Mechanical load induces upregulation of transcripts for a set of genes implicated in secondary wall formation in the supporting tissue of Arabidopsis thaliana

Kento Koizumi · Ryusuke Yokoyama · Kazuhiko Nishitani

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Abstract We examined the effects of mechanical load on transcripts of a set of cell wall related genes that are implicated in the formation of supporting tissues, by applying a 50 mg strip of aluminum foil to the inflorescence stem of Arabidopsis thaliana, a weight roughly half the fresh weight of the stem. Transcript levels of 12 of the 15 genes examined were increased by load application, as were the levels of some transcription factors that regulate secondary wall formation. These findings support the involvement of a load-sensing system in regulation of supporting tissue formation via transcriptional regulation of cell wall related genes.

Keywords Gene expression · Mechanical load · Secondary wall · Supporting tissue · Transcription

Introduction

During the early diversification of land plants, estimated to have begun about 400 million years ago, the ancestors of vascular plants evolved supporting tissue, which confers mechanical support to the plant body, thereby holding their aerial parts upright under normal gravitational (1 g) conditions. This type of tissue is characterized by a well-developed secondary wall, and functions not only to hold the plant’s own weight, but also to cope with a wide spectrum of external mechanical stresses, including those caused by wind and herbivore attacks.

Certain types of mechanical stress affect the growth and morphology of plants. Jaffe and coworkers reported that rubbing the internodes of Bryonia dioica resulted in altered morphogenesis, and termed this response ‘thigmomorphogenesis’ (Chehab et al. 2009; Jaffe 1973; Jaffe and Forbes 1993). Various signaling molecules including phytohormones and intracellular calcium have been implicated in this response (reviewed by Chehab et al. 2009). Braam and Davis (1990) showed that touch stimuli can affect the expression of certain genes, which are termed touch genes (Braam 2005; Braam and Davis 1990; Iliev et al. 2002). Changing gravitational acceleration by centrifugation also affects plant growth and cell wall properties (Hoson et al. 1996; Hoson and Soga 2003; Nishitani et al. 1992; Waldron and Brett 1990). These observations imply that plants have a sensory system for mechanical stimuli, and thereby can modulate their growth and morphogenesis to cope with mechanically stressful environmental conditions.

In view of these considerations, we have hypothesized that land plants use mechanical stress applied to individual cells as a signal to regulate and optimize formation of supporting tissue even during programmed development of the plant shoot (Yokoyama and Nishitani 2006). If this
hypothesis is true, then changes in the plants’ own weight will affect transcriptional regulation of a set of genes required for deposition of secondary wall, which is responsible for formation of supporting tissue. During our comprehensive expression studies on 765 putative cell wall related genes of *Arabidopsis thaliana* based on DNA microarray technology, we identified 45 genes that were preferentially expressed in basal region of the inflorescence stem (Imoto et al. 2005). We also noticed that a certain set of putative cell wall related genes were downregulated when plants were placed horizontally (Yokoyama and Nishitani 2006). Interestingly, most of these “downregulated” genes were considered to be implicated in secondary wall formation, in that they were preferentially expressed in the basal region of the inflorescence. These observations led us to postulate that placing the plant horizontally relieved its body weight from the basal supporting tissue, thereby reducing mechanical stress in the stem, and suppressing the formation of supporting tissue (Yokoyama and Nishitani 2006). To confirm the significance of load stimulus in the formation of supporting tissue, in the present study, we adopted another approach, in which we focused on 15 putative cell wall related genes that were preferentially expressed in the non-growing basal region of the inflorescence stem, and were also downregulated when the inflorescence stem were placed horizontally (Table 1). We applied a 50-mg weight to the inflorescence stem and examined the effect of weight application on the quantitative expression profiles of the 15 putative cell wall related genes. We also examined promoter::β-glucuronidase (GUS) expression patterns of these individual genes within the stem.

### Materials and methods

**Plant materials**

For load-application experiments and observation of GUS staining patterns, seeds of *Arabidopsis thaliana* (L.) Heynh. accession Columbia and transgenic lines expressing promoter::GUS fusion genes were sown and grown on rock wool moistened with MGRL medium (Tsukaya et al. 1991) at 22°C under continuous illumination (50 μmol m⁻² s⁻¹) in an LH200RD incubator (NKsystem, Osaka, Japan). For screening of transgenic lines, Arabidopsis plants were grown under sterile conditions on solid Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 50 mg/L kanamycin under the same light and temperature conditions.

**Load-application experiments**

We applied mechanical load to the inflorescence stems of Arabidopsis or its transgenic lines expressing promoter::GUS genes by clipping a half-folded 50-mg aluminum foil strip (20 mm wide × 20 mm long) around the stem 20–40 mm below the apex of each of ten plants that had been grown until the stems reached a height of 100 (±20) mm (Fig. 1d). For a control treatment, a half-folded 15-mg aluminum foil strip (20 mm wide × 20 mm long) was attached likewise to the same region of the stem of each of a different set of ten plants. After the load-applied plants were allowed to grow for 6 h, stem regions I (40–60 mm below the apex) and II (60–80 mm below the apex) were harvested and examined for quantitative expression profiles of the 15 putative cell wall related genes and promoter::β-glucuronidase (GUS) expression patterns within the stem.

### Table 1 Genes with transcripts downregulated when *Arabidopsis* plants were placed horizontally for 30 min (adapted from Yokoyama and Nishitani 2006)

<table>
<thead>
<tr>
<th>Gene family</th>
<th>Gene name</th>
<th>Relative expression change</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>β-1,3-glucanase</td>
<td>BGL2</td>
<td>-2.25</td>
<td>Zeidler et al. (2004);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wenzel et al. (2008)</td>
</tr>
<tr>
<td>β-1,4-glucanase</td>
<td>CEL2</td>
<td>-0.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOR (IRX2)</td>
<td>-0.70</td>
<td></td>
</tr>
<tr>
<td>Cellulose synthase</td>
<td>CesA7 (IRX3)</td>
<td>-1.14</td>
<td>Taylor et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>CesA4 (IRX5)</td>
<td>-1.29</td>
<td>Taylor et al. (2003)</td>
</tr>
<tr>
<td>Chitinase</td>
<td>CTL2</td>
<td>-1.15</td>
<td>Zhong et al. (2002)</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>BGAL4</td>
<td>-1.24</td>
<td></td>
</tr>
<tr>
<td>Laccase</td>
<td>Lac2</td>
<td>-1.05</td>
<td>Cai et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>IRX12 (Lac4)</td>
<td>-1.02</td>
<td>Brown et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Lac17</td>
<td>-1.00</td>
<td></td>
</tr>
<tr>
<td>Pectin esterase</td>
<td>PME61</td>
<td>-1.04</td>
<td></td>
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<tr>
<td>Peroxidase</td>
<td>PER42</td>
<td>-0.90</td>
<td>Blee et al. (2003)</td>
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<tr>
<td></td>
<td>PER64</td>
<td>-0.73</td>
<td>Wenzel et al. (2008)</td>
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<tr>
<td>Polygalacturonase</td>
<td>PG20</td>
<td>-1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PG43</td>
<td>-0.83</td>
<td></td>
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</table>

*Unless gene names have already been published, names designated in our previous work (Imoto et al. 2005) are shown in italics

Changes in expression levels for transcripts caused by placing whole Arabidopsis plants horizontally for 30 min. Mean values of two independent experiments were calculated and expressed as a log₂ ratio (log₂[(transcripts in plant placed horizontally)/(transcripts in upright control plant)])

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were excised separately, immediately frozen in liquid nitrogen and stored at \(-80^\circ C\) until extraction of mRNA for quantification unless otherwise specified. The experiment was repeated three times using independent samples.

Quantification of transcript levels

To quantify transcript levels of individual genes, total RNA fractions that had been extracted from frozen stem tissue sections were used for one-step real-time PCR on an ABI 7300-RT-PCR system (Applied Biosystems, Foster City, CA) using the SYBR Green RT-PCR reagent kit according to a procedure described previously (Yokoyama and Nishitani 2001). Gene-specific primers were designed using Primer Express software version 3.0 (Applied Biosystems). Reverse transcription was initiated at 48°C for 30 min followed by inactivation of the reverse transcriptase at 95°C for 10 min, and then by PCR (95°C for 15 s, 60°C for 1 min) for 40 cycles. The absolute copy numbers of transcripts per picogram of total RNA were calculated using linear DNA standard samples. To confirm the specificity of PCR amplification using each primer set, we ensured that the dissociation curve for each of the PCR products consisted of a single peak. The quantification was performed three times using total RNA derived from three independent samples, and mean values are shown in Figs. 1 and 3.

Generation of promoter::GUS transgenic lines

To generate transgenic Arabidopsis plants expressing promoter::GUS fusion genes, a 3 kb region upstream of each of the 15 genes was amplified from genomic DNA of Arabidopsis by PCR and then subcloned into vector pBI101 to yield a construct harboring the promoter region fused to the GUS gene (Jefferson et al. 1987). These constructs were transformed into Arabidopsis using *Agrobacterium tumefaciens* C58 strain via the floral-dip transformation method (Clough and Bent 1998). Kanamycin resistance was used as a selectable marker to identify T2 and T3 lines homozygous for the reporter gene construct, and lines homozygous for a single promoter::GUS gene were also confirmed by PCR amplification using primers 5’-GGGAAAGCGCGTACAAAG-3’ and 5’-GCGAAGC GGCTAGATATCA-3’, designed for the GUS gene.
Multiple independent transgenic lines for each individual promoter::GUS construct, except for Lac17 promoter::GUS, were assayed for GUS expression pattern, and a single line exhibiting a typical expression pattern was selected for each construct and used for further GUS expression analyses.

Histochemical analysis of GUS expression

Transgenic plants expressing promoter::GUS fusion genes were grown until inflorescence stems reached around 100 mm in length, and cross-sections 70 μm thick were cut from region I (Fig. 1d) of individual inflorescence stems. These sections were incubated in GUS staining solution containing 1 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-Gluc) and 0.1 M Na2HPO4–NaH2PO4 buffer (pH 7.0) at 37°C for several hours or overnight, followed by washing in 70% ethanol. The GUS-stained sections were cleared in a mixture of chloral hydrate, glycerol and water (8 g, 1 mL and 2 mL, respectively) according to Matsui et al. (2005), and observed under differential interference contrast optics (DMRPX; Leica, Heerbrugg, Switzerland). Images were recorded using a Retiga EXi CCD camera (QImaging, Surrey, Canada).

For GUS staining of the whole rosette leaves of 2-week-old transgenic plants and the floral organs of 5-week-old transgenic plants, whole organ specimens were pretreated with 90% acetone solution for 10 min on ice followed by rinsing and vacuum-infiltration in 0.1 M phosphate buffer solution containing 1 mM X-Gluc, 0.1 M Na2HPO4–NaH2PO4 buffer (pH 7.0), 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6 and 0.5% Triton X-100. The GUS-stained leaves and floral organs were observed under an MZ APO stereoscopic microscope (Leica). Images were recorded using a CCD camera.

Histological analysis

For histological analysis, region I as defined in Fig. 1d of the inflorescence stem of Arabidopsis that had been treated with the 50 mg load-application for 5 days, was cross-sectioned using a vibratome to 70 μm thickness and stained with 2% phloroglucinol-HCl to visualize lignified tissues. The stained sections were observed under differential interference contrast optics and the images were recorded using a CCD camera.

Accession numbers

Sequence data of the genes analyzed in this study can be found in the Arabidopsis Genome Initiative database under the following accession numbers: BGL2 (At3g57260), CEL2 (At1g19940), KOR (At5g49720), CesA7 (At5g17420), CesA4 (At5g44030), CTL2 (At3g16920), BGAL4 (At5g56870), Lac2 (At2g29130), IRX12 (At2g38080), Lac17 (At5g60020), PME61 (At3g59010), PER42 (At4g21960), PER64 (At5g42180), PG20 (At1g80170), PG43 (At3g42950), MYB103 (At1g63910), SND3 (At1g28470) and NST1 (At1g32770).

Results and discussion

Effects of load application on transcript levels of 15 cell wall related genes

This work was undertaken based on the hypothesis that plants monitor the fresh weight of their shoots, and use it as a signal to control shoot growth and morphogenesis. Accordingly, we applied a strip of aluminum weighing 50 mg to a 100 mm tall inflorescence stem of Arabidopsis, a weight roughly equal to the fresh weight of the upper half of the inflorescence stem (Fig. 1e), then allowed the plant to grow for 6 h. To account for the effect of touch and shading caused by the load application, as a control treatment we applied a 15 mg aluminum strip, a weight lighter than a quarter of the fresh weight of the upper part of the stem (Fig. 1e). After weight application, we quantified transcript levels of the 15 genes (Table 1) in two stem regions below the site of load application using real-time RT-PCR.

We found that transcript levels of 12 of the 15 putative cell wall related genes were significantly upregulated in response to the application of the 50 mg weight (Fig. 1). Transcript abundance of the CesA7 and CesA4 genes, which are essential for cellulose synthesis in secondary walls (Taylor et al. 1999, 2003), were elevated about 2.5-fold compared to controls. The transcript levels of eight genes, CesA7, CesA4, CTL2, IRX12, PME61, BGAL4, PG43 and CEL2, were higher in response to the load application in region I, which was situated just below the weight application site, than in region II, which is located along the stem below region I. To our knowledge, this is the first report showing that expression of a set of cell wall related genes is upregulated in response to application of a physiological load equivalent to the fresh weight of the plant stem.

Application of hypergravity by centrifugation at hundreds of times g has often been used to study plant responses to mechanical load (Hoson and Soga 2003; Nishitani et al. 1992; Soga et al. 2004; Waldron and Brett 1990). Under such high gravitational conditions, however, the inflorescence stem of Arabidopsis would receive such an extraordinary mechanical load that it could not remain upright. Under such conditions, the shoot would also suffer from dehydration due to reduced water transport through xylem, and likely exhibit stress responses to drought.
Furthermore, Martzivanou and Hampp (2003) reported that the set of genes with expression levels altered in response to hypergravity conditions of 7×g consisted largely of general stress-responsive genes. In light of this consideration, application of a relatively light load to the plant stem, but not hypergravity by centrifugation, is a more promising method for dissecting the molecular mechanisms by which plants sense and respond to mechanical load under physiological conditions.

Load application did not significantly upregulate the expression of 3 of the 15 genes, PG20, PER64 or BGL2 (Fig. 1), that had been downregulated significantly when plants were placed horizontally for 30 min (Yokoyama and Nishitani 2006). It should be noted that when an intact plant is placed horizontally, the gravitropic response commences within 30 min (Fukaki et al. 1996). Thus, the method used to relieve the shoot’s weight by placing it horizontally cannot distinguish between the response of the plant to load relief and that resulting from gravitropism. In contrast, the light-load application method we used in this study is not affected by gravitropic response and thus allows observation of the effect of mechanical load on the plant shoot. Thus, it is quite likely that transcription of PG20, PER64 and BGL2 is affected during the geotropic response of the plant, but not in response to simple load application.

Tissue specific expression patterns

To examine tissue specificity of the expression pattern of these 15 genes, we generated transgenic plants expressing promoter::GUS lines for the two typical genes CesA7 and CesA4, between the control and load-applied plants. No significant difference in expression pattern was observed between the control and load-applied plants for each of the CesA7 and CesA4 promoter::GUS lines (Fig. S2), despite apparent upregulation of their transcript levels by load application (Fig. 1). This implies that load application upregulated transcript levels specifically only in the supporting tissues, but did not affect gene expression in other tissues. This idea is consistent with the observation that the promoter::GUS expression patterns for these genes are essentially similar among different regions along intact inflorescence stem (Fig. S3).

Ko et al. (2004) demonstrated that application of a weight onto an inflorescence stem of short-day grown Arabidopsis plants resulted in stimulation of the deposition of secondary wall in supporting tissues. Arabidopsis plants grown under short-day conditions develop a thick stem with supporting tissues in which secondary wall is massively deposited (Ko et al. 2004). In contrast, in the present study, we applied a weight around the stem of an intact plant grown under continuous light conditions, under which less secondary wall is deposited, and these plants cannot withstand application of more than a 100 mg load. These results indicate that Arabidopsis has the ability to sense mechanical load in both thick, short-day grown plants and thin, continuous-light grown plants, and that plants that have constructed only a poor secondary wall also have the potential to accelerate secondary wall deposition in response to environmental conditions.

Roles of transcriptional regulation

Recent studies have revealed a set of transcription factors that work coordinately to regulate secondary wall formation and have essential roles in the formation of supporting tissues. We examined the effect of weight application on some transcription factor genes, AtMYB103, SND3 and NST1, and found that the transcript levels of two genes, AtMYB103 and SND3 were upregulated significantly in response to the artificial load application in both regions I and II (Fig. 3). AtMYB103 and SND3 have been demonstrated to activate transcription of certain cell wall related genes that are involved in the secondary wall formation in fiber tissues (Zhong et al. 2008), where all of the load-regulated 12 genes were expressed (Fig. 2). Although we have no direct evidence for it, it is tempting to speculate that the upregulation of the 12 genes by load application is mediated directly or indirectly via the action of certain transcription factors, such as AtMYB103 and SND3. On
the other hand, the transcript level of NST1 was slightly upregulated only in region I (Fig. 3). NST1, together with NST3/SND1, has been suggested to regulate AtMYB103 and SND3 (Zhong et al. 2008), and is considered to function as a master switch to regulate fiber formation (Ko et al. 2007; Mitsuda et al. 2007; Zhong et al. 2006; Zhong et al. 2007). Thus, it seems likely that de novo differentiation of fibers is not required for load-stimulated supporting tissue formation. In support of this view, even a prolonged load application for a duration of 5 days did not change the histological pattern of the supporting tissues (Fig. S4). Taken together, these results imply that a mechanical

Fig. 2 Promoter::GUS expression profiles of 15 genes in Arabidopsis. The genes were categorized as in Fig. 1. Cross-sections of region I (Fig. 1d) of inflorescence stems derived from 100 mm long shoots. Expression of each gene was detected in the xylem, interfascicular fibers, or both. Bar 200 μm
signal is transmitted through a distinct signaling pathway independent of NST1 or NST3/SND1, but mediated by the actions of AtMYB103 and SND3.

Some of the 12 load-regulated genes were expressed not only in interfascicular fibers but also in xylem vessels, whose differentiation and development is regulated via other NAC transcription factors such as VND6, VND7 and XND1 (Kubo et al. 2005; Yamaguchi et al. 2008; Zhao et al. 2008). Given that xylem vessel cell walls also confer mechanical strength on the stem, it is possible that some vessel-specific NAC transcription factors might play a role in the transcriptional regulation of the load-regulated cell wall genes. In the present study, we did not investigate responses of these transcription factors to weight application. Possible roles of these transcription factors, including AtMYB103 and SND3, in weight-regulated supporting tissue formation need further investigation.

Based on the results of a search of AGRIS, an online database of Arabidopsis cis-acting elements (Palaniswamy et al. 2006), putative cis-elements exist commonly in the 5'-upstream regions of most of the 12 genes (Table 2), and it is possible that these elements are involved in transcriptional regulation of this set of genes, although their functions in the formation of supporting tissue have not been demonstrated experimentally. Molecular dissection of the transcriptional network linking the apparatus sensing the mechanical load applied to the stem to transcriptional regulation of the genes responsible for secondary wall deposition would be a promising new approach to explore the load-regulated mechanism for supporting tissue formation.

**Concluding remarks**

Although the whole picture of the load sensory apparatus still remains elusive, it has been hypothesized that the state of the cell wall, including weight loading, is relayed to an intracellular signaling pathway by a stretch-activated calcium pulse (Batiza et al. 1996; Chehab et al. 2009). In yeast, the integral membrane protein Mid1 confers a stretch-activated calcium influx and relays the stretching signal to a downstream target, Cch1p (Iida et al. 1994; Kanzaki et al. 1999; Locke et al. 2000). A Mid1 homolog of Arabidopsis, MCA1, has been shown to correlate mechanical stimuli with Ca^{2+} influx (Nakagawa et al. 2007). Although the mechanism by which load signal is sensed and transmitted to regulate the expression of the 12 cell wall genes and the two transcriptional factors has not been demonstrated experimentally, it is

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**Table 2** Putative cis-acting elements that frequently appear in the 5'-upstream region of load-regulated genes identified in the present study

<table>
<thead>
<tr>
<th>Conserved binding domain</th>
<th>Sequence</th>
<th>Number</th>
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<tr>
<td>RAV1-A BS</td>
<td>CAACA</td>
<td>12</td>
<td>Kagaya et al. (1999)</td>
</tr>
<tr>
<td>GATA promoter motif</td>
<td>TGATAA</td>
<td>12</td>
<td>Teakle et al. (2002)</td>
</tr>
<tr>
<td>T-box promoter motif</td>
<td>ACTTTG</td>
<td>11</td>
<td>Chan et al. (2001)</td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>TTGACT</td>
<td>10</td>
<td>Yu et al. (2001)</td>
</tr>
<tr>
<td>GATA promoter motif (LRE)</td>
<td>AGATAA</td>
<td>10</td>
<td>Le Gourrierec et al. (1999)</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>GGTATA</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>LFY consensus BS</td>
<td>CCATTG</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ibox promoter motif</td>
<td>GATAAG</td>
<td>9</td>
<td>Giuliano et al. (1988)</td>
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<tr>
<td>GATA promoter motif (LRE)</td>
<td>TGATAG</td>
<td>9</td>
<td></td>
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</table>

Known binding sequences were searched using the online tool AGRIS (http://arabidopsis.med.ohio-state.edu)

a Number of genes with putative binding sequences in their 5'-upstream regions of the 12 load-regulated genes shown in Fig. 1
possible that these membrane proteins acts as a component of the sensory apparatus involved in signal transduction that leads to regulation of supporting tissue formation, which is mediated by a number of transcription factors and downstream cell wall related genes.

In conclusion, the results obtained in the present study provide evidence in support of the hypothesis that Arabidopsis possesses the ability to sense subtle changes in the fresh weight of its shoot, and thereby can regulate transcripts levels of a certain set of genes that are suggested to function in the construction and maintenance of secondary wall in supporting tissues. Elucidation of the molecular mechanisms by which the mechanical stimulus is perceived and transmitted to transcriptional regulators that govern supporting tissue formation would help us understand the biological significance of the developmental flexibility of land plants, which would be required not only in adapting to mechanically stressful land conditions, but also in modulating internally programmed normal growth processes.

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References


Braam J, Davis RW (1990) Rain-, wind-, and touch-induced cell formation would help us understand the biological significance of the developmental flexibility of land plants, which would be required not only in adapting to mechanically stressful land conditions, but also in modulating internally programmed normal growth processes.


