Reverse Genetic Approach to Exploring Genes Responsible for Cell-Wall Dynamics in Supporting Tissues of *Arabidopsis thaliana* under Microgravity Conditions

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**Abstract** In 2008, the ‘Cell Wall’ experiment is scheduled to be launched and conducted on the International Space Station with the European Modular Cultivation System (EMCS). The main aim of this in-orbit plant science experiment is to elucidate the effect of gravitational conditions on supporting tissue formation in plants, thereby gaining new insight into the molecular mechanisms by which plants adapted to the land environment. In this first space experiment with in-orbit control experiments, we will specifically aim to elucidate the expression profiles of several candidate genes encoding proteins that are involved in the construction and restructuring of the secondary cell wall in the stem of *Arabidopsis thaliana* grown both in microgravity and 1G conditions. This review article deals with biological background pertinent to the ‘Cell Wall’ experiment, the anticipated experimental procedures to be used, together with a perspective of how this space experiment will extend our knowledge in both pure and applied life sciences.

**Introduction**

**Biological implications of supporting tissues in land plants**

Plants, which are currently found ubiquitously on the surface of the earth and are familiar to us, are actually unusual organisms in that they are extremely flexible in adapting to a wide variety of environmental conditions. It is quite conceivable that this strategic adaptability characteristic of plants has allowed them to pioneer numerous evolutionary paths during the long history of life on Earth. In the invasion of land by plants, estimated to have begun about a half-billion years ago, the aquatic ancestors of plants must have had to overcome several hurdles in adapting to land conditions which, in many aspects, are totally different from those in the sea. Accordingly, land plants have acquired several unique mechanisms for adapting to land during the evolutionary process. First, on land, plants needed to develop conductive tissue to provide them with the capability of transporting water and nutrients long distances from their underground parts to their aerial parts. Second, to protect themselves from water loss, they acquired a cuticular layer on their surface. Third, they had to develop particular mechanisms and structures to support their bodies against the 1G pull of gravity in an atmospheric environment with little or no buoyancy. To meet these requirements in adapting to land, ancestors of land plants have evolved vascular tissue which functions as both supporting and conducting tissues, and epidermal tissue with a cuticular layer. These tissues are all strong but sufficiently flexible to allow plants to send shoots above ground.

Of these three mechanisms, supporting tissue is of particular importance in terms of plant growth under 1G gravity conditions. Understanding how plants utilize gravity signals in regulating supporting-tissue formation would substantially extend our fundamental knowledge of the molecular mechanisms regulating plant growth. Such knowledge is essential especially when mankind will break new ground in near future on other planets or space vessels, where organisms will be exposed to unusual gravity conditions. Despite the biological importance of supporting tissue in land plants, the molecular mechanism by which its structure is generated, assembled, and maintained in response to the gravitational environment is only poorly understood. Our poor understanding is mostly due to the complexity and diversity of the cell wall, which plays a pivotal role in not only the construction but also the regulation of...
supporting tissues.

Structural Features of the Cell Wall

The plant cell wall is a dynamic apparatus composed of many different types of macromolecular components, such as cellulose microfibrils, cross-linking polysaccharides, pectic polysaccharides, aromatic compounds and proteoglycans, which are integrated into an organized super-molecular system (Nishitani 2002; Somerville et al. 2004; Cosgrove 2005). The structural feature of the cell wall varies considerably from cell to cell, and also changes during cell differentiation, for example from cell-plate formation (Yokoyama et al. 2001) through to wall expansion (Nishitani et al. 1981) in the primary wall, and finally, to secondary wall deposition processes (Nishitani et al. 1982) occurring in supporting tissue. Accordingly, individual cell-types, such as those in supporting tissue, have their own cell wall with specific structural features as well as specific mechanical strengths (Nishitani et al. 1979).

In dicotyledonous plants, including Arabidopsis thaliana, the primary cell walls are characterized by the cellulose/xylloglucan framework composed of approximately equal amounts of cellulose microfibrils and xylloglucans (Nishitani 1997). This framework is embedded in a network of abundant pectic polysaccharides which are composed chiefly of homogalacturonans and rhamnogalacturonans (Carpita et al. 1993). In contrast, the secondary walls, which are typical in supporting tissue, are composed of higher proportions of cellulose microfibrils impregnated with lignin, which forms a hydrophobic network that bonds cellulose microfibrils tightly, thereby providing the secondary walls with mechanical strength and hydrophobicity (Carpita et al. 1993).

Several important proteins that affect the structural and functional features of various polymers in cell walls, thereby modifying the mechanical properties, have been identified and their genes cloned. These proteins include β1-4 glucanases (Lashbrook et al. 1994), xylloglucan endotransglucosilase/hydrolases (XTH) (Nishitani et al. 1992), expansins (McQueen-Mason et al. 1992), cellulose synthases (Pear et al. 1996; Taylor et al. 1999), polygalacturonic acid (Gonzalez-Carranza et al. 2002), pectin methyl esterases (Micheli et al. 1998), peroxidases (Veitch 2004) and laccases (Ranocha et al. 1999). Notably, according to the database of plant genome sequences completed to date, these cell wall related proteins are invariably encoded by a large multi-gene family. Such a multi-gene family typically consists of dozens of members. For example, the XTH family of genes, which is central to construction of the basic architectures of the cellulose/xylloglucan framework in plant cell walls consists of 33, 29 and 44 genes in Arabidopsis thaliana, rice and poplar, respectively (Yokoyama et al. 2001; Yokoyama et al. 2004; Geisler-Lee et al. 2006).

Cell-wall-type specific gene-set hypothesis

We demonstrated that each of the XTH family genes was expressed predominantly in a particular organ(s), indicating the organ-, tissue- or cell- specific expression profile of each member of this family of genes (Catalá et al. 1997; Bourquin et al. 2002; Vissenberg et al. 2005; Becnel et al. 2006). Furthermore, individual members responded differently to sets of plant hormones (Chen et al. 2002; Osato et al. 2006) and environmental signals, such as mechanical stimulus (AtXTH22 or TCH4) (Xu et al. 1996) and light (AtXTH15) (Hare et al. 2003). Similar cell-type specific expression patterns are found in other gene families encoding cell-wall related proteins, such as cellulose synthases (Holland et al. 2000) and expansins (Wu et al. 2001).

Plant anatomy tells us that a plant body is comprised of some 40 different cell types, and that each cell type has a specific type of cell wall. This fact, coupled with the notion of ‘cell type-specific expression patterns of each member gene’ as mentioned above, hints at the possibility that a single or a few members from each of the cell wall related gene families is required to characterize a certain cell wall type, and thereby, a certain cell type. We have termed this scheme the ‘Cell Wall-Type Specific Gene-Set Hypothesis’ (Nishitani 2002). To explain this scheme simply, let us suppose that a cell wall specific to supporting tissue A requires the action of XTH-a, Expansin-a and Cellulose Synthase-a, and another cell

![Cell Wall-Type Specific Gene-Set Hypothesis](image_url)

Fig. 1. A schematic illustration of the ‘Cell Wall-Type Specific Gene-Set Hypothesis’. This hypothesis supposes that a plant body consists of several cell types, and each cell type has its own type of cell wall. However, genes involved in constructing and restructuring cell walls are typically encoded by large multi-gene families such as the XTH family, Expansin family and Cellulose Synthase family. A single or a small number of members from each family commit to a certain cell wall type, and consequently, a certain cell type. (adapted from Nishitani, 2002)
wall specific to supporting tissue B requires the action of XTH-b, Expansin-b and Cellulose Synthase-b. When the enzyme group consisting of XTH-a, Expansin-a and Cellulose Synthase-a works cooperatively in a cell wall, the cell wall will be differentiated and will function appropriately for supporting tissue A. (Figure 1) This hypothesis also supposes that land plants have acquired the molecular machinery by which plants can monitor the load on a shoot due to 1G gravity in such a way as to regulate precisely the actions of these genes independently. These genes must work in a coordinated manner so that supporting tissue is not only efficiently formed, but is sufficiently flexible and strong to withstand the 1G environment.

**Gravitational conditions affect cell-wall dynamics**

Two decades ago, Halstead and Dutcher (1987) demonstrated that gravitational conditions could affect the properties of plant cell walls. Growth responses of plants to various gravitational conditions have been investigated by two approaches, namely, ‘hypergravity experiments’ and ‘microgravity experiments’. In the former approach, functions and growth responses of plants are compared between 1G and higher gravity conditions. In contrast, in microgravity experiments, plants which are grown under reduced gravity or microgravity conditions are compared to those grown in 1G conditions. Very high gravity conditions, such as 300G, can inhibit shoot growth and stimulate cell wall stiffening (Waldron et al., 1990; Nishitani et al., 1992; Hoson et al., 1996). In contrast, microgravity conditions have been shown to exert the opposite effect on the mechanical properties of the cell wall. Cellulose and lignin content were reduced in plants exposed to microgravity conditions in space (Cowles et al., 1984; Nedukha, 1996). Furthermore, an experiment carried out during the Space Shuttle STS-95 mission showed that the growth of Arabidopsis thaliana (ecotype: Columbia) hypocotyls was stimulated under microgravity conditions compared to the on-ground controls, although no 1G in-orbit control group was included in this experiment. These preliminary space experiments also showed that reduction in gravity increased the flexibility of the cell wall, with concomitant reduction in the molecular mass of xyloglucans and an increase in xyloglucan-degrading activity in the cell walls of the hypocotyls (Soga et al., 2002).

**Transcriptional regulation of cell-wall related genes by mechanical stimuli**

The name of the TCH4 (AtXTH22) gene is based on the unique response of this gene’s expression to mechanical stimuli. AtXTH22 has been shown to be up-regulated rapidly and drastically by a mechanical stimulus, TouCH (Braam, 1992). Considering the general function of the XTH family of genes, it is likely that AtXTH22 is involved in stiffening or reinforcement of the cell wall in response to touch. Given these characteristics of AtXTH22, the gene may also respond to changes in gravitational strength. However, the cis-acting element responsible for responding to the touch-derived signal has not been identified, and how mechanical stimuli lead to rapid activation of AtXTH22 gene expression is still unknown (Iliev et al., 2002). In addition to this touch-induced XTH member, there are many genes that are up-regulated by mechanical stimuli. Some of these genes encode calcium-binding proteins and cell wall-related proteins (Braam, 2005). Again, the signaling pathways connecting mechanical sensing and transcriptional regulation of these genes remain elusive.

Recently, Ko et al. (2004) showed that the weight of the plant body may act as a mechanical signal. They applied a 2.5 g weight to a 5-cm section of A. thaliana stem and monitored gene expression profiles. Many genes showed significant changes in expression profile in

<table>
<thead>
<tr>
<th>Name of gene family</th>
<th>Number of all genes in each family</th>
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</thead>
<tbody>
<tr>
<td>cellulose synthase</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>callose/glucan synthase</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>α-xylanoyltransferase</td>
<td>8</td>
<td></td>
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<td>α-fucosyltransferase</td>
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<td>glycosyltransferase</td>
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<td>epimerase</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>xyloglucan</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>endotransglucosylase/hydrolase</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>p-1,4-glucanase</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>expansin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>α-fucosidase</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>galactosidase</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>xylodidase</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>p-1,3-glucanase</td>
<td>77</td>
<td>1</td>
</tr>
<tr>
<td>chitinase</td>
<td>23</td>
<td>1</td>
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<tr>
<td>arabinosidase</td>
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<tr>
<td>mannose-hydrolase</td>
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<tr>
<td>mannosidase</td>
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<tr>
<td>pectatelelase</td>
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<td>polygalacturonase</td>
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<tr>
<td>pectinesterase</td>
<td>111</td>
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<td>3</td>
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<tr>
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<tr>
<td>extensin</td>
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<td>30</td>
<td>1</td>
</tr>
<tr>
<td>wall-associated receptor kinase</td>
<td>5</td>
<td></td>
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</table>

a: number of gene(s) down-regulated in response to gravitational stimuli (see Fig. 2). These are therefore considered ‘Key Gene(s)’ in the reaction of plants to gravitational signals.
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response to weight application. For example, AtXTH22 expression was up-regulated 34.6-fold following weight application compared with the unloaded control.

This load-application experiment was conducted as part of a series of ‘hypergravity experiments’. The results also showed that load-application stimulated the deposition of secondary cell walls in xylem tissue. In 5’ upstream regions of most genes up-regulated by load-application, putative auxin responsive cis-acting elements were found, suggesting the involvement of auxin signaling in transcriptional regulation of load-sensitive secondary cell wall depositions (Ko et al., 2004). On the other hand, as part of a series of

‘microgravity experiments’, we recently demonstrated that relieving plants of their weight by placing them horizontally caused reduced expression levels of most cell wall-related genes, especially Type C genes (Table 1) (Yokoyama et al., 2006). Type C genes are preferentially expressed at the basal region of inflorescence stems and are thought to provide supporting tissue with mechanical strength (Imoto et al., 2005) (Figure 2). This result suggests the involvement of translational regulation of these genes in the cell wall during changes in supporting tissues triggered by changing gravity conditions (Nishitani et al., 2004). These genes are as follows:

- β-1,3-glucanase/41, β-1,4-glucanase/2, β-1,4-glucanase/24, cellulose synthase/8, cellulose synthase/9, chitinase/12, galactosidase/21, glycine-rich protein/9, laccase/1, laccase/3, laccase/17, pectinesterase/61, peroxidase/38, peroxidase/60, polygalacturonase/20 and polygalacturonase/43.

Objectives of the ‘Cell Wall’ experiment

The ‘Cell Wall’ experiment is scheduled to be launched in February 2008 and conducted on the International Space Station with the European Modular Cultivation System (EMCS). The main aim of the experiment is to elucidate the effect of gravitational conditions on cell wall-related genes preferentially expressed in the supporting tissue of Arabidopsis inflorescence stems. The results will provide new insight into the molecular mechanisms underlying the gravity-responsive formation of supporting tissue in land plants.

In the present research, we specifically aim to elucidate the expression profiles of several candidate genes encoding proteins that are involved in the construction and restructuring of the secondary cell wall in Arabidopsis grown under microgravity and 1G conditions. Based on these expression profiles, we will examine whether or not a defined subset of cell wall-related genes are specifically responsible for the formation of supporting tissue in land plants, and thereby examine the cell wall-type specific gene-set hypothesis. This specific aim forms part of our long-term experimental strategy as follows:

1. Identification of gene sets that are responsible for gravity-dependent formation of cell walls in supporting tissue.
2. Characterization of expression profiles of genes that have been demonstrated to play a central role in the formation of gravity-dependent supporting tissue.
3. Functional analysis of the genes described in (2).
4. Based on the molecular mechanisms underlying gravity-dependent supporting tissue formation, we will deduce the capability of vascular plants to respond to a wide spectrum of gravitational signals in space.
Experimental procedures

Plant material

Throughout the ‘Cell Wall’ experiments, we will use *Arabidopsis thaliana* (L.) Heynh. (Ecotype: Columbia). To monitor promoter activity of the candidate genes shown in Table 1, we have generated transgenic *Arabidopsis* plants expressing promoter::GUS fusion genes. Briefly, 3 kb 5’-upstream regions of individual genes were amplified from genomic DNA of *A. thaliana* Col. 0 by PCR and cloned in-frame upstream of the *GUS* gene in the respective restriction sites of pBI101 (Clontech, Pal Alto, CA) to generate *GUS* fusion gene constructs (Jefferson *et al.*, 1987). This was followed by transformation into *A. thaliana* Col. 0 via Agrobacterium tumefaciens C58 strain using the floral-dip transformation technique (Clough *et al.*, 1998). For in-orbit experiments the wild type and a transgenic line expressing one of the GUS fusion genes will be selected and used.

On-board experimental conditions

Prior to launch, seeds of each of these *A. thaliana* lines will be sterilized and placed individually in each of the seven pores of a Plant Cultivation Chamber (PCC). After launch, the seeded PCC will be installed into the EMCS on the ISS and the plant seeds will be watered to initiate germination synchronously. All plant material will be cultivated uniformly both under 1G and microgravity conditions at 23 ±1˚C under light (50 - 75 Wm⁻²) / Dark (16/8 h) conditions at 60 ± 10 % humidity. During cultivation, parameters of plant growth including over-all images of the plants, shoot length, morphological traits, water supply, temperature and humidity in the PCC will be monitored and recorded.

Sampling and storage in-orbit

After the stems grow to 10 cm or longer (equal to 30-35 days growth after germination), individual whole shoots will be harvested by cutting the base of the stems with scissors. Individual whole stems will be stored intact in a KFT container either containing RNAlater (Ambion, Inc., Austin, TX) or 1/10 formalin solution (Sigma-Aldrich, Inc., St.Louis, MO; HT501128). Samples fixed with RNAlater will be kept at +2˚C for 5 days, then at −95˚C during the flight, while samples fixed with 1/10 formalin solution will be frozen immediately and kept at −95˚C during the flight.

Post-flight analysis

Two approaches will be employed to analyze the effects of gravity on cell walls in supporting tissues of *A. thaliana* shoots: a histological approach and a quantitative approach for cell wall structure and expression profiles of genes involved in the construction and modification of cell walls. Formalin-fixed samples will be subjected to histochemical staining with toluidine blue to examine morphological differences in supporting tissue between

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**Fig. 3.** (a) Plant samples before (left panel) or after (right panel) preservation in a formalin solution. Samples were stored in 1/10 formalin solution for three months at various temperatures indicated at the bottom of the panels. (b) GUS activity after storage in formalin solution. After the storage in the 1/10 formalin solution for three months at various temperatures, samples were washed with phosphate buffer (pH7.0) at room temperature, incubated in a X-Gluc solution overnight at 37˚C, dehydrated with 70% ethanol, clarified with chloral hydrate, and observed under a binocular light microscope.
1G-grown and microgravity-grown plants. This sample will also be subjected to GUS activity staining to localize promoter activities of several of the 16 key genes. Our preliminary on-ground experiments indicate that promoter-GUS activity can be discerned clearly even after 3 months storage in 1/10 formalin solution provided the samples are kept at −20°C (Figure 3). We will also examine immunohistochemical localization of some of the 16 key genes using specific antibodies raised against individual gene products. However, samples fixed with RINAlater will be used for characterization of mRNA expression levels. Using quantitative real-time RT-PCR, we will compare transcript levels of the 16 genes quantitatively in various parts of A. thaliana stems from plants grown in 1G and microgravity conditions. We will also characterize the histochemical localization of their mRNA by hybridization analysis of several of the 16 genes. By comparing the effect of gravity on the expression profiles of respective key genes and gene products, we will examine the cell wall-type specific gene set hypothesis.

**Perspective**

During the evolution of land plants, the development of an efficient, functional plant axis consisting of supporting tissues and conducting tissues was essential in resolving the serious problems encountered by plants as they evolved into large land-based organisms. The direction of evolution towards large organisms was inevitable, given competition with neighboring individuals for solar energy. It is generally believed that acquiring the ability to construct rigid and flexible cell walls was a pivotal step in the evolution of the axis of vascular plants. The ‘Cell Wall’ project offers the first opportunity to gain insight into cell wall dynamics as regulated by gravity signals. Accordingly, it is expected that the ‘Cell Wall’ experiment will help extend our understanding of the regulatory systems by which plants construct an efficient axis in the stem under different gravity conditions. Elucidation of these regulatory systems, in turn, will help unveil the evolutionary path by which vascular plants acquired rigid and flexible cell walls. This project will particularly contribute to two basic research fields: evolutionary biology and the developmental biology of land plants.

In addition to its biological importance, the ‘Cell Wall’ experiment will also provide invaluable data with respect to the cultivation of plants in space, which will be useful as we begin utilizing space. Exploring other planets in an attempt to find new habitats for mankind is no longer science fiction. Mars is one of the most promising candidates for future habitation beyond Earth. Given that exploration is part of the true nature of mankind, it is inevitable that we will explore space and colonize other planets in future. Plants will be important companion organisms during the colonization process because of their usefulness to mankind. They provide human beings with many resources such as oxygen, food, fiber and medicinal materials. For the more, plants are indispensable entity in mental aspects of human life. The results from the ‘Cell Wall’ experiment will help in the genetic design of plants optimized for growth and function under unusual gravity conditions. From this viewpoint, our project is not only pure science, but also applied research. Contributions from the ‘Cell Wall’ experiment to both basic and applied research will provide vitality and prosperity for life in the future.

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