Note

Estimating the flexural rigidity of *Arabidopsis* inflorescence stems: Free-vibration test vs. three-point bending test

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Received August 31, 2020; accepted December 14, 2020 (Edited by S. Sawa)

Abstract The mechanical strength of a plant stem (a load-bearing organ) helps the plant resist drooping, buckling and fracturing. We previously proposed a method for quickly evaluating the stiffness of an inflorescence stem in the model plant *Arabidopsis thaliana* based on measuring its natural frequency in a free-vibration test. However, the relationship between the stiffness and flexural rigidity of inflorescence stems was unclear. Here, we compared our previously described free-vibration test with the three-point bending test, the most popular method for calculating the flexural rigidity of *A. thaliana* stems, and examined the extent to which the results were correlated. Finally, to expand the application range, we present an example of a modified free-vibration test. Our results provide a reference for improving estimates of the flexural rigidity of *A. thaliana* inflorescence stems.

Key words: *Arabidopsis thaliana*, flexural rigidity, free-vibration test, inflorescence stem, three-point bending test.

The aerial parts of a land plant are constantly exposed to mechanical stress from the external environment, e.g., gravity, wind, animals, and surrounding plants. A stem is a load-bearing organ whose mechanical strength provides the plant with resistance to drooping, buckling and fracture. The extent of resistance against deformation by the bending force of stems is determined by flexural rigidity (EI). Vibration characteristics are determined by stiffness (k) of bending (de Langre 2019). These values are related, as greater stiffness causes a higher natural frequency of vibration. Stem vibration is caused by the bending force, and thus the stiffness value k is associated with EI . Several methods are used to estimate the EI values of plant organs, including the three-point bending test and the free-vibration method (Niklas and Spatz 2012; Shah et al. 2017; Spatz and Speck 2002; Zebrowski 1991). The bending test allows EI to be calculated directly but requires an expensive machine equipped with a load cell and a linear actuator (for example, a material testing machine and a creep meter). By contrast, a vibration test can be performed using relatively inexpensive tools, but EI is estimated indirectly based on the natural frequency of vibration.

Arabidopsis thaliana is the most widely studied model land plant. Although *A. thaliana* is an annual herbaceous plant that forms rosette leaves, it is used

as a model for woody plants due to its advantages for investigating molecular mechanisms (e.g., its short life cycle, availability of genetic information and easy genetic manipulation). The inflorescence stem of *A. thaliana* is a load-bearing organ, making it suitable for studying the mechanical aspects of plants (Strabala and Macmillan 2013). The mechanical properties of wild-type, mutant and transgenic *A. thaliana* plants have been analyzed using various machine-mediated tests (reviewed by Brulé et al. 2016), including the three-point bending test to calculate EI and the bending modulus in cell wall-related mutants (Bichet et al. 2001; Jones et al. 2001; MacMillan et al. 2010, 2013; Paul-Victor and Rowe 2011; Turner and Somerville 1997; Zhu et al. 2015). A more accessible method for quickly evaluating k of an inflorescence stem was recently proposed based on natural frequency, as determined from a free-vibration test (Nakata et al. 2018). However, the relationship between the k and EI values of *A. thaliana* inflorescence stems remains to be solved.

Here, we compare the free-vibration test and three-point bending test. We generated a regression equation for calculating EI based on k determined by a free-vibration test (experiment A: exA) and modified the free-vibration test method for use in stems with various phenotypes (experiment B: exB).

We performed regression analysis of the relationship between EI and k using 53 natural accessions of *A. thaliana* from the 1001 Genomes Project (CS78942; Supplementary Table S1; The 1001 Genomes Consortium 2016) established to have wide mechanical variability (MacMillan et al. 2013). We used *A. thaliana* accession Columbia-0 (Col-0) for the modified free-vibration test. The seeds were surface sterilized, sown on solid MS medium (1x Murashige and Skoog salts, 1% sucrose, pH 5.7) containing 0.05% MES-KOH, vitamin mix and 0.25% gellan gum (for exA) or 0.5 M MES-KOH, 0.01% myo-inositol and 0.5% gellan gum (for exB). The plates were stored at 4°C in the dark for 3 days, and the plants were grown at 22°C under long-day conditions (16-h light/8-h dark) (for exA) or under continuous light (for exB). Two-week-old plants were transplanted to culture soil with vermiculite (for exA) or vermiculite alone (for exB) and grown under long-day conditions at 22°C until their height exceeded 20 cm (for exA) or reached 15–20 cm (for exB). The primary stems were harvested and immediately used for the free-vibration test. For exA, after performing the original version of the free-vibration test, the stems were soaked in water and their surfaces wiped briefly with a paper towel before performing the three-point bending test.

For exA, the free-vibration test was performed as previously described (Nakata et al. 2018) using 10-cm fragments from the base of each inflorescence stem. The camera of an iPhone 6s plus was used to capture 240-fps (frame per second) videos with the high speed-shooting function (which equals the slo-mo mode) of Photo App. The captured videos were analyzed using the Python program AraVib, and damped natural frequency f_d was automatically calculated. We previously reported that there is only a very small difference between natural frequency and damped natural frequency in *A. thaliana* stems (Nakata et al. 2018). In this study, we treated damped natural frequency f_d as equivalent to natural frequency f_n for convenience. Stiffness k was calculated using the following equation:

$$k = (2\pi f_d)^2 m_{eq}$$

where m_{eq} represents the equivalent mass of the oscillator. m_{eq} was calculated using the following equation:

$$m_{eq} = 0.245 \times 0.9 \times [\text{Fresh weight}]$$

where [Fresh weight] represents the fresh weight of the 10-cm fragment weighed with a precision balance before capturing the video. The weight of the 10-cm fragment was the order of mg, and when k was calculated, the unit of m_{eq} was converted from mg to kg.

For the three-point bending test, a Rheoner II Creep Meter (RE2-33005C) equipped with 20 N load cell (Yamaden, Tokyo, Japan) was used. For the three-point

Figure 1. Overview of the three-point bending test. (A) Schematic diagrams (the upper panels) and an image (the lower panel) of the three-point bending test. (B) Example of a load–deformation curve generated using the three-point bending test.

bending test, the optional jig FX-3305B-3L was attached to the creep meter. The sensitivity of the load cell was set to 10x amplification. The 4-cm fragment from the base was attached orthogonally to a plunger on the upper side and two supports (3 cm apart from each other) on the lower side (Figure 1A). The lower supports were moved upwards at a constant speed (0.05 mm s^{-1}). During this time, the load was sequentially measured by the plunger connected to the load cell (Figure 1A). The values were used to generate a load–deformation curve. Since we commonly observed a linear correlation between the load and the deformation value during 0.0–0.6 mm of deformation of the tested stems, we used a Python program to automatically calculate the coefficient based on the linear regression model (Figure 1B). Based on this coefficient, we estimated EI using the following equation (Niklas and Spatz 2012; Onoda et al. 2015):

$$EI = \frac{L^3}{48} \frac{F}{\delta}$$

where F represents the load, δ represents the displacement, and L represents the span (3 cm in this study). F/δ corresponds to the initial slope from the load–deformation curve.

To generate a regression equation between k and EI , we examined 137 plants from the 1001 Genomes collection using the free-vibration test and the three-

point bending test. As expected, the k and EI values showed wide variations (Figure 2A). The k ranged from 0.027–0.810 (N m^{-1}) and the EI ranged from 5.18–148.16 (N mm^2), indicating that both parameters varied approximately 30-fold across the plants. Importantly, we detected a strong positive correlation between k and EI (Figure 2A; $r^2=0.8582$). The regression equation was estimated as $EI=154.8k+6.2$ by the least square method. To evaluate the precision of the estimation, we calculated the relative error between EI derived from the three-point bending test and EI predicted based on k . The relative error was calculated using the following equation:

$$[\text{Relative error}] = \frac{[\text{Measured } EI] - [\text{Predicted } EI]}{[\text{Predicted } EI]}$$

The distribution of relative error is shown in Figure 2B. In this experiment, 50% of the samples fell within an error range of approximately 15%, and 75% fell within an error range of approximately 25%, indicating that the error range was much smaller than the range of individual differences.

Our data thus revealed a strong correlation between k and EI . Using regression analysis, we generated an equation to estimate EI from k . The EI values calculated from the three-point bending test differed from those predicted from k . There are several possible reasons for this. Estimations of both EI and k could have artificial errors. In the vibration test, the prerequisite for estimating k is that the tested stems are straight and have a uniform distribution of mass. According to Nakata et al. (2018), the basal 10-cm fragments of 20–30-cm tall Col-0 plants tend to be linear in shape and have little bias in terms of mass. However, whether this is true for the other accessions examined in the current study has not been investigated. Indeed, natural variations in mechanical and cell wall properties were previously observed in a study of 12 *A. thaliana* accessions (MacMillan et al. 2013). In addition, in the three-point bending test, the ovalization of an inflorescence stem in the transverse plane might negatively affect EI estimations (Niklas and Spatz 2012). Finally, the properties of the samples used in the two tests might have differed: because we needed to use the same samples in both tests, we used freshly harvested stems in the free-vibration test and water-soaked samples in the three-point bending test. Freshly harvested samples are usually used for bending tests of *A. thaliana* stems to reduce the effects of water loss (Paul-Victor and Rowe 2011; Turner and Somerville 1997). Nevertheless, the errors of the predicted EI values were much smaller than the individual differences in these values. Therefore, the regression equation generated in this study provided a rough prediction of the EI values obtained thus far.

Of the artificial errors described above, we focused on reducing the negative effects of variations in shape

Figure 2. Correlation between stiffness k and flexural rigidity EI . (A) Results of regression analysis of stiffness k and flexural rigidity EI . (B) Density plot of the relative differences between predicted EI and measured EI values.

and mass distribution, as well as the use of our method to examine various traits of *A. thaliana* stems. We developed a modified free-vibration test using shorter stem fragments (which equals exB). The following modifications were made to the free-vibration test: we used 6-cm fragments, we marked the 1-cm position from the apical end with red dye, we sandwiched the 1-cm region from the base between two metal blocks (Supplementary Figure S1A) and we pulled the apical end of the stem sideways with a bamboo skewer (Supplementary Figure S1B) and released it after filming began (Figure 3A, Supplementary Figure S1C). Using the modified method, it appeared that the vibration speed increased (Figure 3A, B) and the oscillation was quickly damped (Figure 3B). Based on these observations, we altered the size of the Hanning window from 240 frames (1 s) to 80 frames (1/3 s) in the AraVib program. In the original version, the damped natural frequency was <30 Hz (Nakata et al. 2018), whereas in the modified version, the damped natural frequency was 30–50 Hz (Figure 3C). The ratio of the damped natural frequency to the sampling frequency (30–50:240) was reasonable for frequency analysis according to the Nyquist–Shannon sampling theorem (Nyquist 1928; Shannon 1949). These data indicate that the natural frequency of shorter stems could be analyzed using the modified version of the free-

Figure 3. The modified free-vibration test shows the higher-speed and quickly-damped oscillation. (A) Frames of a high-speed video obtained during the free vibration of stems. “t” represents time after the start of vibration. (B) Examples of vibration waveforms. (C) Examples of FFT power spectra. In (A–C), the upper panels show the results of the original test using 10-cm fragments and the lower panels show the results of the modified test using 6-cm fragments.

vibration test. We plan to compare k from this modified method with that from the original version for the usage to reduce the negative effects of variations in shape and mass distribution and expand its application range.

Acknowledgements

We thank the Arabidopsis Biological Resource Center for providing the seeds of natural accessions from the 1001 Genomes Project, Eriko Tanaka (NAIST, Japan) for technical support and Ko Kato (NAIST, Japan), Tadashi Kunieda (NAIST, Japan) and Satoru Tsugawa (NAIST, Japan) for technical advice.

This work was supported by MEXT KAKENHI Grant-in-Aid for Scientific Research on Innovative Areas “Plant-Structure Optimization Strategy” Grant Numbers JP19H05365 (to Y.O.), JP18H05496 (to H.U.), JP18H05484 and JP18H05489 (to T.D.) and by JSPS KAKENHI Grant Numbers JP19K23753 (to M.N.) and JP19K06732 (to H.U.).

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