. The production of nitric oxide (NO) and reactive oxygen species (ROS) induced by the activation of MAPK subsequently lead to cell death (Overmyer et al., 2003, for review).

3) Stabilization of plasma membrane

Ca is one of the components of plasma membrane. It has been known that Ca^{2+} stabilizes plasma membrane by binding to the phosphate groups of phospholipids, a component of plasma membrane. In *Arabidopsis thaliana*, it has been reported that a low-Ca condition causes electrolyte leakage, an indicator of damages in plasma membrane (Schapire et al., 2008). In addition, a mutation on *synaptotagmin 1*, a protein considered to be involved in repairing the damage of plasma membrane, further enhances electrolyte leakage induced by low-Ca (Schapire et al., 2008). The above-mentioned evidence suggests the importance of Ca^{2+} in alleviation of plasma membrane damages caused by low-Ca.

It has been known that, under low-Ca condition, Ca deprivation from the phospholids of plasma membrane precede the deprivation from Ca pectate (Mostafa and Ulrich, 1976; Marschner, 2011), suggesting that low-Ca condition negatively affects stability of plasma membrane.

Transport of Ca^{2+} in plants

Plants absorb nutrients from soil via roots and transport them to shoots. Nutrients in soil solution enter into xylem via symplastic and/or apoplastic pathway. The symplastic pathway is route via cytosol connected with plasmodesmata, and apoplastic pathway is route via apoplastic space including cell wall matrix. There is a barrier in apoplastic routes in endodermis. This barrier consists of lignin and called Casparian strip. Casparian strip is formed from elongation zone and fill the gap between endodermal cells preventing solutes entering into stele. Therefore, nutrients must enter into symplastic pathway in the region where Casparian strip is formed. On the other hand, in the root tip region, Casparian strip is not formed and it has been considered that nutrients and other components can enter into stele through apoplastic pathway (White 2001).

Ca^{2+} functions as a second messenger by transient increase of its concentration in cytosol, therefore, Ca^{2+} concentration in cytosol is kept in the level of submicromolar in basal state. Ca^{2+} is mainly transported to shoot via the apoplastic pathway. It has been considered that Ca^{2+} is hardly transported via symplastic pathway. In other words, Ca^{2+} is absorbed from the root tip where Casparian strip is not formed, and is transported by transpiration stream to shoots through xylem,

hardly on phloem, because Ca^{2+} binds to pectin in cell wall and becomes almost immobile (Clarkson *et al.*, 1984; White and Broadley., 2003). Consequently, Ca^{2+} tends to accumulate much in older leaves and less in new leaves with lower transpiration rate.

The absorption of Ca^{2+} from soil is competitive with that of other nutrients such as Mg^{2+}, K^+ and NH_4^+ (Walker et al., 1955; Overstreet et al., 1952; Kirkby and Mengel, 1967). It has been reported that plants grown on serpentine soil which has higher Mg concentration showed the low Ca concentrations (Proctor, 1971). Thus, transport of Ca can be restricted when concentrations of other cations are high in soil.

It has been reported that several genes encode Ca^{2+} transporters in Arabidopsis, such as Ca^{2+}/H^+ antiporter (*CAX*), *MCA* (Ca^{2+}-permeable mechanosensitive channel), *ECA* (ER-type Ca^{2+}-ATPase), *CNGC* and *ACA* (autoinhibited Ca^{2+}-ATPase). Although Ca^{2+} transport from root to shoot is mainly mediated by transpiration stream through xylem, several transporters have been shown to be involved in Ca^{2+} concentration or uptake in shoot. CAX1 and 3 are reported as vacuole-localized Ca^{2+}/H^+ antiporter and the Ca^{2+} concentration in shoots of *cax1 cax3* double mutant is reduced to about 20% compared to that of wild-type plants (Cheng et al., 2005). It has been reported that the double mutant of plasma membrane localized MCA1 and 2 showed the reduced Ca^{2+} uptake activity (Yamanaka et al., 2010). It has been also known that ECA1 is involved in the Ca^{2+} concentration in shoot (Wu et al., 2002). A plasma membrane localized CNGC2 has been suggested to be involved in influx of Ca^{2+} from apoplast into cytosol and responsible for Ca^{2+} concentration in shoot under excess- Ca^{2+} condition (Wang et al., 2017).

Besides the involvement of the transporters in Ca^{2+} accumulation in shoot, Ca^{2+} transporters also have important roles in signal transduction. For example, a vacuole-localized ACA4 and ACA11 have been shown to be involved in cellular signal transduction including salicylic acid (SA) signaling pathway, suggesting the importance of compartmentation of cytosolic Ca^{2+} in vacuole for proper cellular process (Boursiac et al., 2010).

Ca deficiency symptoms in crops and its molecular mechanisms

In agriculture, Ca deficiency symptom is one of the often-occurring physiological disorder which is observed as blossom end rot (BER) in fruits, tip burn in younger leaves, or core rot in enclosed younger leaves. Ca has a tendency to be accumulated much in the old and large tissues with high transpiration rate and less in the new and small tissues with low transpiration rate. Ca is almost immobile because of the binding to pectin in cell wall with egg-box structure (Powell et al., 1982). Due to these characteristics of Ca, Ca tends to be deficient in new tissues. Furthermore, newly developing tissues require a large amount of Ca for the component of cell wall. Thus, Ca deficiency symptoms can occur in new tissues even if Ca concentration in soil is sufficient.

One of most well-known Ca deficiency symptoms in crops is BER. BER is a phenomenon of development of necrotic lesions in the tip of fruits such as tomato and green pepper. It has been considered that the local shortage of Ca causes the necrosis in these tissues. de Freitas et al (2012) generated RNAi lines of pectin metylestrase (PME) genes in tomato. The generated RNAi lines showed lower de-metylesterified rate of HG, which decreased the region of HG required for the binding to Ca^{2+}. In these lines, the concentration of apoplastic free Ca^{2+} increased and the plasma membrane stability was increased. They observed the inverse correlation between the plasma membrane stability and the rate of incident of BER. They also generated transgenic tomato overexpressing CAX1, a vacuole Ca^{2+}/H^{+} antiporter, and these transgenic tomatoes showed the lower concentration of apoplastic free Ca^{2+} and increased the rate of incident of BER (de Freitas et al., 2011). From these results, they propose that the development of BER is associated with the reduced plasma membrane stability probably caused by the lower concentration of apoplastic free Ca^{2+}.

Wu et al (2012) showed that overexpression of *Calreticulin 1* (*CRT1*), encoding ER localized Ca^{2+} binding protein, alleviate the BER induced by overexpression of CAX1. They speculate that the increased Ca^{2+} capacity in ER could realize proper Ca^{2+} distribution in cellular levels in spite of enhanced intake of Ca^{2+} into vacuole by overexpression of CAX1.

Tip burn or core rot in foliar vegetables is also one of the well-known Ca deficiency symptoms in plants. Tip burn appears necrotic lesions in the tip of leaves, such as cabbage or Chinese cabbage. In Chinese cabbage, it has been known that the rate of incident of tip burn increased when the plants were grown under low Ca (Kuo et al., 1981). In addition, in new leaves, the concentration of water soluble Ca^{2+} in edges of leaves is lower than that in leaf blade or midrib. Furthermore, the concentration of water soluble Ca in leaves with tip burn is lower than that in leaves without tip burn (Kuo et al., 1981). Contrary, another study reported that there is no correlation between Ca^{2+} concentration in leaves and the rate of tip burn among a double haploid population of Chinese cabbage: the authors presented positive correlation between tip burn and SA signaling induced by low-Ca condition (Su et al., 2016).

About the gene involved into tip burn, it has been shown that the overexpression of *CAX1* can increase the tip burn in tobacco (Hirschi 1999), consistent with the case of transgenic tomato overexpressing *CAX1* which exhibits BER (de Freitas et al., 2011).

Genes involved in low-Ca tolerance in Arabidopsis

Other than the above mentioned genes (*PME*, *CAX1*, *CRT1*) in the subsection "Ca deficiency symptom in plants", several genes have been shown to be involved in low-Ca tolerance in Arabidopsis. A T-DNA insertion line of ECA1 has been shown to exhibit poor growth under low-Ca condition (Wu et al., 2002). Considering the case of CRT1, these results may suggest the importance of ER pool of Ca^{2+}. In addition, Yamaguchi et al (2006) showed that a spermine deficient double mutant, *acl5*/*spms*, showed poor growth on low-Ca condition, suggesting the involvement of polycation including spermine in low-Ca adaptation.

So far, also in our laboratory, several genes has been identifiedas genes involved in the low-Ca tolerance in Arabidopsis. I and co-authors have revealed the involvement of *pleiotropic regulatory locus 1* (*PRL1*) in the low-Ca tolerance (Shikanai et al., 2015). It has been reported that PRL1 is a regulatory protein of sugar metabolism (Németh et al, 1998). This data suggests a close relationship between energy metabolism and the low-Ca tolerance in Arabidopsis. Oda and Kamiya et al (2016) identified a magnesium transporter gene, *MRS2-4*, as an essential gene for the low-Mg, high-Mg and low-Ca tolerance, suggesting the relationship between Mg and Ca homeostasis. Li and Kamiya et al (2017) identified a novel gene affecting Casparian strip, suberin accumulation, Ca^{2+} concentration in shoot and low-Ca tolerance. This study revealed suberin has a function as apoplastic barrier and affecting Ca concentration in shoot.

Cell wall and callose in plants

One of the most significant differences between plant cells and animal cells is the existence of cell wall. Cell wall in plants is mainly composed of polysaccharide, such as cellulose, pectin, xyloglucan, and xylan or phenolic compounds such as lignin. In addition, it has been known that one of minor polysaccharides, callose is essential for plant growth. In the following subsections, the function of cellulose, pectin and callose are described for proper understanding of this present thesis.

Cellulose

Cellulose is one of the polysaccharide of β-1,4 glucan with β-1,4 linkage of glucose. β-1,4 glucan is synthesized by plants and some kind of microbes (Siró and Plackett, 2010). It is estimated that cellulose is the most abundant carbohydrate on the earth (Siró and Plackett, 2010). Cellulose is one of the essential component of plant cell wall and it has been known that the complete disruption of celloluse synthase leads to lethality (Persson et al., 2007).

It has been known that cellulose deficient mutants often showed increased pectin or ectopic callose deposition (Lukowitz et al., 2001), suggesting the interaction between cellulose and other cell wall polysaccharide (Robert et al., 2004).

Pectin

Pectin is one of the cell wall matrix polysaccharide (Mohnen, 2008, for review). Pectin is composed of homogalacturonan (HG, as shown in page 13), rhamnogalacturonan I (RG I, as shown in page 13) and rhamnogalacturonan II (RG II, as shown in page 13) (Mohnen, 2008). HG comprise about 65% of pectin and is consisted of polymer of galacturonic acid with α-1,4 linkage (Mohnen, 2008, for review). It has been known that pectin is synthesized in Golgi and secreted to the outside of plasma membrane, subsequently de-esterified by PME (Pelloux et al, 2007, for review). De-esterified HG can bind to Ca^{2+} with its carboxyl groups, then forms Ca^{2+}-pectin crosslinks, which is called as egg-box structure (Powell et al., 1982). As I mentioned in page 12, the gelation of pectin is essential for many biological processes: for examples, a mutant of *pectin methylesterase 35* showed a pendant stem, which is associated with the reduced de-methylesterification of the homogalacturonan of cortex of basal part of stem (Hongo et al., 2012). In addition, overexpression of pectin methylesterase inhibitor gene inhibited the formation of inflorescence (Peaucelle et al., 2008).

Moreover, oligogalacturonan, a fragment of pectin, has been known to induce immune response and ectopic callose deposition in Arabidopsis (Denoux et al., 2008).

Callose

Callose is one of the cell wall polymers whose main chain is consisted of β-1,3 glucan, a linear

polymer of glucose with β-1,3 linkage. β-1,3 glucan widely spreads in plant, algae, fungi, yeast and bacteria (Cui et al., 2001; Stone and Clarke., 1992, for review).

Callose is essential for plants, especially for cell plate and pollen. In Arabidopsis, it has been suggested that there are 12 callose synthase (GSL) (Hong et al., 2001). It has been reported that *gsl8* showed decreased accumulation of callose in cell plate and seedling lethality in homozygous plants (Thiele et al., 2009). GSL6 is also suggested to contribute to callose accumulation in cell plate (Hong et al., 2001). Pollen maturation also requires callose, and *gsl1gsl5* double mutant and *gsl10* mutant are known as gametophytic lethality (Enns et al., 2005; Töller et al., 2008). *GSL2* has been also shown to be involved in callose synthesis in pollen and contribute to fertility (Dong et al., 2005).

It has been suggested that callose synthesized by GSL7 in sieve plate is required for efficient transport of sugar through phloem tissue (Barratt et al., 2011). *GSL8* and *GSL12* have been shown to be involved in the callose accumulation required for regulation of permeability of plasmodesmata (Vatén et al., 2011; Han et al., 2014). *gsl8* mutant showed the reduced plasmadesmal callose and increased plasmodesmata permeability (Han et al., 2014). The gain of function alleles of *GSL12* showed the increased calllose accumulation in plasmodesmata and reduced plasmodesmata permeability. These studies showed the significant role of callose in the symplastic trafficking.

One of the well-known characteristics of callose is its accumulation during pathogen infection, and this characteristic is used for the quantification of the activity of plant immunity (Luna et al., 2011). It has been known that plants accumulate callose, called papilla, at the site of pathogen infection, and this callose deposition is considered to protect the subsequent invasion of pathogen. Contrary to this understanding, Nishimura et al (2003) showed the enhanced tolerance of *gsl5* to pathogen which lacked callose deposition during infection. This enhancement of tolerance to pathogen of *gsl5* is caused by the enhanced salicylic acid (SA) signaling pathway in defense response (Nishimura et al., 2003). Ellinger et al (2013) had shown that the overexpression of *GSL5* increased the tolerance to pathogen because of elevated callose deposition rather than SA signaling. Overexpression of *GSL5* resulted in the suppression of SA signaling pathway, consistent with the result of Nishimura et al (2003) showing that disruption of *GSL5* resulted in the enhanced SA signaling pathway (Ellinger et al., 2013). From these evidence, it has been considered that there is feedback regulation between callose deposition and SA signaling pathway, and both of them contribute to defense against pathogen.

Declaration

In present thesis, the citations of results from other researchers are described in main body and marked in red lines in figures, and the citations of results from my master thesis are described in main body and marked in blue lines in figures. The experiments performed by other persons are stated.

I declare that the research described in the present thesis was performed by Yusuke Shikanai, unless otherwise stated.

Chapter 1: Callose synthesis suppresses cell death induced by low calcium condition in *Arabidopsis thaliana*

Contents of this chapter cannot be published here because there is a plan to publish contents included in this chapter in 5 years.

Chapter 2: Contributions of *GSL* family to low-Ca tolerance in *Arabidopsis thaliana*

Contents of this chapter cannot be published here because there is a plan to publish contents included in this chapter in 5 years.

Chapter 3: Analysis of the involvement of callose in a low-Ca sensitive mutant, *lcs7*

Contents of this chapter cannot be published here because there is a plan to publish contents included in this chapter in 5 years.

General discussion

In this thesis, I clarified the mechanisms of the low-Ca adaptation and the development of Ca deficiency symptom in *Arabidopsis thaliana*.

Previous studies on Ca deficiency symptoms in plants

As I described in Introduction, several studies have been performed on the molecular mechanism of the development of Ca deficiency symptoms in plants. For examples, it has been demonstrated that the overexpression of vacuolar-localized Ca^{2+}/H^{+} antiporter leads to Ca deficiency symptoms (Hirschi., 1999): the disruption of ER-localized Ca^{2+} pump makes *A. thaliana* sensitive to low Ca (Wu et al., 2002); the overexpression of ER-localized Ca^{2+} binding proteins (CRT1) confers plants tolerance to Ca deficiency (Wu et al., 2012); down-regulation of pectin metylestrase (PME) decreases the incidence of Ca deficiency symptom possibly due to the increase of Ca^{2+} in apoplastic space (de Freitas et al., 2012). These reports suggest the importance of Ca distribution in tissue and/or cell in the development of Ca deficiency symptoms. Although several studies have shown that disruption of gene (s) enhance low-Ca sensitivity in plants, to my knowledge, no report identified the molecular mechanism (s) of adaptation in response to low-Ca condition in plants.

Issues that I clarified in my thesis

Before my Ph.D. study, it had been clarified that callose synthase genes *GSL1, 8* and *10* are essential for low-Ca tolerance in *A. thaliana*. In addition, it had been revealed that the low-Ca sensitivity of a transcription regulation factor mutant, *lcs7*, is enhanced by *gsl* mutations.

In my thesis, I demonstrated that the wild-type plants accumulate ectopic callose in response to low-Ca condition, and that the callose accumulation in *gsl* mutants are reduced (Fig. 1-4C, 1-7, 2-7A, B). Cell death under the low-Ca condition is enhanced in *gsl* mutants and wild-type plants treated with callose synthase inhibitor (Fig. 1-1C, D, 1-8B, C, 2-9A, B). These results showed that callose synthesis is required for the prevention of cell death and the low-Ca adaptation. In addition, I revealed that the low-Ca sensitivity of *lcs7* is not associated with the reduction of ectopic callose accumulation (Fig. 3-4, 3-5) and associated with the differential expressions of defense response genes (Fig. 3-6, 3-7). This result suggests the involvement of defense responses in development of Ca deficiency symptoms in plants.

Novelty of my thesis over previous studies

I found that the ectopic callose accumulation in response to low Ca is responsible for the prevention of Ca deficiency symptoms. I believe that this is a brand-new knowledge on the behavior of plants against low-Ca condition. Moreover this is the first description of plants' strategy to overcome low-Ca conditions by preventing cell death through callose synthesis. My thesis differs from the

previous reports in that I described the molecular mechanisms for the adaptation to low-Ca condition.

In the research field of plant immunity, it has been widely accepted that ectopic callose accumulation is to reinforce cell wall to prevent invasion of pathogens (Ellinger et al., 2013). My thesis suggests that, irrespective of pathogen invasion, the ectopic callose accumulation is required for the prevention of cell death. My finding lead us to re-interpret the significance of ectopic callose accumulation during pathogen invasion, in terms of prevention of cell death.

Common characteristics between low-Ca response and defense response

Through my thesis, I found at least two common characteristics between low-Ca response and defense response. One is, as I mentioned in the above paragraph, the accumulation of ectopic callose accumulation (Fig. 1-4C, 1-7, 2-7A, B). Another is the transcriptome pattern. Defense response genes are altered in Col-0 under low-Ca condition, *gsl10-5*, and *lcs7* (Fig. 1-S9, Fig. 3-6).

It is probable that these common characteristics between low-Ca response and defense response are caused by the cell wall damage. During pathogen infection, cell wall of plants is damaged by pathogens. Pathogen infection is recognized by host plants by receptors for pathogen-associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) (Denoux et al., 2008). The fragment of pectin, oligogalacturonic acid (OG), is known as one of the DAMPs (Denoux et al., 2008). Considering that pectin-Ca^{2+} crosslink is required for cell wall, low-Ca condition may de-stabilize pectin in cell wall and de-stabilized pectin may be fragmented into OG, leading to trigger defense responses. These possible physical phenomena under low-Ca or pathogen infection may cause similar physiological and transcriptional responses.

Toward application to breeding of low-Ca tolerant crops

Breeding for enhancing callose synthesis is one of the promising ways for low-Ca tolerant crops, because the enhancement of callose synthesis should also contribute to plant immunity (Ellinger et al., 2013).

On the other hand, it seems to be difficult to use the down-regulation of defense responses for the prevention of necrosis under low-Ca condition because the down-regulation of defense responses may lead to the enhancement susceptibility to pathogen and subsequent loss of products. Further clarification of the relationship between low-Ca response and defense response are needed for the utilization of defense response genes for breeding of low-Ca tolerant crops.

Conclusion

Plants are the basis of survival of humanity. Thus, the question how plants adapt to surrounding environments consists of the question how human exist in the world. Furthermore, I believe that

breeding of crops is one of the promising solution to the problems, such as global population growth and economic growth in rural area. I pray that my thesis can consist of the answer to the above questions and contribute to the solutions to the problems.

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