Studies on the Genes Involved in the Regulation of Cell Wall Mechanical Properties in Arabidopsis

（細胞壁の力学的性質を制御するシロイヌナズナ遺伝子に関する研究）

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CONTENTS

GENERAL INTRODUCTION

The functions of plant cell walls

Plants are subjected to a great variety of environmental signals, such as light, temperature, water, and gravity, which influence their processes of growth and morphogenesis. In addition to environmental signals, plants are under continuous threat of biotic stresses caused by pathogenic bacteria, fungi, and viruses. Plants have adapted to various biotic or abiotic stresses during their evolution. Plant cell walls are the outermost structure, where the cell encounters the change of their environments. In plants, unlike animals, the cell wall surrounds the cell membrane and provides these cells with structural support and mechanical strengths. The plant cell wall plays a principal role in diverse plant physiological functions, as mentioned above. Thus, the cell wall is responsible for environmental responses, as well as growth regulation and morphogenesis (Hoson, 2002; Cosgrove, 2005; Wolf et al., 2012).

On the other hand, the presence of a rigid cell wall poses a problem for plant cells. The cell wall may present an obstacle to movement of large molecules into and out of the cell, however, the plant cell makes the transport pathway with the cell wall. The cell walls of neighboring cells are in direct contact, and so the plant's cell walls combine to form a major transport pathway, called the apoplast. Apoplastic transport involves not

only the movement through the cell walls but also across the plasma membrane. Apoplast also functions as a place of the synthesis of the plant hormone and as a transfer location of the signal from the external environment (Turusaki at al., 1997; Hoson, 1998).

In addition to these biological functions, the plant cell wall is the source of raw material for many products of paper and textiles, such as cotton. Plant cell walls are also used to make synthetic fibers, such as rayon. The plant cell walls are the most abundant source of organic carbon on the planet. This photosynthetically-fixed carbon is reused by microbial enzymes that convert cell wall polysaccharides to monosaccharides and oligosaccharides, a process that is of biological and industrial importance (Sticklen, 2008; Himmel and Bayer, 2009). Today, main trials are underway worldwide to develop cost-effective methods for converting "cellulosic biomass" into biofuels to replace fossil fuels. Thus, the plant cell wall is critical for essential processes in not only plant growth and development, but also daily function of human living.

The structure of the cell wall

The plant cell walls vary greatly in appearance and composition in different cell types. For instance, cell walls of cortex and parenchyma in the pith are commonly thin. By contrast, epidermal cells and xylem vessels have thicker cell walls. In guard cells, the walls adjacent to the stomatal

pore are much thicker than the walls on the other sides of the cell. Despite this morphological variation, cell walls are generally classified into two major types: primary walls and secondary walls. Primary walls are defined as walls formed during cell growth. Primary walls are unique structures that are strong but usually thin and flexible. Its shape and size dictate most of the externally recognizable features of plant development (Brett and Waldron, 1996; Fry, 2000). Primary walls control the rate and direction of cell expansion, thus the final size and shape of the cell (Fry, 2004a). Secondary walls are formed after cell enlargement stops. Secondary walls are deposited between the plasma membrane and the primary walls. Secondary walls may be highly specialized in structure and composition, reflecting the differentiated state of the cell. Secondary walls are mainly found in tracheary elements and fibers in the water-conducting tissue (such as xylem). They provide mechanical strength to these cell types, which serve as mechanical tissues to enable vascular plants to grow to a great height. Deposition of lignified secondary walls in tracheary elements not only reinforces these water conduits to resist the negative pressure generated during transpiration, but also renders them waterproof for efficient water transport (Zhong and Ye, 2015).

Primary walls contain several types of polysaccharides, smaller ratio of glycoproteins and, in some specialized cell-types, various noncarbohydrate substances such as lignin. Cell wall polysaccharides are

classified into three categories: pectins, hemicelluloses, and cellulose. Pectins and hemicelluloses are also called matrix polysaccharides. Most pectins are solubilized by aqueous buffers and dilute acidic solutions or calcium chelators. Pectins are diverse group of hydrophilic, gel-forming polysaccharides rich in acidic sugar residues. Pectins are galacturonate (GalA)-rich, acidic polysaccharides partly methylesterified galacturonan, linked to the rhamnogalacturonans RG- I and RG- II, which are rich in arabinose, galactose, rhamnose (Fry, 2004a). On the other hand, hemicelluloses are GalA-free, neutral or slightly acidic polysaccharides that usually require a strong alkali extractant. Major hemicelluloses are the xylogluans and xylans. Xyloglucans are composed mainly of glucose, xylose, galactose, fucose residues. Xylans include arabinoxylans, glucuronoarabinoxylans, and so on. Cellulose is the major fibriform component of the cell wall. Cellulose is compsed of β-(1→4)-D-glucan chains coalesced to for a microfibril with well- or less-ordered regions, which is insoluble in water. Cellulose microfibrils have high tensile strength. Matrix polysaccharides and cellulose are produced via different pathways. Matrix polysaccharides are synthesized in Golgi apparatus and cellulose are synthesized by plasma membrane complexes. The ratio and composition of the cell wall polysaccharides differs phylogenetically among plant taxa (Popper and Fry, 2003). For example, the primary walls of flowering plants are classified into two major groups. Type I cell walls

are components of the cell walls of dicotyledons and the non-comelinoid monocotyledons. Type I cell walls are characterized by a cellulose-xyloglucan framework with approximately equal amounts of cellulose microfibrils and xyloglucans (Fry, 2004a). On the other hand, type II cell walls are found only in commelinoid monocotyledons, which include rice (*Oryza sativa*), barley (*Hordeum vulgare*) and oats (*Avena sativa*). Unlike type I cell walls, type II cell walls have less xyloglucans than cellulose. The major polysaccharides that cross-link the cellulose microfibrils are glucuronoarabinoxylan and β -(1→3):(1→4) mixed glucans (Carpita and Gibeaut, 1993; Kato et al. 1982). Compared with type I cell walls, type II cell walls contains higher amount of phenylpropanoids.

Primary walls also contain glycoproteins whose exact functions are uncertain. These proteins are localized in the cell walls of specific cell types. For instance, hydroxyproline-rich glycoproteins are localized in cambium and vascular parenchyma and proline-rich glycoproteins are localized in xylem and cortex. Such glycoproteins are usually identified by short motifs or repeating sequences of amino acids or high degree of glycosylation. In addition to these glycoproteins with repetitive motifs, cell walls also contain arabinogalactan proteins (AGPs). AGPs consist of a core-protein backbone O-glycosylated by one or more complex carbohydrates consisting of arabinose and galactose as main components. More than 90% of the mass of AGPs may be sugar residues. Multiple AGP

forms are found in several plant tissues, either in the cell wall or associated with the external face of the plasma membrane, and they express in specific cells and tissues. AGPs may function in cell division, growth, receptors, and modulators of cell wall mechanics (Seifert and Roberts, 2007).

In addition to matrix polysaccharides, cellulose and glycoproteins, phenolics are also one of the major components of call wall. Phenolics are included about 5 to 12% of cell wall components in different taxa (Brett and Waldron, 1996). Lignin, a phenolic polymer, is the major phenolic in secondary walls. The lignin precursors are the three aromatic alcohls (*p*-coumarly, coniferyl and sinapyl alcohols). These precursors are linked by a wide variation of bonds in the final polymer, and the linkage pattern of these precursors is irregular. Polymerization of precursors can continue to occur as long as activated precursors and space in the cell wall not occupied by macromolecules. The result is a very strong, hydrophobic meshwork, which surrounds the other wall components and cements them in place. The comprehensive structures give the cell wall to constructive strength. In addition to lignin, other phenolic compounds may be present. Primary wall polysaccharides contain not only sugar residues but also certain phenolic esters. Some polysaccharide chains may be cross-linked to each other by oxidatively coupled phenolic side-chains (Markwalder and Neukom, 1976; Fry, 1979; Ishii, 1991; Wende et al., 1999; Fry et al., 2000). The most important of these is ferulic acid. In cereals, which have only low

amounts of pectins, ferulic acid appears to be esterified to arabinose and may have an important role in the cross-linking of arabinoxylans. Two ferulic acid units can be linked by peroxidase activity to form a diphenyl bond. Ether links between ferulic acid and pectin may also occur (Brett and Waldron, 1996; Fry, 2004a). Pectins also contain feruloyl esters in spinach cell walls (Fry, 1982). Taken together, the cell wall consists of a cellulose microfibril phase and matrix polysaccharide phase, and also contains glycoproteins and phenolic compounds. In addition, many cross-links occur among these constituents of the cell wall.

Plant growth and the mechanical properties of the cell wall

Plant cells typically expand ten to a thousand-fold in volume before reaching maturity. The cell wall undergoes this wide range expansion without losing its mechanical integrity and without becoming thinner. Many factors influence the rate of cell wall expansion. Type and age of cell are important developmental factors. Various plant hormones such as auxin and gibberellin are also important factors. Environmental conditions such as water and light efficacy may identically modulate cell expansion. These internal and external factors are most likely to modify cell expansion by altering the way in which the cell wall is loosened, so that it yields (irreversibly stretches) differently. Because the cell wall is the major mechanical restraint that limits cell expansion, much attention has

been given to its physical properties. Using the bending method, technique of measurement of cell wall mechanical properties, Heyn and van Overveek (1931) reported that cell elongation associates with cell wall properties: An irreversible deformation of the cell wall is needed for cell elongation and auxin causes an increase in a reversible and an irreversible extensibilities of cell wall that is, cell wall loosening. Not just using the bending method, many plant researchers have measured the mechanical properties of cell walls using various techniques: the plasmolysis method (Kohji et al., 1981) and the stretching method (Masuda and Wada, 1966). Olson et al. (1965) first adopted an automatic Instron-type tensile-tester for load extension analysis. Since then, the use of this technique has prevailed. Using Instron-type tensile-tester, Yamamoto et al. (1970) developed a stress-relaxation analysis to evaluate the rheological properties of the cell wall and showed that auxin-induced cell expansion is closely correlated with alteration of a reological parameter of the cell wall, designated as the minimum stress relaxation time (T_0) (Yamamoto and Masuda, 1971; Sakurai, 1991). Upon application of auxin, the minimum stress relaxation time of the cell wall decrease within 15 min. This change in the mechanical properties of the cell wall is followed by cell elongation exerted by turgor pressure. Based on the findings from many investigations using these methods, it has been postulated that the cell wall extensibility determines the growth rate of plant cells.

The purpose of the present study

The architecture and mechanical properties of plants depend on the structure of the cell wall. For cell growth control, plants regulate mechanical deformations and changes in cell wall structure or compositions. The cell wall extensibility is an emergent property of complex network interactions between cell wall polymers. The thickness of cell walls and the molecular mass of matrix polysaccharides underlie the regulation of the cell wall extensibility (Taiz, 1984; Sakurai, 1991). Briefly, the mechanical properties of the cell wall are determined by the concentrations at which that cell wall constituents, such as matrix polysaccharides, cellulose, proteins, and phenolics, are present by, their molecular size, and chemical structure, as well as by the complex interactions among them. The synthesis, modification, and degradation of cell wall constituents are mediated by various cell wall-related enzymes whose activities are regulated by the expression of their genes. Intensive studies on the regulation of cell wall metabolism have been conducted, but the mechanisms underlying the regulation of the mechanical properties of the cell wall remain to be clarified.

Genome-wide loss-of-function screening is a powerful approach to clarify unknown gene functions. T-DNA insertion mutants are widely used in Arabidopsis to disrupt gene functioning. The confirmed homozygous T-DNA insertion lines in Arabidopsis are available as a suitable resource

(O'Malley and Ecker, 2010). In Chapter 1, I first established an effective approach to explore Arabidopsis T-DNA insertion lines relative to their cell wall mechanical properties. I also performed the first round screening of randomly selected Arabidopsis T-DNA insertion lines to find the lines showed modifications of cell wall extensibility using established protocol.

In Chapter 2, based on the results in Chapter 1, I focused on *ANTHOCYANINLESS2* (*ANL2*), a gene involved in the regulation of cell wall mechanical properties. I examined the changes in cell wall extensibility, growth behavior, and amounts of cell wall polysaccharides in hypocotyls of *ANL2* knockout mutant, *anl2*. I also examined the expression of the class III peroxidase gene family and activity of the ionically wall-bound peroxidase in *anl2*. In addition to these analyses, growth phenotypes of *anl2* were analyzed using plants grown under continuous white light conditions. From these results, I concluded that *ANL2* as a novel cell wall-related gene.

CHAPTER 1

Phenotypic screening of Arabidopsis T-DNA insertion lines for cell wall mechanical properties

ABSTRACT

I performed a phenotypic screening of confirmed homozygous T-DNA insertion lines in Arabidopsis for cell wall extensibility, in an attempt to identify genes involved in the regulation of cell wall mechanical properties. Seedlings of each line were cultivated and the cell wall extensibility of their hypocotyls was measured with a tensile tester. Hypocotyls of lines with known cell wall-related genes (*cesa*/*csl*, *xth*, *lac*, *agp*, *sus*) showed higher or lower extensibility than those of the wild-type at high frequency, indicating that the protocol used was effective. In the first round of screening of randomly selected T-DNA insertion lines, I found that 22 lines showed modifications of cell wall extensibility. Out of the 22 lines, 11 were classified as "already known" cell wall-related lines, whereas the other 11 were classified as "non-cell wall-related" lines. These results suggest that screening of lines for cell wall extensibility is effective to understand the mechanism underlying the regulation of cell wall properties.

INTRODUCTION

In plants, the cell wall surrounds the cell membrane and provides these cells with structural support and mechanical strength, thereby playing a principal role in diverse plant physiological functions such as growth regulation and environmental responses (Hoson, 2002; Cosgrove, 2005; Wolf et al., 2012). The plant cell wall is composed of various constituents, including cellulose, matrix polysaccharides, phenolics, and structural proteins (Carpita and Gibeaut, 1993). The mechanical properties of the cell wall are determined by the concentrations at which these constituents are present, their molecular size, chemical structure, as well as the complex interactions among them. The synthesis, modification, and degradation of cell wall constituents are mediated by various wall-related enzymes whose activities are regulated by the expression of their genes. Intensive studies have been conducted on cell wall architecture and gene-regulated metabolism of cell wall constituents. Nevertheless, the molecular mechanisms underlying the regulation of the mechanical properties of the cell wall remain to be elucidated.

Plants have several thousand genes that encode the proteins for the regulations of cell wall metabolisms. Tentative annotations reveal more than 1000 genes encoding cell wall-related proteins in Arabidopsis genome, and an equal number of unannotated genes are suspected of encoding

proteins that function in cell wall metabolism (Carpita et al., 2001, Yong et al., 2005). A powerful way to probe function of the individual components of the cell wall is through identification and characterization of mutants (Reiter et al., 1997). Mutations in known cell wall-related genes show the visible and invisible phenotypes (Carpita and McCann, 2015). For example, mutants exhibiting etiolated dwarf hypocotyls included *procuste1*, a mutation in *CESA6* leading to a deficiency in primary wall cellulose (Fagard et al., 2000). *pom-pom1-1*, *pom-pom2-1*, and *cobra1-1* are three conditional root-tip swelling mutants when seedlings are exposed to high concentrations of sucrose (Hauser et al., 1995). These cell wall mutants with visible phenotypes are easily identifiable, but growth conditions that visible phenotypes appeared are not the same between several mutants. So, characterizing visible mutant phenotypes might be inefficient in genome-wide screening. On the other hand, fourier-transform infrared (FTIR) microspectroscopy had been used in the analysis of invisible cell wall mutant phenotypes. FTIR was shown to be a powerful high-throughput method of distinguishing different features of cell wall polysaccharide architecture (Sene et al., 1994), and therefore to be able to screen for mutants with altered abundances of diagnostic wavenumbers (Chen et al., 1998). However, Yong et al. (2005) suggested that a simple or direct relationship is unlikely to exist between a mutant genotype and its impact on wall architecture as revealed by a mutant spectrotype.

Genome-wide loss-of-function screening is a powerful approach to clarify unknown gene functions. T-DNA insertion mutants are widely used in Arabidopsis to disrupt gene functioning. The confirmed homozygous T-DNA insertion lines in Arabidopsis, generated by a project at the SALK institute (the Salk Unimutant Collection), are a suitable resource for this purpose (O'Malley and Ecker, 2010). In the present study, I developed the method to explore these T-DNA insertion lines for cell wall mechanical properties by efficiently cultivating seedlings and measuring cell wall extensibility of their hypocotyls with a tensile tester. A preliminary screening of the T-DNA insertion lines for known cell wall-related genes using the established protocol revealed the utility of this approach.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia-0) and the confirmed homozygous T-DNA insertion lines of Arabidopsis were used in the present study. T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). Arabidopsis seeds were planted on 1% (w/v) agar in a 24-well culture plate, and incubated at 4°C for 7 d. The seeds were then exposed to white light for 1 d at 23°C to induce germination, and cultivated at 24°C in the dark. After cultivation, seedlings were fixed by pouring hot 80% ethanol into each well, and stored in fresh 80% ethanol until further use.

Measurement of hypocotyl length and cell wall extensibility

Before measuring cell wall mechanical properties, seedlings fixed in 80% ethanol were rehydrated with several changes of water and the lengths of their hypocotyls were measured using a scale. The cell wall extensibility of hypocotyls was measured with a tensile tester (Tensilon RTM-25; Toyo Baldwin, Tokyo, Japan) (Parvez et al., 1996). Hypocotyls were fixed between two clamps (distance between clamps was 1 mm) and

stretched by lowering the bottom clamp at a speed of 20 mm/min until a load of 0.8 g was produced. Cell wall extensibility (strain/load, μm/g) was determined by measuring the load's rate of increase just before it reached 0.8 g. I selected-T-DNA insertion lines whose cell wall extensibility had increased or decreased by more than 30% from the wild-type, irrespective of significant statistical differences.

RESULTS

A protocol was developed to explore the confirmed homozygous T-DNA insertion lines for cell wall mechanical properties. This consisted of efficient seedling cultivation and sample fixation, and measurement of cell wall extensibility with a tensile tester. The preliminary screening was performed for the T-DNA insertion lines of genes known to encode cell wall proteins, cellulose synthase/cellulose synthase-like protein (*CESA*/*CSL*), xyloglucan endotransglucosylase/hydrolase (*XTH*), laccase (*LAC*), arabinogalactan protein (*AGP*), sucrose synthase (*SUS*). Hypocotyls of lines with *CSLB2* and *CSLD6* genes showed lower cell wall extensibility than the wild-type (Fig. 1). On the other hand, lines with *CSLG2* genes showed higher cell wall extensibility than the wild-type. In the T-DNA insertion lines of *XTH* genes, the cell wall extensibility of hypocotyls with lines of *xth17-1* and *xth17-2* was largely higher than that of the wild-type (Fig. 2). Hypocotyls of lines with *LAC* genes showed higher cell wall extensibility, in particular *lac7*, *lac11*, and *lac15*, than the wild-type (Fig. 3). Similar trend to higher cell wall extensibility of hypocotyls was found in the T-DNA insertion lines of *AGP* genes (Fig. 4). Unlike the *LAC* and *AGP* genes, hypocotyls of *SUS* lines showed lower cell wall extensibility, in particular *sus3-2*, than the wild-type (Fig. 5).

The employed protocol was then used to screen randomly selected T-DNA insertion lines. I selected lines where cell wall extensibility was increased or decreased by more than 30% from that of the wild-type, irrespective of statistically significant differences (Fig. 6). The lines were also selected for hypocotyl length based on the same standard. In some lines (represented by * in Fig. 6), the change in cell wall extensibility was accompanied by elongation growth. However, other mutants (represented by **) showed changes only in cell wall extensibility.

I performed the first round screening of 405 randomly selected lines, and found that 22 lines showed modifications of cell wall extensibility. I searched the Arabidopsis Information Resource (TAIR) database to determine whether the selected lines were involved in the regulation of cell wall metabolism. Out of the 22 lines, 11 were classified as "already known" cell wall-related lines, whereas the other 11 were classified as "non-cell wall-related lines" (Table 1). Of the 11 known cell wall-related lines, eight displayed higher cell wall extensibility and three lines had lower extensibility than the wild-type. On the other hand, of the 11 non-cell wall-related lines, seven had higher extensibility and four were lower than the wild-type (Table 1).

DISCUSSION

A reliable source of mutant lines and an efficient analytical protocol are required for identifying novel genes involved in the regulation of the cell wall properties in plants. The confirmed homozygous T-DNA insertion lines in Arabidopsis are available as a suitable resource for this purpose (O'Malley and Ecker, 2010). Ryden et al. (2003) developed a sophisticated method to analyze the mechanical properties of Arabidopsis mutants. However, no effective approaches to genome-wide screening for the cell wall properties previously existed. In the present study, I established an efficient protocol to explore Arabidopsis T-DNA insertion lines relative to their cell wall mechanical properties. I first selected etiolated hypocotyls as a study material because the effects of light are avoidable and they are usable in the short term. The use of a 24-well culture plate also enabled efficient seedling cultivation and sample fixation. Indirect analytical methods, such as curvature or osmotic shock, were first used to examine the mechanical properties of the cell wall. However, these methods were shown to be unreliable when applied to the tiny Arabidopsis seedlings. Thus, I decided to use a conventional tensile tester and a simple stress-strain method for the analysis.

The preliminary screening of T-DNA insertion lines for the known cell wall-related genes were performed. Hypocotyls of lines with some

CESA/*CSL* genes showed higher or lower cell wall extensibility than the wild-type (Fig. 1). Cellulose synthase in plants are encoded by a gene family named cellulose synthase A, which is a multigene family found in all land plants. The Arabidopsis genome holds 10 *CESA* genes (*CESA1-10*) that encode proteins with homology to bacterial cellulose synthases (Pear et al., 1996). In Arabidopsis, *CESA1*, *CESA3*, *CESA6*, *CESA2*, *CESA5*, and *CESA9* have been associated with the CesA complexes active during primary wall formation, while *CESA4*, *CESA7*, and *CESA8* have been reported to be part of the CesA complex responsible for secondary wall cellulose synthesis. In addition, the role of the remaining CesA family member, *CESA10*, is at this point not clear (Endler and Persson, 2011). The *CESA* genes are members of a superfamily that includes genes with a sequence similarity with *CESA* genes and are named cellulose synthase like (*CSL*). The *CSL* genes have themselves been grouped into nine families designated *CSLA*, *-B, -C, -D, -E, -F* and *-G* (Richmond and Somerville, 2000). *CSL* genes encode various polysaccharides synthases. For example, *CSLA* genes encode synthases of mannan and glucomannan (Dhugga et al., 2004; Liepman et al., 2005; Goubet et al., 2009). The members of the *CSLC* family have been implicated in synthesis of the xyloglucan backbone (Cocuron et al., 2007). Thus, the changes of cell wall extensibility in cesa/csl may be involved in differences of synthesis of cell wall polysaccharides catalyzed many synthases which *CESA*/*CSL* genes encode.

The xyloglucan is thought to play an important structural role in the primary cell wall. The two known activities of xyloglucan endotransglucosylase/hydrolase (*XTH*) are referred to enzymologically as xyloglucan endotransglucosylase activity and xyloglucan endohydrolase activity. *XTH* represent potentially important agents for controlling wall strength and extensibility (Fry, 1989). Yokoyama and Nishitani (2001) identified 33 genes as members of the *XTH* gene family, and revealed that most members exhibit distinct expression profiles in terms of tissue specificity and responses to hormonal signals, with some members exhibiting similar expression patterns. Out of the 33 *XTH* genes, 11 genes (*XTH1-11*) were classified into Group 1, 15 genes (*XTH12-26*) were into Group 2, and 7 genes (*XTH27-33*) into Group 3 (Rose et al., 2002). Group 1 and Group 2 catalyzed xyloglucan endotransglucosylase reaction and Group 3 showed xyloglucan endotransglucosylase and endohydrolase activities. In the T-DNA insertion lines of *XTH* genes, the cell wall extensibility of hypocotyls with lines of *xth17-1* and *xth17-2* was largely higher than that of the wild-type (Fig. 2). Because of *XTH17* gene encodes xyloglucan endotransglucosylase, molecular mass of xyloglucan may be reduced in *xth17*, while may cause an increase of cell wall extensibility in *xth17*.

Lignin is formed from three aromatic alcohols: *p*-coumaryl, coniferyl and sinapyl alcohols. Laccase is necessary for these monolignol

polymerization (Brett and Waldron, 1996). 17 putative laccase genes were identified in Arabidopsis (McCaig et al., 2005; Hoegger et al., 2006). Hypocotyls of lines with *LAC* genes, in particular *lac7*, *lac11*, and *lac15*, showed higher cell wall extensibility than the wild-type (Fig. 3). Zhao et al. (2013) observed that simultaneous disruption of *LAC4*, *LAC11*, and *LAC17* almost completely abolished lignin deposition, and identified *LAC11* as a laccase involved in monolignol polymerization. Reduction of cell wall phenolics such as lignin may be responsible for the increase in cell wall extensibility of hypocotyls of lines with *LAC* genes.

Higher cell wall extensibility of hypocotyls was found in the T-DNA insertion lines of *AGP8*, *AGP24* and *AGP40* genes (Fig. 4). AGPs may function in cell division, growth, receptors, and modulators of cell wall mechanics (Seifert and Roberts, 2007). These AGP genes may especially involve in the regulation of cell wall extensibility.

In the lines of *SUS* genes, hypocotyls of lines showed lower cell wall extensibility than the wild-type (Fig. 5). Sucrose synthase is a key enzyme involved in sucrose metabolism. This enzyme catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose. *SUS* is located around the plasma membrane and cell wall in a pattern similar to cellulose microfibril orientation (Amor et al., 1995; Haigler et al., 2001). In developing wood, deficient sucrose synthase activity did not specifically affect cellulose biosynthesis, but causes an overall decrease in

cell wall polymers (Gerber et al., 2014). Knockout of *SUS* gene may cause the metabolic changes of sucrose that may affect not only cellulose microfibril synthesis but also that of the cell wall polymer.

The preliminary screening of T-DNA insertion lines for the known cell wall-related genes, *CESA*/*CSL*, *XTH*, *LAC*, *AGP*, and *SUS* showed the presence of lines whose cell wall extensibility was modified at high frequency (Fig. 1 - 5), indicating the protocol's effectiveness. My results also suggest that these genes regulate the metabolism of cell wall constituents, thereby contributing to the regulation of cell wall extensibility. Out of these cell wall-related lines, *xth17-1* and *xth17-2* had higher cell wall extensibility in etiolated hypocotyls, whereas the *xth17* line showed reduced petiolar growth rates in response to both low R/FR and green shade (Sasidharan et al., 2010). This discrepancy may be caused by differences in organs and growth conditions.

The modification of cell wall extensibility was also observed at a certain rate in the screening of randomly selected T-DNA insertion lines (Fig. 6). In the screening, variations in length among lines were shown to be smaller than those in cell wall extensibility. Growth may be limited by factors other than cell wall mechanical properties, such as osmotic potential, in lines that show changes only in cell wall extensibility. The cell wall plays a principal role not only in growth regulation, but also in many other physiological processes such as environmental responses. Thus, screening

of lines for cell wall extensibility needs to be conducted to understand the mechanism underlying the regulation of cell wall properties.

Table 1. A list of the T-DNA insertion lines selected in the study.

Locus	Stock number	Gene description	Cell wall extensibility (% of WT)
Cell wall-related genes			
Higher cell wall extensibility			
AT1G65310	SALK_008429C	Xyloglucan endotrans-glucosylase/hydrolase 17	140
AT2G32540	SALK_067582C	Cellulose synthase-like protein B4	128
AT2G41905	SALK_148775C	Protein match is Arabino-galactan protein 23	127
AT3G20865	SALK_089247C	Arabinogalactan protein 40	129
AT4G24000	SALK_008597C	Cellulose synthase-like protein G2	150
AT5G10430	SALK_024865C	Arabinogalactan protein 8	146
AT5G40730	SALK_046558C	Arabinogalactan protein 24	121
AT5G48100	SALK_002972C	Laccase-like 15	131
Lower cell wall extensibility			
AT1G32180	SALK_037261C	Cellulose synthase-like protein D6	66
AT2G32530	SALK_025333C	Cellulose synthase-like protein B3	76
AT4G02280	SALK_016906C	Sucrose synthase 3	63
Non-cell wall-related genes			
Higher cell wall extensibility			
AT1G32950	SALK_013864C	Subtilase family protein	148
AT1G55020	SALK_000058C	Lipoxygenase	120

AT1G66860	SALK_014047C	Class I glutamine amido-transferase-like superfamily protein	131
AT1G67960	SALK_000924C	Pollen defective in guidence 1	127
AT3G02810	SALK_000019C	Receptor-like cyto-plasmic kinase	167
AT4G17780	SALK_000834C	F-box and associated interaction domains-containing protein	150
AT5G03070	SALK_005888C	Putative importin alpha isoform	147
Lower cell wall extensibility			
AT3G10090	SALK_006148C	Nucleic acid-binding, OB-fold-like protein	73
AT3G30570	SALK_000350C	Non-LTR retrotrans-poson family	68
AT4G00730	SALK_000196C	Anthocyaninless 2	57
AT4G08050	SALK_000344C	Gypsy-like retrotran-sposon family	73

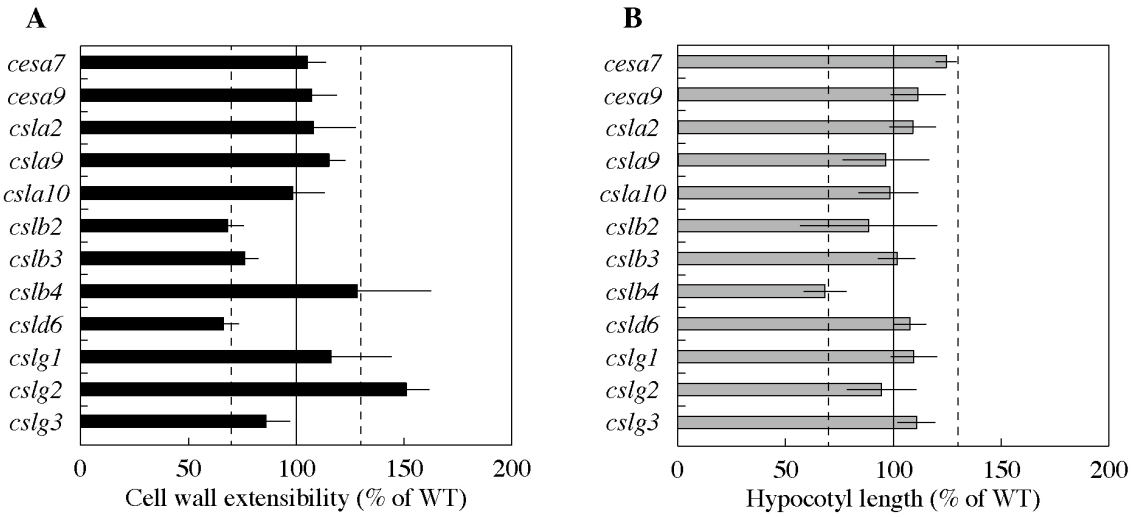


Fig. 1. Cell wall extensibility (A) and length (B) of hypocotyls in Arabidopsis T-DNA insertion lines of cellulose synthase/cellulose synthase-like protein (*CESA*/*CSL*) genes. Seedlings grown for 48 h in the dark were harvested, and the length and cell wall extensibility of hypocotyls was measured. Values of cell wall extensibility and length of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

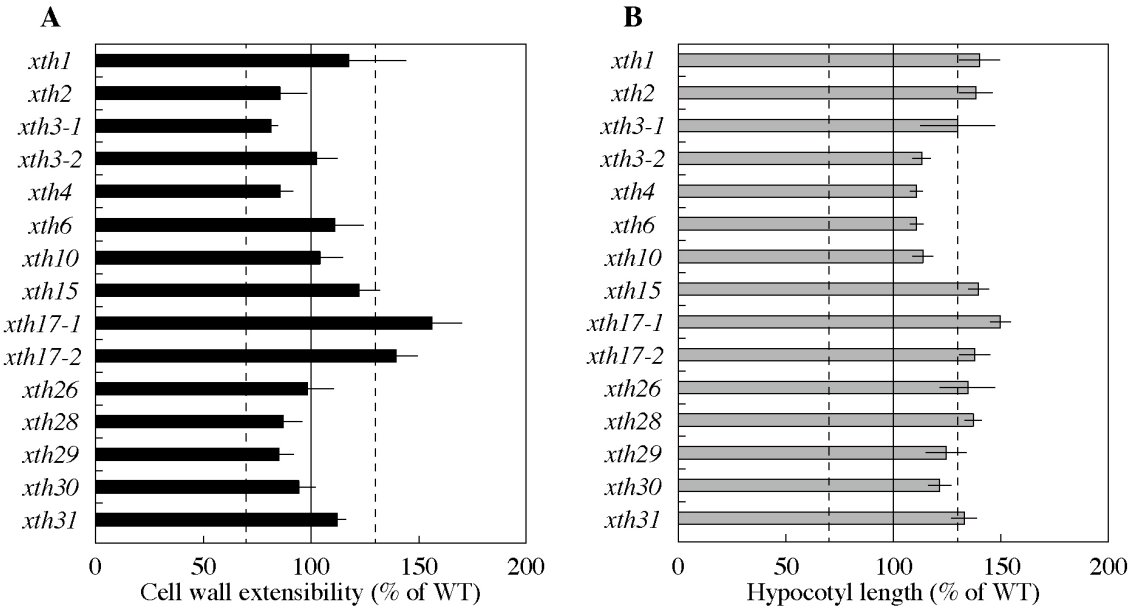


Fig. 2. Cell wall extensibility (A) and length (B) of hypocotyls in Arabidopsis T-DNA insertion lines of xyloglucan endotransglucosylase/hydrolase (*XTH*) genes. Cell wall extensibility and the length of hypocotyls were measured. Seedlings were grown as described in Fig. 1. Values of cell wall extensibility and length of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

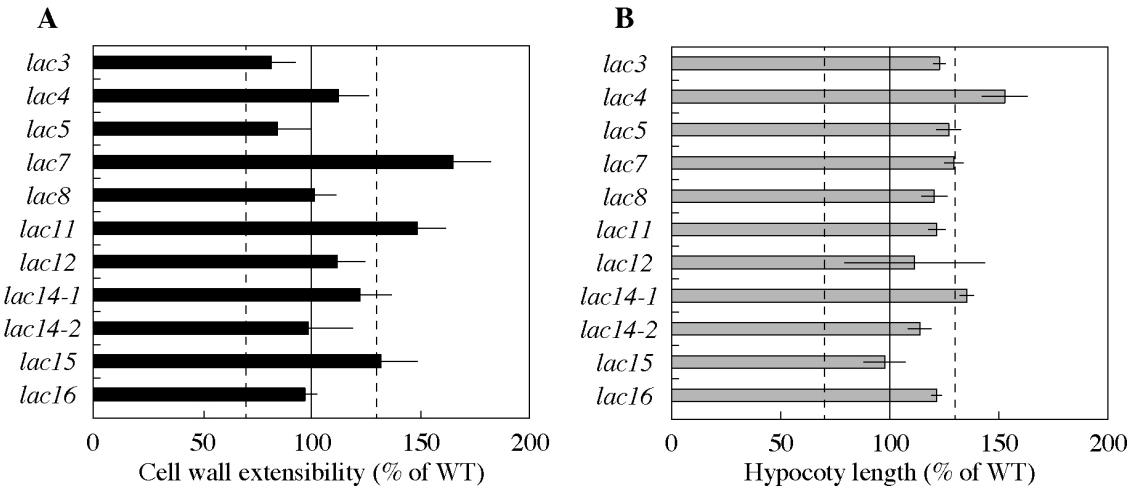


Fig. 3. Cell wall extensibility (A) and length (B) of hypocotyls in Arabidopsis T-DNA insertion lines of laccase (*LAC*) genes. Cell wall extensibility and the length of hypocotyls were measured. Seedlings were grown as described in Fig. 1. Values of cell wall extensibility and length of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

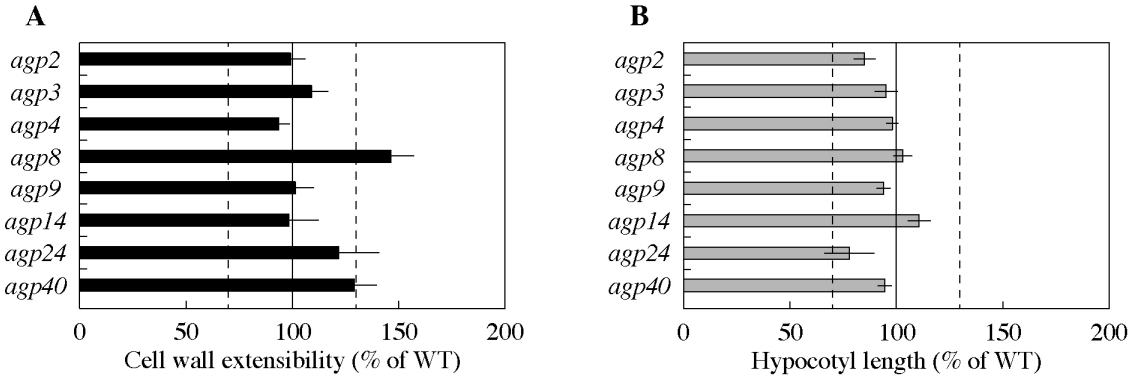


Fig. 4. Cell wall extensibility (A) and length (B) of hypocotyls in Arabidopsis T-DNA insertion lines of arabinogalactan protein (*AGP*) genes. Cell wall extensibility and the length of hypocotyls were measured. Seedlings were grown as described in Fig. 1. Values of cell wall extensibility and length of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

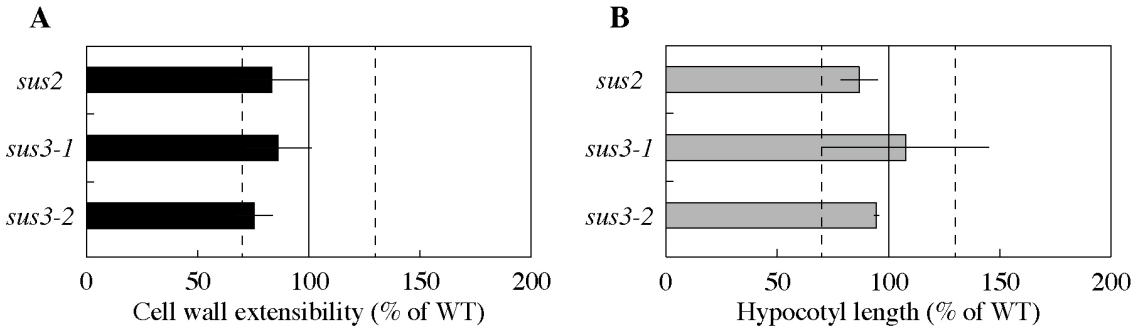


Fig. 5. Cell wall extensibility (A) and length (B) of hypocotyls in Arabidopsis T-DNA insertion lines of sucrose synthase (*SUS*) genes. Cell wall extensibility and the length of hypocotyls were measured. Seedlings were grown as described in Fig. 1. Values of cell wall extensibility and length of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

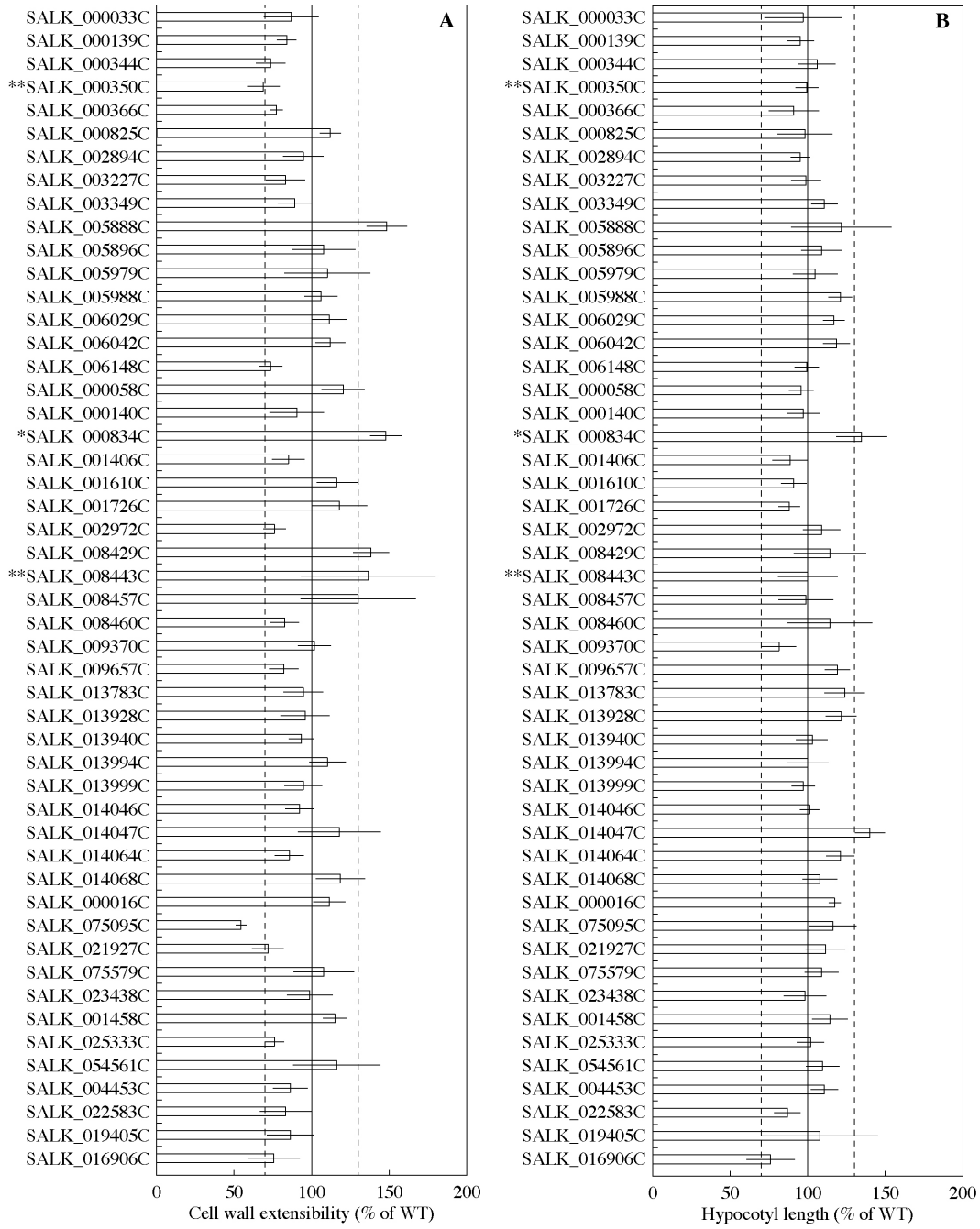


Fig. 6. Example of screening randomly selected T-DNA insertion lines. Cell wall extensibility (A) and the length (B) of hypocotyls were measured. Seedlings were grown as described in Fig. 1. Cell wall extensibility values and the lengths of T-DNA insertion lines are represented as the relative value against those of the wild-type. Broken lines represent 70% and 130% levels of the wild-type value. Data are means ± SE (n = 3).

CHAPTER 2

Functional analysis of ANTHOCYANINLESS2 in relation to the cell wall properties

ABSTRACT

In the first round of screening of randomly selected T-DNA insertion lines, I identified *ANTHOCYANINLESS2* (*ANL2*), a gene involved in the regulation of cell wall mechanical properties. In the *anl2* mutant, the cell wall extensibility of hypocotyls was significantly lower than that of the wild-type. Diameter of hypocotyls increased at upper region of *anl2* hypocotyls than that of the wild-type. Levels of cell wall polysaccharides per hypocotyl, particularly cellulose, increased in *anl2*. Microarray analysis showed that in *anl2*, expression levels of the major peroxidase genes also increased. Moreover, the activity of ionically wall-bound peroxidases clearly increased in *anl2*. The activation of peroxidases as well as the accumulation of cell wall polysaccharides may be involved in decreased cell wall extensibility. The approach employed in the present study could contribute to our understanding of the mechanisms underlying the regulation of cell wall mechanical properties.

INTRODUCTION

I found that 22 lines showed modifications of cell wall extensibility by the first round of screening of randomly selected T-DNA insertion lines, as shown in Chapter 1. Out of the 22 lines, 11 were classified as "already known" cell wall-related lines, whereas the other 11 were classified as "non-cell wall-related" lines. Out of the 11 lines of non-cell wall-related lines, I focused on *ANTHOCYANINLESS2* (*ANL2*), a gene involved in the regulation of cell wall mechanical properties.

Two ANTHOCYANINLESS loci, *ANL1* and *ANL2*, have been reported in Arabidopsis (Kubo et al., 1999, 2007). Both genes are expressed in leaves, stems, flower buds, and roots. *ANL1* encodes UDP-glucose:flavonoid-3-*O*-glucosyltransferase (Kubo et al., 2007), whereas *ANL2* encodes a homeodomain protein belonging to the HD-GLABRA2 group (Kubo et al., 1999). Kubo et al. (1999) suggested that *ANL2* regulates two independent pathways: an anthocyanin accumulation in subepidermal tissues and cellular organization in the primary root. The *ANL2* gene is also involved in cuticle biosynthesis. In rosette leaves of *anl2*, Nadakuduti et al. (2012) observed a 40% reduction in the cutin monomer load and a 25% reduction in the alkane load of cuticle waxes. However, a decrease in the cutin level does not explain the decrease in cell wall extensibility of *anl2*. Therefore, I examined the

possible mechanism underlying modifications of cell wall mechanical properties in *anl2*.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia-0) and the confirmed homozygous T-DNA insertion lines of Arabidopsis were used in the present study. T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). Arabidopsis seeds were planted on 1% (w/v) agar in a 24-well culture plate, and incubated at 4°C for 7 d. The seeds were then exposed to white light for 1 d at 23°C to induce germination, and cultivated at 24°C in the dark. After cultivation, seedlings were fixed by pouring hot 80% ethanol into each well, and stored in fresh 80% ethanol until further use.

Measurement of growth behaviors and cell wall extensibility

Before measuring cell wall mechanical properties, seedlings fixed in 80% ethanol were rehydrated with several changes of water and the lengths of their hypocotyls were measured using a scale. The diameters of their hypocotyls were measured using a digital multi-angle stereoscopic microscope system (VB-G25; Keyence, Osaka, Japan) and determined using ImageJ software (http://rsbweb.nih.gov/ij/, NIH). The cell wall

extensibility of hypocotyls was measured with a tensile tester (Tensilon RTM-25; Toyo Baldwin, Tokyo, Japan) (Parvez et al., 1996). Hypocotyls were fixed between two clamps (distance between clamps was 1 mm) and stretched by lowering the bottom clamp at a speed of 20 mm/min until a load of 0.8 g was produced. Cell wall extensibility (strain/load, μm/g) was determined by measuring the load's rate of increase just before it reached 0.8 g.

Quantification of cell wall polysaccharides

Seedlings (120 per batch) were immediately boiled for 10 min in 80% ethanol after cultivation for quantification of cell wall polysaccharides. Cell wall polysaccharides were fractionated by the methods described by Nishitani and Masuda (1979) and Sakurai et al. (1987). Hypocotyls excised from rehydrated seedlings were homogenized in water, and then washed with water, acetone and a methanol:chloroform mixture (1:1, v/v), and treated with 2 units/mL porcine pancreatic a-amylase (EC 3.2.1.1; type I-A; Sigma, St. Louis, Mo., USA) in 50 mM sodium acetate buffer (pH 6.5) at 37°C for 3 h. After amylase treatment, pectic substances were extracted from the cell wall material three times (15 min each) with 50 mM EDTA (pH 6.8) at 95°C. Hemicellulose was extracted three times (12 h each) with 4% (w/v) KOH, and three times (12 h each) with 24% (w/v) KOH

containing 0.02% $NaBH_4$ at 25°C. The fractions extracted with 4% and 24% KOH were designated as hemicellulose-I and hemicellulose-II, respectively; these fractions were neutralized with acetic acid. The alkali-insoluble fraction (cellulose fraction) was then washed with 0.03 M acetic acid and ethanol, and dried at 40°C. The cellulose fraction was dissolved in 72% (v/v) sulfuric acid for 1 h at 25°C, and then diluted with a 29-fold volume of water. The sugar content in each fraction was determined by the phenol-sulfuric acid method (Dubois et al., 1956) and expressed as glucose equivalents.

Extraction and assay of cell wall-bound peroxidase activity

The collected seedlings (20 per batch) were immediately frozen with liquid nitrogen and kept at -80°C until further use. The frozen seedlings were homogenized with ice-cold 10 mM sodium phosphate buffer (pH 6.8). The homogenate was poured into a plastic column (mini column CC07; Sarstedt, Tokyo, Japan) and then thoroughly washed with the same buffer. Cell wall residues were then suspended in 10 mM sodium phosphate buffer (pH 6.0) containing 1.5 M NaCl. The suspension was kept for 24 h at 4°C and then filtered through the mini column. The filtrate was used for the assay of the ionically wall-bound peroxidase activity (Wakabayashi et al., 2012). Activity was measured according to the

methods outlined in Sato et al. (1993). The reaction mixture (1 mL) contained 50 μL of the enzyme preparation, 13 mM guaiacol, and 5 mM H_2O_2 in 40 mM MES-KOH buffer (pH 6.0). The reaction was initiated by the addition of H_2O_2 at 25°C. The absorbance at 470 nm was recorded at 30 s intervals, and enzyme activity was quantified as the increase in absorbance 1.5 to 2.0 min after the addition of H_2O_2.

Microarray analysis

Seedlings were collected and immediately frozen with liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA). cDNA and fluorescently labeled cRNA were synthesized using an Affymetrix GeneChip WT PLUS Reagent Kit (Agilent Technologies, Santa Clara, CA, USA). Microarray analysis was performed by Kurabo Industries (Osaka, Japan) with an Arabidopsis Gene 1.0ST Array (Agilent Technologies).

In plants, Class III peroxidases comprise secretory plant peroxidases, and the Arabidopsis genome contains 73 class III peroxidase genes (Tognolli et al., 2002; Welinder et al., 2002). I analyzed the expression levels of class III peroxidase genes using the values of fluorescence intensities obtained by the microarray analysis.

Phenotype analysis

Wild-type and *anl2* seeds were sown on rockwool blocks moistened with 2000-fold diluted Hyponex solution (Hyponex Japan, Osaka, Japan), and then cultivated under continuous white light (43 ± 2 μmol s^{-1} m^{-2}) at 23°C for 30 d to analyze rosette leaf morphology. Rosette leaves were numbered from the first true leaf that emerged after the cotyledons. Anatomical analysis was carried out using the methods of Kozuka et al. (2011). The leaf blades of the sixth to tenth rosette leaves were detached from the plants. An approximately 2-mm-thick transverse section was sliced at the middle region of the leaf blade by hand-sectioning with a razor blade. Photographs of these sections were taken using a digital multi-angle stereoscopic microscope system (VB-G25; Keyence, Osaka, Japan). An index of leaf flatness was calculated as the ratio of the straight-line distance between the two edges of the leaf blade to the actual width of the leaf blade along the curved surface, as determined using ImageJ software. Leaf blades of the first to fourth rosette leaves were observed with a scanning electron microscope (TM-1000; Hitachi, Tokyo, Japan) to analyze trichome morphology.

RESULTS

Identification of ANL2 as a cell wall-related gene

ANTHOCYANINLESS2 (*ANL2*; AT4G00730, SALK_000196C) was one of 11 non-cell wall-related genes identified as that involved in the regulation of cell wall mechanical properties. Cell wall extensibility of hypocotyls for *anl2* was clearly lower than that of the wild-type (Fig. 1). However, no clear differences were detected in the hypocotyl length between the wild-type and *anl2*. Diameter of hypocotyls significantly increased at upper region in *anl2* (Fig. 2). Diameters of hypocotyls at middle and lower region for *anl2* was no clear difference between the wild-type and *anl2*.

Quantitative changes in cell wall polysaccharides may be one of the possible mechanisms inducing the modification of cell wall extensibility. Cell wall polysaccharides were fractionated into pectin, hemicellulose-I, hemicellulose-II, and cellulose fractions, and their quantities were determined in etiolated hypocotyls of *anl2*. The quantities of all four fractions per hypocotyl or per unit length of hypocotyl significantly increased in *anl2* compared to the wild-type (Fig. 3). In particular, the increase in cellulose level was prominent. Total cell wall polysaccharides in *anl2* increased by about 60%.

Gene expression levels and activity of peroxidases in anl2

To obtain further a clue to the mechanism underlying the decrease in cell wall extensibility in *anl2*, transcriptional profiles were analyzed by microarray. In *anl2*, the quantity of 13 transcripts was more than twice that of the wild-type (Table 1), whereas that of 49 transcripts was less than 50% of that of the wild-type (Table 2). Because peroxidase genes were included in both categories, I analyzed the expression of class III peroxidase family genes in more detail. In total, 73 sequences encoding class III peroxidase genes have been identified in Arabidopsis (Tognolli et al., 2002; Welinder et al., 2002). Of these, 16 transcripts significantly changed in *anl2*; seven genes were up-regulated, and nine were down-regulated (Fig. 4). The expression level of the major peroxidase gene (i.e., 69) significantly increased in *anl2*. The levels of relatively abundant peroxidase genes, 10, 39, and 59, also increased significantly, whereas those of peroxidase genes 16, 21, and 73 decreased in *anl2*.

I measured the activity of ionically wall-bound peroxidases to determine whether modifications in gene expression reflected its activity because the expression levels of the major, and some other abundant peroxidase genes, were changed in *anl2*. The cell wall-bound peroxidase activity per seedling and per total cell wall polysaccharides significantly increased in *anl2* compared to the wild-type (Fig. 5).

Phenotypic analysis of anl2

Growth phenotypes of *anl2* were analyzed using plants grown under continuous white light conditions. There were no clear differences in the germination rate, plant body height, or the number of lateral branches. However, alterations of trichome morphology and rosette leaves were observed in *anl2* (Fig. 6). The number of trichome branching points was changed in *anl2*, where the number of trichomes with three branching points was increased, and trichomes with two branching points decreased (Fig. 6A, B). No changes were observed in the number of trichomes with one or four branching points (Fig. 6B). On the other hand, rosette leaves were much more curved in *anl2* compared to the wild-type (Fig. 6C). The morphological analysis revealed that leaf flatness was clearly reduced in *anl2* (Fig. 6D).

DISCUSSION

ANL2 is a gene involved in the regulation of the cell wall mechanical properties that I identified in the first round screening of randomly selected T-DNA insertion lines. The cell wall extensibility of *anl2* hypocotyls was significantly lower than that of the wild-type (Fig. 1). *ANL2* encodes a homeodomain protein belonging to the HD-GLABRA2 group and may have pleiotropic functions such as an anthocyanin accumulation (Kubo et al., 1999). In rosette leaves of an *anl2* mutant, Nadakuduti et al. (2012) observed a 40% reduction in cutin accumulation. *GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 4* (*GPAT4*) and *GPAT6* are involved in cutin biosynthesis in Arabidopsis (Yang et al., 2010). In gene ontology, the term 'cutin biosynthetic process' (GO:0010143) has been classified. I analyzed the expression levels of 16 genes with this classification by a microarray analysis (Table 3). Of these genes, *GPAT5* and *GPAT7* were significantly down-regulated, which may be involved in the inhibition of cutin biosynthesis. In general, a reduced cutin level may lead to softening of the cell wall. Thus, I need to determine other causes contributing to decreases in cell wall extensibility.

Homeodomain proteins also regulate the expression of genes related to the metabolism of cell wall polysaccharides. For example, GLABRA2 (GL2), a member of the HD-GLABRA2 group homeodomain

protein family, directly up-regulated the expression of *CESA5* and down-regulated the expression of *XTH17* during root development (Tominaga-Wada et al., 2009). However, the involvement of *ANL2* in the metabolism of cell wall polysaccharides had not yet been reported. In the present study, diameter of hypocotyls for anl2 was increased than that of the wild-type (Fig. 2). Moreover, I showed that the quantity of all four polysaccharide fractions, particularly cellulose, per hypocotyl or per unit length of hypocotyl significantly increased in *anl2*, compared to the wild-type (Fig. 3). The accumulation of cell wall polysaccharides may be involved in a decrease in cell wall extensibility in *anl2*.

Expression levels of major and other abundant peroxidase genes were significantly higher in *anl2* compared to the wild-type (Fig. 4). The ionically wall-bound peroxidase activity per seedling and per total cell wall polysaccharides also increased in *anl2* (Fig. 5). Cell growth is tightly associated with cell wall stiffening and loosing, and the balance between these two processes can be precisely controlled by the antagonistic activities of class III peroxidases (Francoz et al., 2015). Plant class III peroxidases are responsible for oxidizing lignin precursors, including monolignols, lignin oligomers, and polymers (Whetten and Sederoff, 1995; Hoson, 2000; Sasaki et al., 2004; Fagerstedt et al., 2010). Peroxidases are also involved in cross-linking of the structural hydroxyproline-rich glycoproteins and polysaccharide-bound ferulic acid residues in the cell

wall (Fry, 2004b; Bunzel, 2010). Changes in the expression of peroxidase genes are known to affect the cell wall architecture. Plants over-expressing *PEROXIDASE 37* (*PRX37*) showed an increase in the amount of esterified phenolic material associated with their cell wall (Pedreira et al., 2011). The over-expression of *PRX37* may also decrease cell wall extensibility. In the present study, the signal intensity of *PRX37* increased significantly in *anl2*. In addition, Shigeto et al. (2015) reported that double knockout mutants of *PRX2*, *PRX25*, and *PRX71* had decreased cell wall volume in Arabidopsis stems. On the other hand, hypergravity has been shown to increase cell wall-bound peroxidase activity as well as levels of lignin, thereby decreasing cell wall extensibility (Soga et al., 2001; Wakabayashi et al., 2009; Hoson and Wakabayashi, 2015). Tamaoki et al. (2009) reported that the expression of some peroxidase genes had increased more than two-fold following a hypergravity stimulus. Thus, changes in the expression of class III peroxidases genes, as well as the accumulation of cell wall polysaccharides, may be responsible for the decrease in cell wall extensibility.

No clear differences were noted in appearance of light-grown *anl2* plants. However, morphological alterations in trichomes and rosette leaves were observed in *anl2* (Fig. 6). Trichome branching in Arabidopsis is thought to be mediated by transiently stabilized microtubular structures (Mathur and Chua, 2000). In *lefty1* and *lefty2* mutants, which are caused by

dominant-negative amino acid substitutions in α-tubulin, branching of leaf trichomes were highly reduced (Abe et al., 2004). The mutant of katanin, a protein shown to be involved in regulating microtubule disassembly by severing microtubules, was also observed to decrease trichome branching (Burk et al., 2001). In *anl2*, expression levels of β-tubulin genes were lower compared to the wild-type (Table 4). So, changes in trichome branching in *anl2* may have been caused by alternations of microtubule structures, and *ANL2* may be involved in the regulation of microtubule stability. On the other hand, stiffness of epidermal layers is important for the retention of leaf flatness, and stiffness of epidermal layers is strongly associated with cuticle thickness (Onoda et al., 2015). The preliminary analysis of cell wall extensibility was performed in the rosette leaves of *anl2*. Cell wall extensibility of rosette leaves was clearly increased in *anl2* (Fig. 7). As mentioned above, the *ANL2* gene is involved in cuticle biosynthesis. My results suggest that a reduction of cutin accumulation in rosette leaves may lead to softening of the rosette leaves and cause a decrease in leaf flatness in *anl2*.

Table 1. Transcripts whose expression level was more than twice that of the wild-type in *anl2*.

Locus	Gene description	Log_2 (Fold change)
AT5G19890	Peroxidase 59	1.5*
AT4G12470	Azelaic acid induced 1	1.5*
AT1G32290	Unknown protein	1.5
AT4G03410	Peroxisomal membrane family protein	1.2*
AT5G38940	RmlC-like cupins superfamily protein	1.2
AT5G63660	Scorpion toxin-like knottin superfamily protein	1.2*
AT5G33390	Glycine-rich protein	1.1*
AT2G43140	Basic helix-loop-helix DNA-binding superfamily protein	1.1*
AT2G24990	Serine/threonine-protein kinase Rio1	1.1*
AT5G42530	Unknown protein	1.0*
AT5G57890	Glutamine amidotransferase type 1 family protein	1.0
AT1G52060	Mannose-binding lectin superfamily protein	1.0
AT4G11393	Defensin-like family protein	1.0

Fold changes (signal intensity in *anl2*/signal intensity of the wild type) represent the means of four independent measurements. * Mean value was significantly different between the wild-type and *anl2* at the 5% level (Student's t-test).

Table 2. Transcripts whose expression level was less than 50% of that of the wild-type in *anl2*.

Locus	Gene description	Log_2 (Fold change)
AT1G29395	Cold regulated 314 inner membrane 1	-1.0*
AT1G48720	Unknown protein	-1.0
AT3G17640	Leucine-rich repeat family protein	-1.0*
AT4G08040	1-Aminocyclopropane-1-carboxylate synthase 11	-1.0*
AT1G31710	Copper amine oxidase family protein	-1.0*
AT5G46920	Intron maturase, type II family protein	-1.0
AT1G08005	SIT4 phosphatase-associated family protein	-1.0
AT5G09480	Hydroxyproline-rich glycoprotein family protein	-1.0*
AT4G11320	Papain family cysteine protease	-1.0*
AT5G39520	Unknown protein	-1.0
AT5G58860	Cytochrome P450, family 86, subfamily A, polypeptide 1	-1.0*
AT2G03200	Eukaryotic aspartyl protease family protein	-1.0*
AT1G74460	GDSL-like Lipase/Acylhydrolase superfamily protein	-1.0*
AT1G68760	Arabidopsis thaliana nudix hydrase homolog 1	-1.0
AT2G20670	Unknown protein	-1.1*
AT5G48010	Arabidopsis thaliana thalianol synthase 1	-1.1
AT4G24140	Alpha/beta-Hydrolases superfamily protein	-1.1*
AT5G09530	Proline-rich protein 10	-1.1*
AT2G40370	Laccase 5	-1.1*
AT2G44130	Kiss me deadly 3	-1.1*
AT5G12420	O-acyltransferase family protein	-1.1*
AT1G25141	F-box associated ubiquitination effector family protein	-1.2*
AT5G55180	O-Glycosyl hydrolases family 17 protein	-1.2*

AT2G35380	Peroxidase 20	-1.2*
AT1G55990	Glycine-rich protein	-1.2
AT5G44550	Unknown protein	-1.2*
AT5G07870	HXXXD-type acyl-transferase family protein	-1.2*
AT2G41190	Transmembrane amino acid transporter family	-1.3*
AT1G49960	Xanthine/uracil permease family protein	-1.3*
AT5G38910	RmlC-like cupins superfamily protein	-1.3*
AT4G17470	Alpha/beta-Hydrolases superfamily protein	-1.3*
AT5G06090	Glycerol-3-phosphate sn-2-acyltransferase 7	-1.4*
AT5G13580	ABC-2 type transporter family protein	-1.4*
AT3G06390	Unknown protein	-1.4*
AT2G34490	Cytochrome P450, family 710, subfamily A, polypeptide 2	-1.4*
AT4G00730	Anthocyaninless 2	-1.4*
AT1G29910	Chlorophyll a/b binding protein 3	-1.5*
AT1G74670	Gibberellin-regulated family protein	-1.5*
AT2G23540	GDSL-like Lipase/Acylhydrolase superfamily protein	-1.6*
AT5G38000	Zinc-binding dehydrogenase family protein	-1.6*
AT1G53480	Arabidopsis mto 1 responding down 1	-1.7*
AT1G72140	Major facilitator superfamily protein	-1.7*
AT2G22510	Hydroxyproline-rich glycoprotein family protein	-1.7*
AT4G33610	Glycine-rich protein	-1.7*
AT4G20390	Unknown protein	-1.8*
AT4G38080	Hydroxyproline-rich glycoprotein family protein	-1.8*
AT3G60260	ELMO/CED-12 family protein	-1.9*
AT3G60140	Beta glucosidase 30	-2.0*
AT2G34430	Light-harvesting chlorophyll-protein complex II subunit B1	-2.7*

Fold changes (signal intensity in *anl2*/signal intensity of the wild type) represent the means of four independent measurements. * Mean value was significantly different between the wild-type and *anl2* at the 5% level (Student's t-test).

Table 3. Expression levels of transcripts classified in gene ontology as 'cutin biosynthetic process' (GO:0010143).

Locus	Gene description	Log_2 (Fold change)
AT2G47240	Long-chain acyl-CoA synthetase 1	0.2
AT1G15360	Ethylene-responsive transcription factor	0.1
AT5G23940	BAHD acyltransferase	0.1
AT1G64670	Alpha/beta-hydrolase domain-containing protein	0.0
AT1G06520	Glycerol-3-phosphate sn-2-acyltransferase 1	-0.1
AT4G00400	Glycerol-3-phosphate sn-2-acyltransferase 8	-0.1
AT4G34100	RING/U-box domain-containing protein	-0.1
AT1G49430	Long-chain acyl-CoA synthetase 2	-0.1
AT3G10570	Cytochrome P450, family 77, subfamily A, polypeptide 6	-0.1
AT4G01950	Glycerol-3-phosphate sn-2-acyltransferase 3	-0.3
AT1G01610	Glycerol-3-phosphate sn-2-acyltransferase 4	-0.3
AT3G48720	HXXXD-type acyltransferase-like protein	-0.4
AT2G38110	Glycerol-3-phosphate sn-2-acyltransferase 6	-0.4
AT1G02390	Glycerol-3-phosphate sn-2-acyltransferase 2	-0.6
AT3G11430	Glycerol-3-phosphate sn-2-acyltransferase 5	-0.7*
AT5G06090	Glycerol-3-phosphate sn-2-acyltransferase 7	-1.4*

Fold changes (signal intensity in *anl2*/signal intensity of the wild type) represent the means of four independent measurements. * Mean value was significantly different between the wild type and *anl2* at the 5% level (Student's t-test).

Table 4. Relative expression levels of transcripts of α- and β-tubulin in *anl2*.

	Relative gene expression level	
	α-tubulin	β-tubulin
WT	100	100
anl2	101	85

Values of gene expression level of α- and β-tubulin are represented as the relative value against those of the wild-type. Relative gene expression level represent the means of four independent measurements.

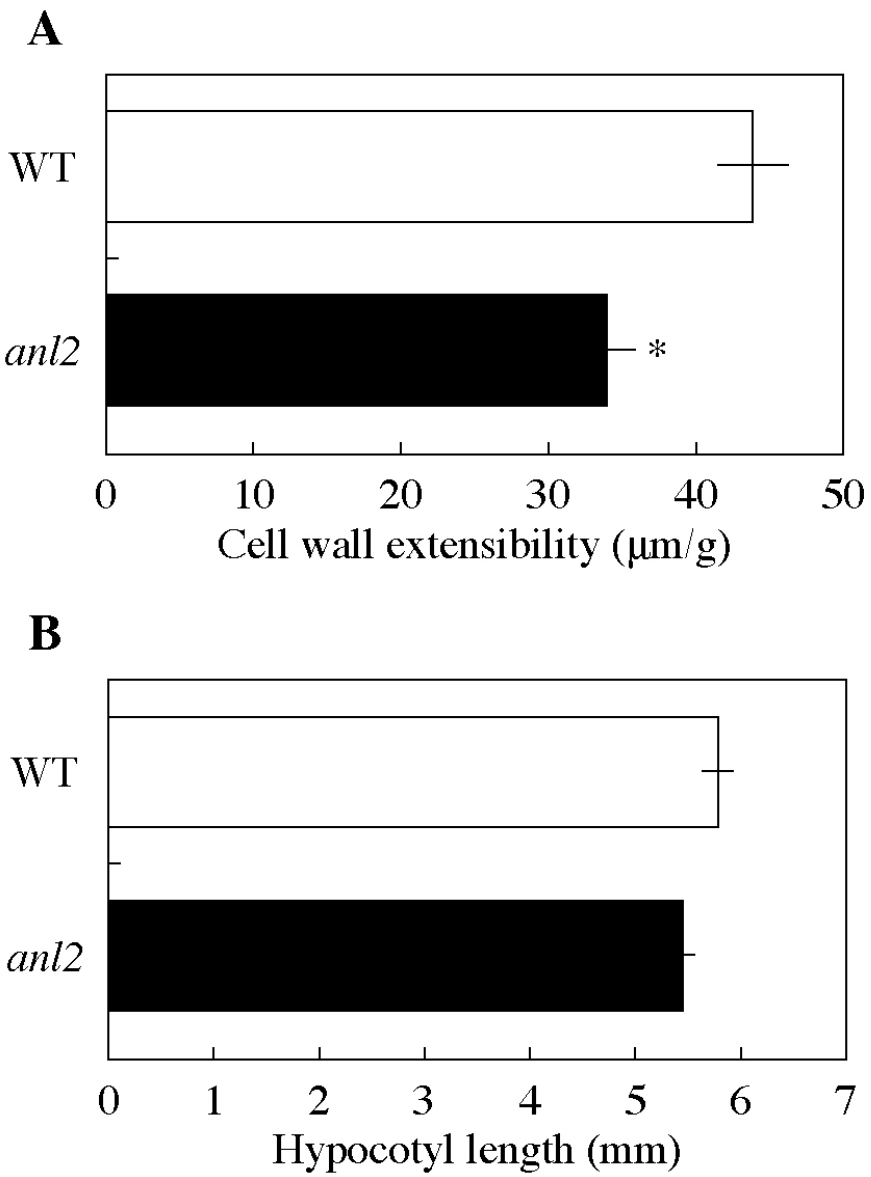


Fig. 1. Cell wall extensibility (A) and length (B) of hypocotyls of an *anl2* mutant. Seedlings grown for 36 h in the dark were harvested and the length and cell wall extensibility of hypocotyls was measured. Data are means ± SE (n = 23). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test).

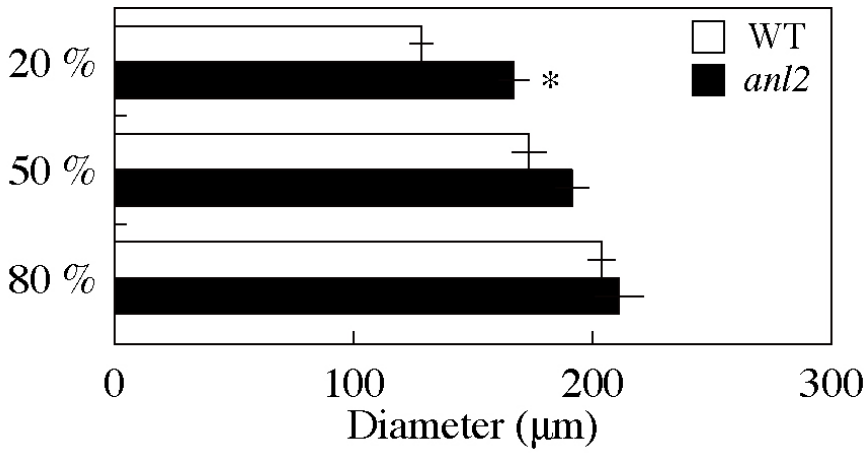


Fig. 2. Diameter of hypocotyls of an *anl2* mutant. Seedlings were grown as described in Fig. 1, and the diameter of hypocotyls was measured. Data are means ± SE (n = 30). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test).

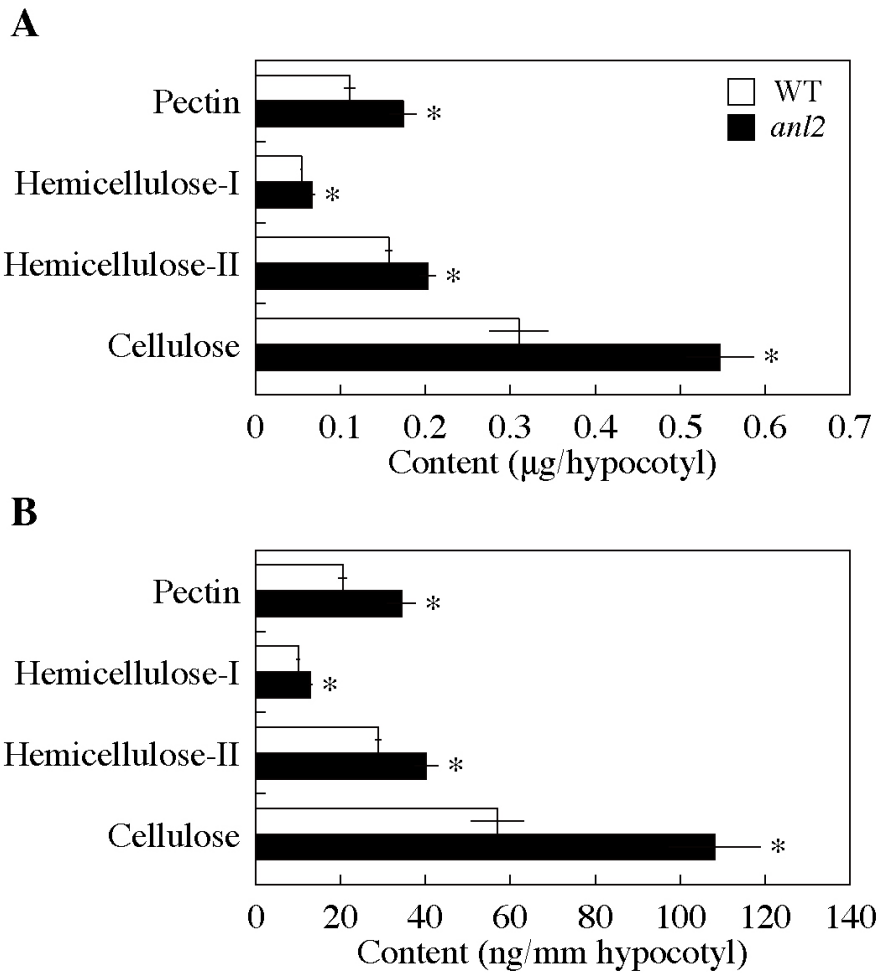


Fig. 3. Amounts of cell wall polysaccharides in *anl2*. Seedlings were grown as described in Fig.1, and cell wall polysaccharides were fractionated into four fractions. Sugar contents in each fraction per hypocotyl (A) and per unit length of hypocotyl (B) were determined by phenol-sulfuric acid method using glucose as the standard. Data are means ± SE (n = 4). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test).

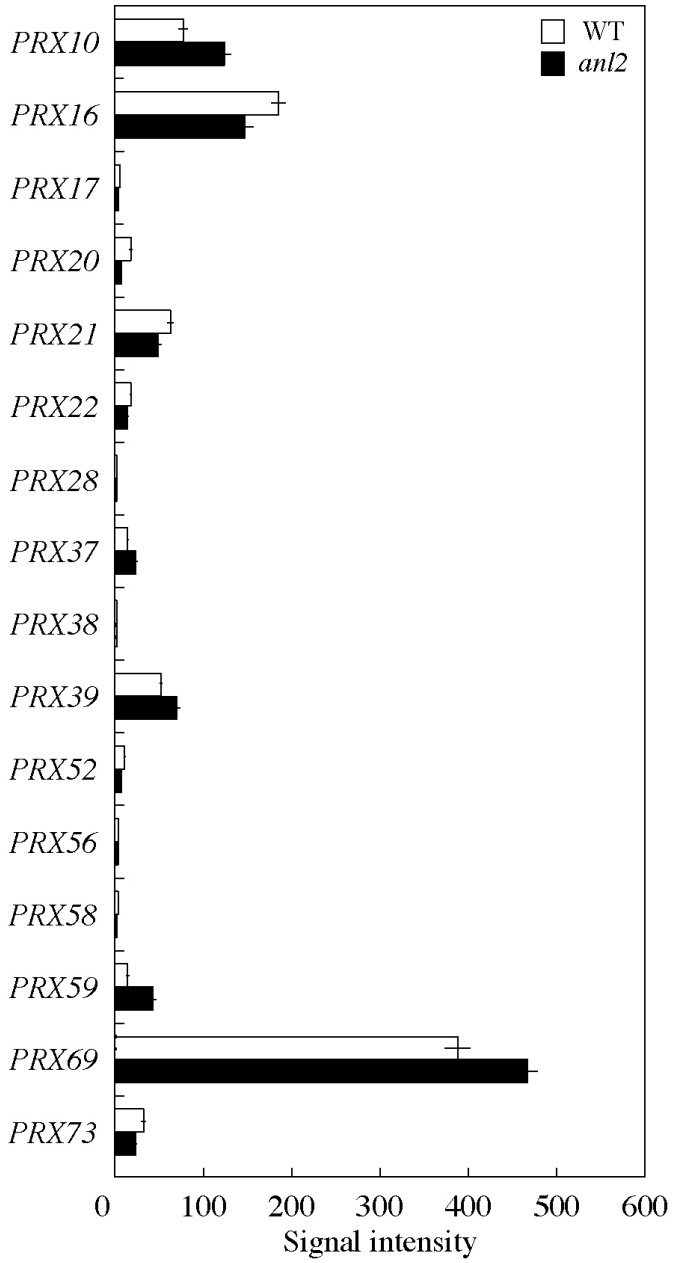


Fig. 4. Expression levels of class III peroxidase genes. Seedlings were grown as described in Fig.1, and the expression levels of class III peroxidase genes were analyzed using fluorescence intensity values obtained through a microarray analysis described in the Materials and Methods. Peroxidase genes whose intensity values are significantly different between the wild-type and *anl2* plants are presented (i.e., *PRX10*, AT1G49570; *PRX16*, AT2G18980; *PRX17*, AT2G22420; *PRX20*, AT2G35380; *PRX21*, AT2G37130; *PRX22*, AT2G38380; *PRX28*, AT3G03670; *PRX37*, AT4G08770; *PRX38*, AT4G08780; *PRX39*, AT4G11290; *PRX52*, AT5G05340; *PRX56*, AT5G15180; *PRX59*, AT5G19890; *PRX69*, AT5G64100; *PRX73*, AT5G67400). Data are means ± SE (n = 4).

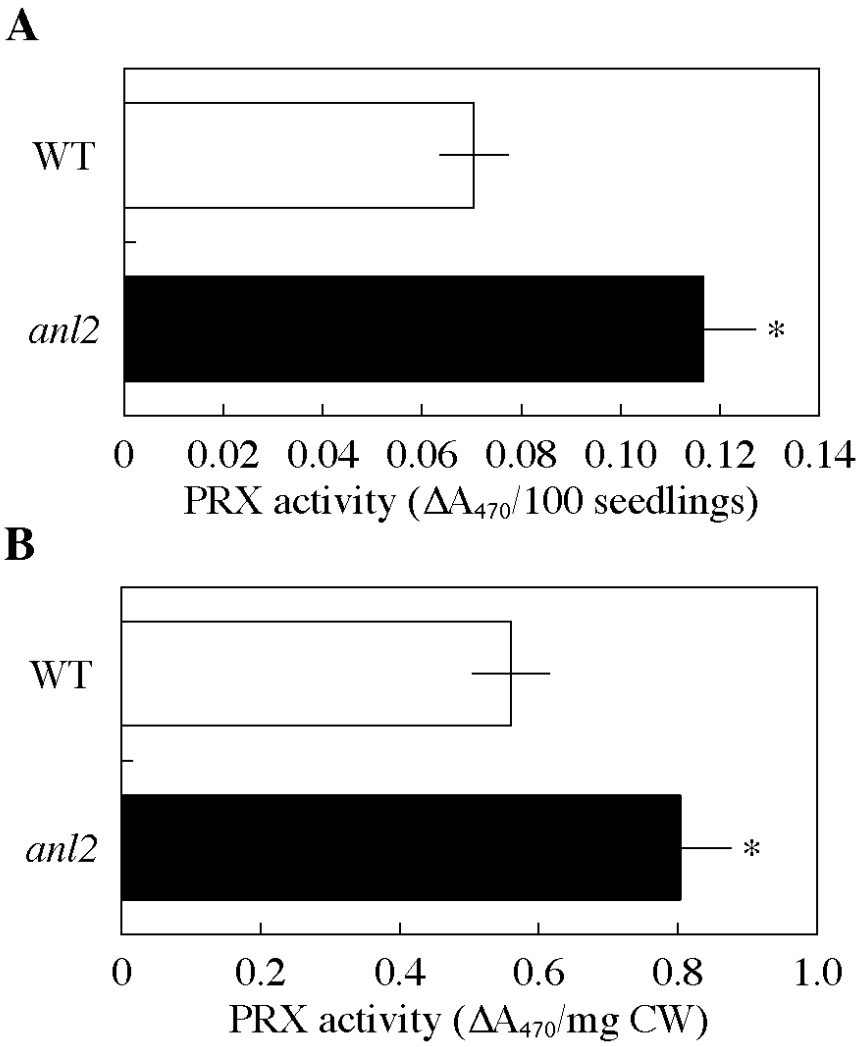


Fig. 5. Activity of cell wall-bound peroxidases (PRX) in *anl2*. Seedlings were grown as described in Fig. 1. Activities per seedling (A) and per total cell wall polysaccharides (B) were measured using guaiacol as the substrate and expressed as the increase in the absorbance at 470 nm. Data are means ± SE (n = 5). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test). CW, cell wall.

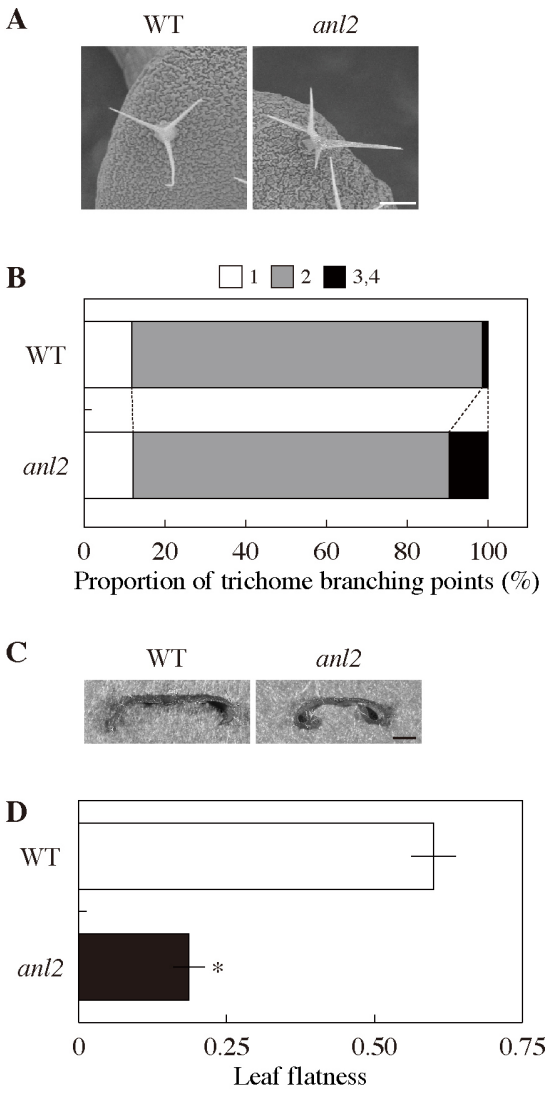
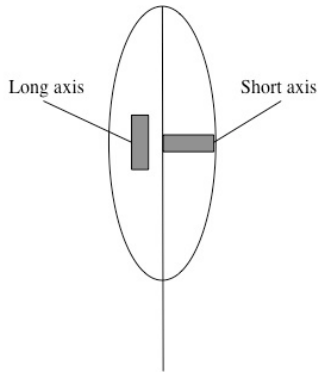


Fig. 6. Morphology of rosette leaves of *anl2*. Rosette leaves obtained from 30-d-grown plants were used for the analysis Trichome morphology (A) was observedusing a scanning electron microscope; scale bar = 100 μm. Proportion of trichome branching points (B). The percentage of branching points was calculated for approximately 800 trichomes from five rosette leaves. Curling of a rosette leaf, where the transverse section of the rosette leaf blade was observed using a stereoscopic microscope; scale bar = 1mm (C). Rosette leaf flatness was analyzed according to the methods described in the Materials and Methods (D). Data are means ± SE (n = 25). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test).

(A)



(B)

	Extensibility (μm/g)	
	Long axis	Short axis
WT	4.9 ± 0.2	6.7 ± 0.2
anl2	5.9 ± 0.3*	8.9 ± 0.8*

Fig. 7. Cell wall extensibility of rosette leaves in *anl2*. The leaf blades of the sixth to tenth rosette leaves were detached from the plants. An approximately 2-mm-thick long axis and short axis section was cut at the middle region of the leaf blade by hand-sectioning with a razor blade (A). Cell wall extensibility of these sections was measured using a tensile tester (B). Date are means ± SE (n = 6). * Mean value was significantly different between the wild-type (WT) and *anl2* mutants at the 5% level (Student's t-test).

GENERAL DISCUSSION

The preliminary screening of T-DNA insertion lines for the known cell wall-related genes showed the presence of lines whose cell wall extensibility was modified at high frequency (Chapter 1 Fig. 1 - 5). The modification of cell wall extensibility was also observed at a certain rate in the screening of randomly selected T-DNA insertion lines (Chapter 1 Fig. 6). I performed the first round screening of 405 randomly selected lines, and found that 22 lines showed modifications of cell wall extensibility. Out of the 22 lines, 11 were classified as "cell wall-related lines", whereas the other 11 were classified as "non-cell wall-related lines" (Chapter 1 Table 1).

In the 11 non-cell wall-related lines, I focused on *ANL2* gene involved in the regulation of cell wall mechanical properties. Hypocotyls of *anl2* showed lower cell wall extensibility and increased diameter than the wild-type. However, no clear differences were detected in the hypocotyl length between the wild-type and *anl2* (Chapter 2 Fig. 1, 2). The quantities of all four fractions of cell wall polysaccharides, particularly cellulose, per hypocotyl or per unit length of hypocotyl significantly increased in *anl2* compared to the wild-type (Chapter 2 Fig. 3). So, the accumulation of cell wall polysaccharides may be caused an increase in cell wall volume, and involved in a decrease in cell wall extensibility in *anl2*.

Not only the accumulation of cell wall polysaccharides, but regulations of other cell wall constituents also were involved in a decrease in cell wall extensibility in *anl2*. In *anl2*, expression levels of major and other abundant peroxidase genes were significantly higher, and the ionically wall-bound peroxidase activity per seedling and per total cell wall polysaccharides increased compared to the wild-type (Chapter 2 Fig. 4, 5). Plant class III peroxidases are responsible for oxidizing lignin precursors, including monolignols, lignin oligomers, and polymers (Whetten and Sederoff, 1995; Hoson, 2000; Sasaki et al., 2004; Fagerstedt et al., 2010). Unfortunately, no clear differences were detected in lignin content of cellulose fraction between the wild-type and *anl2* (data not shown). Peroxidases are also involved in cross-linking of the structural hydroxyproline-rich glycoproteins and polysaccharide-bound ferulic acid residues in the cell wall (Fry, 2004b; Bunzel, 2010). The native target of increased peroxidases in the cell wall of *anl2* should be determined in future studies.

I also found morphological alterations in trichomes and rosette leaves in *anl2*. The number of trichomes with three branching points was increased, and trichomes with two branching points decreased in *anl2* (Chapter 2 Fig. 6A, B). Trichome branching in Arabidopsis is thought to be mediated by transiently stabilized microtubular structures (Mathur and Chua, 2000). Expression levels of β-tubulin genes were lower in *anl2*

compared to the wild-type (Chapter 2 Table 4). *ANL2* may be involved in the regulation of microtubule stability through the regulations of β-tubulin genes expression. On the other hand, rosette leaves were much more curved in *anl2* compared to the wild-type (Chapter 2 Fig. 6C, D). In rosette leaves of *anl2*, Nadakuduti et al. (2012) observed a 40% reduction in the cutin monomer load and a 25% reduction in the alkane load of cuticle waxes. I analyzed the expression levels of 16 genes classified in gene ontology as 'cutin biosynthetic process'. Of these, 12 transcripts decreaed in *anl2*; two genes were significantly down-regulated (Chapter 2 Table 3). Thus, *ANL2* may be also involved in the cutin accumulation through the regulations of cutin biosynthetic genes expression. A reduced cutin level may lead to softening of the rosette leaves in *anl2* (Chapter 2 Fig. 7).

Based upon above results of analysis in *anl2*, the possible mechanism of functions of *ANL2* gene was summarized in Fig. 1. *ANL2* gene mainly regulates three regulation pathways: regulation of wall polysaccharide synthesis, that of phenolic substance formation, and of cortical microtubule formation. Among the regulation of phenolic substance formation, *ANL2* gene is specifically involved in regulation of class III peroxidase and cutin biosynthesis. To obtain a clue to the mechanism underlying the regulation of wall polysaccharide synthesis, transcriptional profiles of major cell wall-related genes were analyzed by microarray. No clear differences were detected in expression levels of

CES/*CSL* and *XTH* genes between the wild-type and *anl2* (data not shown), indicating *ANL2* gene does not directly regulate the expressions of *CES*/*CSL* and *XTH* genes. These results also suggest that *ANL2* gene regulates wall polysaccharide synthesis via many indirect pathways, thereby contributing to the regulation of wall polysaccharide synthesis. *ANL2* gene is involved in regulation of cell wall mechanical properties, which is mediated by the accumulation of cell wall polysaccharides and enhanced peroxidase activity. *ANL2* gene is also involved in regulation of not only rosette leaf morphology but also rosette leaf trichome morphology. Thus, I confirmed *ANL2* as a novel cell wall-related gene.

As mentioned above, plants are subjected to a great variety of environmental signals, such as light, temperature, water, and gravity, which influence their processes of growth and morphogenesis. The cell wall is responsible for environmental responses, as well as growth regulation and morphogenesis (Hoson, 2002; Cosgrove, 2005; Wolf et al., 2012). When plants are subjected to various environmental signals, the mechanical properties of the cell wall are greatly modified. Changes in the properties of cell wall constituents induced by environmental signals have been reported (Hoson, 1998). Gravity is unique among the environmental signals important for plant life in that it is always present in a constant direction and magnitude on earth. Plants have utilized gravity as the most stable and reliable signal for their survival. Plants show two principal responses to

gravity: one is gravimorphogenesis and the other is gravity resistance (Hoson and Fujii, 2009). Gravitropism is a typical response of gravimorphogenesis. Gravity resistance is aresponse that enables the plant to resist the gravitational force by developing a tough body. Plants increase the rigidity of their body locally in gravimorphogenesis and entirely in gravity resistance. Thus, the regulations of cell wall mechanical properties play an important role in both gravity responses to provide the mechanical strength to plant body. In Chapter 1, I found that 22 lines showed modifications of cell wall extensibility. To identify genes involved in gravity resistance, I analyzed the growth behavior of seven selected lines (*anl2*, *xth17*, *lac15*, *sus3*, *agp8*, *agp24* and *agp40*) under hypergravity conditions. Elongation growth of hypocotyls was suppressed by hypergravity at 300 *g* (Table 1). In *anl2*, *xth17*, *lac15*, *sus3*, and *agp40*, elongation growth was suppressed even under 1 *g* conditions. In particular in *agp8* and *agp24*, elongation growth was not further suppressed by hypergravity (Table 1). Cell wall rigidity of hypocotyls for *agp8* and *agp24* was lower than that of the wild-type under 1 *g* conditions. (Chapter 1 Fig. 4). Tamaoki et al. (2009) have reported the up-regulated expression of genes encording core proteins of AGPs in the inflorescence stems of Arabidopsis under hypergravity. Kotake et al. (2009) suggested that the acquisition of AGPs of the present forms may be involved in the development of resistance to gravitational force. To analyze the

hypergravity-induced changes in gene expression in hypocotyls of wild-type, microarray analysis was performed. Under hypergravity conditions at 300 *g*, the quantities of approximately 60% of transcripts in the *AGP* family were more than twice that of under 1 *g* conditions in wild-type (data not shown). These results suggest that *AGPs* were involved in the regulation of cell wall rigidity by contributing to the construction of cell walls. So, *AGPs* genes may be gravity resistance-related ones.

Conclusions

In the present study, I established an efficient protocol to explore T-DNA insertion lines for cell wall mechanical properties in Arabidopsis. I found 22 lines showed modifications of cell wall extensibility, and I confirmed *ANL2* as a novel cell wall-related gene. The possible cause of a decrease in cell wall extensibility in *anl2*, mediated by the accumulation of cell wall polysaccharides and enhanced peroxidase activity. I also confirmed *AGPs* as a gravity resistance-related gene. Novel cell wall-related genes with novel mechanisms may be identified in future studies using the protocol established herein, leading to an understanding of the mechanism underlying the regulation of the cell wall mechanical properties.

Table 1. Effects on hypergravity on elongation growth in hypocotyls of seven selected mutants showed modifications of cell wall extensibility.

	Elongation (mm)	
	1 *g*	300 *g*
WT	6.7 ± 0.2	4.7 ± 0.2*
anl2	6.3 ± 0.2	4.6 ± 0.2*
xth17	6.0 ± 0.3	5.3 ± 0.2*
lac15	6.1 ± 0.2	4.7 ± 0.1*
sus3	6.7 ± 0.2	5.2 ± 0.2*
agp8	5.1 ± 0.4	4.3 ± 0.3
agp24	5.2 ± 0.2	5.1 ± 0.2
agp40	5.2 ± 0.3	4.4 ± 0.2*

Wild-type (WT) and seven selected mutants were grown on agar medium at 1 g for 60 h at 25°C. Seedlings were then transferred to 1 g or 300 g conditions, and grown for a further 24 h at 25°C. Data are means ± SE (n = 8-23). * Mean value was significantly different between the 1 g and *300 g* treatments at the 5% level (Student's t-test).

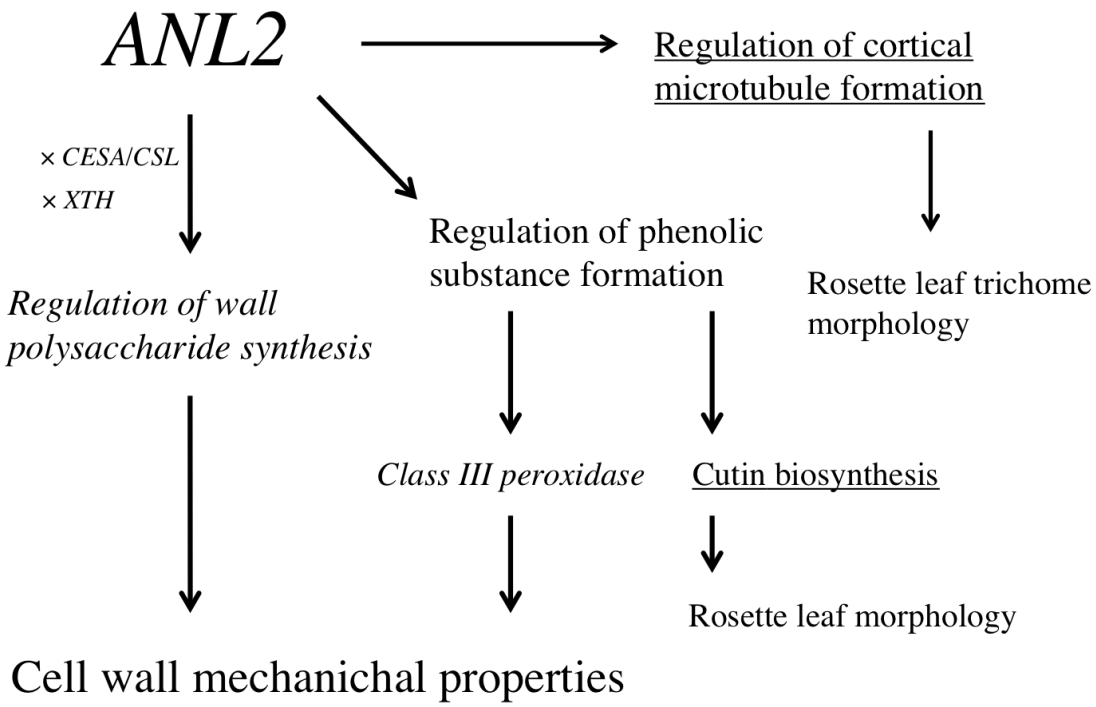


Fig. 1 Possible functions in regulations of *ANL2* gene. Underlines indicate the positive regulation of *ANL2*. Italic faces indicate the negative regulation of *ANL2*.

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REFERENCES

Abe T, Thitamadee S, Hashimoto T. Microtubule defects and cell morphogenesis in the *left1left2* tubulin mutant of *Arabidopsis thaliana*. Plant Cell Physiol 2004;45:211-20.

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA 1995;92:9353-7.

Brett CT, Waldron WK. Physiology and biochemistry of plant cell walls, 2nd edn. London: UK Chapman and Hall, 1996.

Burk DH, Liu B, Zhong R, Morrison WH, Ye ZH. A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell 2001;13:5157-62.

Bunzel M. Chemistry and occurrence of hydroxycinnamate oligomers. Phytochem Rev 2010;9:47-64.

Carpita NC, Gibeaut DM. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 1993;3:1-30.

Carpita NC, Tierney M, Campbell M. Molecular biology of the plant cell wall: searching for the genes that define structure, architecture, and dynamics. Plant Mol Biol 2001;47:1-5.

Chen LM, Carpita NC, Reiter WD, Wilson RW, Jeffries C, MaCann MC. A rapid method to screen for cell wall mutants using discriminant analysis of Fourier transform infrared spectra. The Plant Journal 1998;8:375-82.

Cocuron JC, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Railhel N, Wilkerson CG. A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. Proc Natl Acad Sci USA 2007;104:8550-5.

Cosgrove DJ. Growth of the plant cell wall. Nat Rev Mol Cell Biol 2005;11:850-61.

Dubois M, Gilles KA, Hamilton JK, Robers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P. Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. Science 2004;303:363-66.

Endler A, Persson S. Cellulose synthase and synthesis in *Arabidopsis*. Mol Plant 2001;4:199-211.

Fagard M, Desnos T, Desperz T, Goubet F, Refregier G, Mouille G, MaCann M, Rayon C, Vernhettes S, Hofte H. *PROCUSTE1* encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. The Plant Cell 2000;12:2409-23.

Fagerstedt KV, Kukkola EM, Koistinen VV, Takahashi J, Marajamma K. Cell wall lignin is polymerized by class III secretable plant peroxidases in Norway spruce. J Integr Plant Biol 2010;52:186-94.

Francoz E, Ranocha P, Nguyen-kim H, Jamet E, Burlat V, Dunand C. Roles of cell wall peroxidases in plant development. Phytochem 2015;112:15-21.

Fry SC. Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. Planta 1979;146:343-51.

Fry SC. Phenolic components of the primary cell wall. Feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. Biochem J 1982;203:493-504.

Fry SC. Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. Physiol Plant 1989;75:532-6.

Fry SC. The growing plant cell wall: chemical and metabolic analysis, 2nd impression. NJ: USA Blackburn Press, 2000.

Fry SC. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytologist 2004a;161:641-75.

Fry SC. Oxidative coupling of tyrosine and ferulic acid residues: Intra- and extra protoplasmic occurrence, predominance of trimmers and larger

products, and possible role in inter-polymeric cross-linking. Phytochem Rev 2004b;3:97-111.

Fry SC, Willis SC, Paterson AEJ. Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell suspension cultures. Planta 2000;211:679-92.

Gerber L, Zhang B, Roach M, Rende U, Gorzsas A, Kumar M, Burgert I, Niittyla T, Sundberg B. Deficient sucrose synthase activity in developing wood does not specifically affect cellulose biosynthesis, but causes an overall decrease in cell wall polymers. New Phytol 2014;203:1220-30.

Goubet F, Barton CJ, Mortimer JC, Yu X, Zhang Z, Miles GP, Richens J, Liepman AH, Seffen K, Dupree P. Cell wall glucomannan in Arabidopsis is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. Plant J 2009;60:527-38.

Haigler CH, Ivanova-Datcheva M, Hogan PS, Salnikov VV, Hwang S, Martin K, Delmer DP. Carbon partitioning to cellulose synthesis. Plant Molecular Biology 2001;47:29-51.

Hauser MT, Morikami A, Benfey PN. Conditional root expansion mutants of Arabidopsis. Development 1995;121:1237-52.

Heyn ANJ, Overbeek J. Weitres Versuchsmaterial zur plastischen und elastischen Dehnbarkeit der Zellmembran. Kon Akad Wet Amsterdam 1931;34:1190-5.

Himmel ME, Bayer EA. Lignocellulose conversion to biofuels: current challenges, global perspectives. Curr Opin Biotechnol 2009;20:316-7.

Hoegger PJ, Kilaru S, James TY, Thacker JR, Kües U. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J 2006;273:2308-26.

Hoson T. Apoplast as the site of response to environmental signals. J Plant Res 1998;111:167-77.

Hoson T. Peroxidases. In: Ohnishi M, editor. Glycoenzymes. Tokyo: Japan Scientific Societies Press, 2000. p 137-47.

Hoson T. Physiological functions of plant cell coverings. J Plant Res 2002;115:277-82.

Hoson T, Fujii N. Preface: Gravity responses and the cell wall in plants. Biol Sci Space 2009;23:113.

Hoson T, Wakabayashi K. Role of the plant cell wall in gravity resistance. Phytochem 2015;112:84-90.

Ishii T. Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell walls. Carbohydr Res 1991;219:15-22.

Kato Y, Ito S, Iki K, Matsuda K. Xyloglucan and rβ-D-glucan in cell walls of rice seedlings. Plant Cell Physiol 1982;23:351-64.

Kohji J, Nishitani K, Masuda Y. A study on the mechanism of nodding initiation of the flower stalk in a poppy, *Papaver rhoes* L. Plant Cell Physiol 1981;22:413-22.

Kotake T, Hirata N, Kitazawa K, Soga K, Tsumuraya Y. Arabinogalactan-proteins in the evolution of gravity resistance in land plants. Biol Sci Space 2009;23:143-9.

Kozuka T, Kong SG, Doi M, Shimazaki K, Nagatani A. Tissue-autonomous promotion of palisade cell development by phototropin 2 in *Arabidopsis*. Plant Cell 2011;23:3684-95.

Kubo H, Peeters AJM, Aarts MGM, Pereira A, Koornneef M. *Anthocyaninless2*. A homeobox gene affecting anthocyanin distributions and root development in Arabidopsis. Plant Cell 1999;11:1217-26.

Kubo H, Nawa N, Lupsea SA. *Anthocyaninless1* gene of *Arabidopsis thaliana* encodes a UDP-glucose:flavonoid-3-*O*-glucosyltransferase. J Plant Res 2007;120:445-9.

Liepman AH, Wilkerson CG, Keegstra K. Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proc Natl Acad Sci USA 2005;102:2221-26.

Markwalder HU, Neukom H. Diferulic acid as a possible crosslink in hemicelluloses from wheat germ. Phytochem 1976;15:836-7.

Masuda Y, Wada S. Requirement of RNA for the auxin-induced elongation of oat coleoptile. Physiol Plant 1966;19:1055-63.

Mathur J, Chuna NH. Microtubule stabilization leads to growth reorientation in Arabidopsis trichomes. Plant Cell 2000;12:465-77.

McCaig BC, Meagher RB, Dean JF. Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in Arabidopsis thaliana. Planta 2005;221:619-36.

Nadakuduti SS, Pollard M, Kosma DK, Allen C Jr, Ohlrogge JB, Barry CS. Pleiotropic phenotypes of the *sticky peel* mutant provide new insight into the role of *CUTIN DEFICIENT2* in epidermal cell function in Tomato. Plant Physiol 2012;159:945-60.

Nishitani K, Masuda Y. Growth and cell wall changes in azuki bean epicotyls. I. Changes in wall polysaccharides during intact growth. Plant Cell Physiol 1979;20:63-74.

O'Malley RC, Ecker JR. Linking genotype to phenotype using the Arabidopsis unimutant collection. Plant J 2010;61:928-40.

Olson AC, Bonner J, Morre DJ. Force extension analysis of *Avena* coleoptile cell walls. Planta 1965;66:126-34.

Onoda Y, Schieving F, Anten NP. A novel method of measuring leaf epidermis and mesophyll stiffness shows the ubiquitous nature of the sandwich structure of leaf laminas in broad-leaved angiosperm species. J Exp Bot 2015;66:2487-99.

Parvez MM, Wakabayashi K, Hoson T, Kamisaka S. Changes in cellular osmotic potential and mechanical properties of cell walls during light-induced inhibition of cell elongation in maize coleoptiles. Physiol Plant 1996;96:179-85.

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM. Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proc. Natl Acad Sci USA 1996;93:12637-42.

Pedreira J, Herrera MT, Zarra I, Revilla G. The overexpression of *AtPrx37*, an apoplastic peroxidase, reduces growth in *Arabidopsis*. Physiol Plant 2011;141:177-87.

Popper ZA, Fry SC. Primary cell wall composition of bryophytes and charophytes. Ann Bot 2003;91:1-12.

Reiter W-D, Chapple C, Somerville CR. Mutants of Arabidopsis thaliana with altered cell wall polysaccharide composition. Plant J 1997;12:335-45.

Richmond TA, Somerville CR. The cellulose synthase superfamily. Plant Physiol 2000;124:495-8.

Rose JK, Braam J, Fry SC, Nishitani K. The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. Plant Cell Physiol 2002;43:1421-35.

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter W-D, McCann MC. Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. Plant Physiol 2003;132:1033-40.

Sakurai N. Cell wall functions in growth and development. A physical and chemical point of view. Bot Mag 1991;104:235-51.

Sakurai N, Tanaka S, Kuraishi S. Changes in wall polysaccharides of squash (*Cucurbita maxima* Duch.) hypocotyls under water stress condition.

I. Wall sugar composition and growth as affected by water stress. Plant Cell Physiol 1987;28:1051-8.

Sasaki S, Nishida T, Tsutsumi Y, Kondo R. Lignin dehydrogenative polymerization mechanism: A poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in beta-*O*-4 linkage. FEBS Lett 2004;562:197-201.

Sasidharan R, Chinnappa CC, Staal M, Elzenga JTM, Yokoyama R, Nishitani K, Voesenek LACJ, Pierik R. Light quality-mediated petiole elongation in Arabidopsis during shade avoidance involves cell wall modification by xyloglucan endotransglucosylase/hydrolases. Plant Physiol 2010;154:978-90.

Sato Y, Sugiyama M, Gorecki RJ, Fukuda H, Komamine A. Interrelationship between lignin deposition and the activities of peroxidase isoenzymes in differentiating tracheary elements of *Zinnia*. Planta 1993;189:584-9.

Seifert GJ, Roberts K. The biology of arabinogalactan proteins. Annu Rev Plant Biol 2007;58:137-61.

Sene CFB, MaCann MC, Wilson RH, Grinter R. Fourier-transform Raman and Fourier-transform infrared-spectroscopy –an investigation of 5 higher-plant cell-walls and their components. Plant Physiol 1994;106:1623-31.

Shigeto J, Itoh Y, Hirao S, Ohira K, Fujita K, Tsutsumi Y. Simultaneously disrupting *AtPrx2*, *AtPrx25* and *AtPrx71* alters lignin content and structure in *Arabidopsis* stem. J Integr Plant Biol 2015;57:349-56.

Soga K, Wakabayashi K, Hoson T, Kamisaka S. Gravitational force regulates elongation growth of Arabidopsis hypocotyls by modifying xyloglucan metabolism. Adv Space Res 2001;27:1011–6.

Sticklen MB. Plant genetic engineering for biofuel production: towords affordable cellulosic ethanol. Nat Rev Genet 2008;9:433-43.

Taiz S. Plant cell expansion: Regulation of cell wall mechanical properties. Annu Rev Plant Physiol 1984;35:585-657.

Tamaoki D, Karahara I, Nishiuchi T, De Oliveira S, Schreiber L, Wakasugi T, Yamada K, Yamaguchi K, Kamisaka S. Transcriptome profiling in

Arabidopsis inflorescence stems grown under hypergravity in term of cell walls and plant hormones. Adv Space Res 2009;44:245-53.

Tognolli M, Penel C, Greppin H, Simon P. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene 2002;288:129-38.

Tominaga-Wada R, Iwata M, Sugiyama J, Kotake T, Ishida T, Yokoyama R, Nishitani K, Okada K, Wada T. The GLABRA2 homeodomain protein directly regulates *CESA5* and *XTH17* gene expression in Arabidopsis roots. Plant J 2009;60:564-74.

Tsurusaki K, Takeda K, Sakurai N. Conversion of indole-3-acetaldehyde to indole-3-acetic acid in cell wall fraction of barley (*Hordeum vulgare*) seedlings. Plant Cell Physiol 1997;38:268-73.

Wakabayashi K, Nakano S, Soga K, Hoson T. Cell wall-bound peroxidase activity and lignin formation in azuki bean epicotyls grown under hypergravity conditions. J Plant Physiol 2009;166:947-54.

Wakabayashi K, Soga K, Hoson T. Phenylalanine ammonia-lyase and cell wall peroxidase are cooperatively involved in the extensive formation of

ferulate network in cell walls of developing rice shoots. J Plant Physiol 2012;169:262-7.

Welinder KG, Justesen AF, Kjærsgård IVH, Jensen RB, Rasmussen SK, Jerpersen HM, Duroux L. Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. Eur J Biochem 2002;269:6063-81.

Wende G, Waldron WK, Smith AC, Brett CT. Developmental changes in cell-wall ferulate and dehydrodiferulates in sugar beet. Phytochem 1999;52:819-27.

Whetten R, Sederoff R. Lignin biosynthesis. Plant Cell 1995;7:1001-13.

Wolf S, Hématy K, Höfte H. Growth control and cell wall signaling in plants. Annu Rev Plant Biol 2012;63:381-407.

Yamamoto R, Masuda Y. Stress-relaxation properties of the *Avena* coleoptile cell wall. Physiol Plant 1971;25:330-5.

Yamamoto R, Shinozaki K, Masuda Y. Stress-relaxation properties of plant cell walls with special reference to auxin action. Plant and Cell Physiol 1970;11:947-56.

Yang W, Pollard M, Li-beisson Y, Beisson F, Feig M, Ohlrogge J. A distinct type of glycerol-3-phosphate acyltransferase with sn-2 preference and phosphatase activity producing 2-monoacylglycerol. Proc Natl Acad Sci USA 2010;107:12040-5.

Yokoyama R, Nishitani K. A comprehensive expression analysis of all members of a gene family encording cell-wall enzymes allowed us to predict *cis*-regulatory regions involved in cell wall construction in specific organs of Arabidopsis. Plant Cell Physiol 2001;42:1025-33.

Yong W, Link B, O'Malley R, Tewari J, Hunter CT, Lu CA, Li X, Bleecker AB, Koch KE, McCann MC, McCarty DR, Patterson SE, Reiter WD, Staiger C, Thomas SR, Vermerris W, Carpita NC. Genomics of plant cell wall biogenesis. Planta 2005;221:747-51.

Zhao Q, Nakashima J, Chen F, Yin YB, Fu CX, Yun JF, Shao H, Wang XQ, Wang ZY, Dixon RA. *LACCASE* is necessary and nonredundant with *PEROXIDASE* for lignin polymerization during vascular development in Arabidopsis. Plant Cell. 2013;25:3976-87.

Zhong R, Ye ZH. Secondary cell walls: biosynthesis, patterned deposition and transcriptional regulation. Plant Cell Physiol 2015;56:195-214.