The Bulk Elastic Modulus and the Reversible Properties of Cell Walls in Developing Quercus Leaves

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We examined the relationship between the bulk elastic modulus (ε) of an individual leaf obtained by the pressure-volume (P–V) technique and the mechanical properties of cell walls in the leaf. The plants used were Quercus glauca and Q. serrata, an evergreen and a deciduous broad-leaved tree species, respectively. We compared ε and Young’s modulus of leaf specimens determined by the stretch technique at various stages of their leaf development. The results showed that ε increased from approximately 5 to 20 MPa during leaf development, although other potential determinants of ε such as the apoplastic water content in the leaf and the diameter of a palisade tissue cells remained almost constant. ε in these two species was similar at every developmental stage, although the apparent mechanical strength of the leaf lamina and thickness of mesophyll cell walls were greater in Q. glauca. There were significant linear relationships between Young’s modulus and ε (P < 0.01; R² = 0.78 and 0.84 in Q. glauca and Q. serrata, respectively) with small y-intercepts. From these results, we conclude that ε is closely related to the reversible properties of the cell walls. From the estimation of ε based on a physical model, we suggest that the effective thickness of cell walls responsible for ε is smaller than the observed wall thickness.

Keywords: Bulk elastic modulus — Instron technique — Pressure–volume (P–V) curve — Reversible properties of cell walls — Stress–strain curve — Young’s modulus.

Introduction

The bulk elastic modulus (ε) of an individual leaf represents the ratio of the change in cell turgor (P) to that in the relative cell volume (ΔV/V) of the leaf [ε = ΔP/(ΔV/V)]. This parameter is obtained by the pressure–volume (P–V) technique (Tyree and Hammel 1972, Cheung et al. 1976). ε determines how cell turgor decreases with loss of water in the leaf. The decrease in turgor connects directly with the decrease in leaf water potential and produces the driving force for water flow in the soil–plant–atmosphere continuum (SPAC). In this sense, ε has a role in water relationships of the whole plant. However, the eco-physiological behavior of ε is not clear. In our previous study, ε did not correlate with the daily maximum transpiration rate or osmotic potential of cell sap in the leaves of eight tree species (Saito et al. 2003). Also, there have been conflicting reports concerning changes in ε under drought conditions. Some studies reported that ε in the leaves developed under dry conditions was greater than that in the leaves developed under wet conditions (Sobrado 1986, Ayoub et al. 1992, Clifford et al. 1998). Other studies reported that ε in mature leaves decreased when the leaves were subject to water stress (Davies and Lakso 1998). These uncertainties regarding ε are partially due to our ignorance of the nature of ε in the leaf.

ε has been recognized as an indicator of the elastic properties of cell walls. A leaf with low ε is considered to have more elastic cell walls than that with high ε (Cheung et al. 1976, Tyree and Jarvis 1982, Kramer and Boyer 1995). Let us assume a balloon with a soft rubber skin and a soccer ball with a rigid skin. Then, it is evident that the change in the internal pressure for a given change in their volume should be smaller in the balloon. Similarly, in single plant cells which have a spherical or cylindrical structure, theoretical equations predict that ε is proportional to Young’s modulus of cell walls (Vinters et al. 1976, Tyree and Jarvis 1982). In giant algal cells, the theoretical assumption of the material structure works perfectly
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ε represents the elastic properties of cell walls by necessity (Ortega 1990, Proseus et al. 1999).

However, there is no evidence showing that ε for the whole leaf determined by the P–V technique expresses the elastic properties of cell walls. In other words, the relationship between ε and the cell wall elasticity has not been established at the leaf level yet. The meaning of ε for a leaf is not evident, because a leaf consists of as many as $100 \times 10^6$ cells and has a complicated structure, unlike giant algal cells. Leaves have several tissues such as vascular bundles, epidermis, palisade and spongy tissues. Cell structure and function are different among these tissues. Consequently, ε could be affected by the multicellularity of the leaf. For example, heterogeneity in cell turgor among the leaf cells could cause an apparent decrease in the value of ε (Tyree and Jarvis 1982). Also, the interactions among the cells, which are pushing each other, could cause an apparent increase of ε (Tyree and Jarvis 1982). Moreover, if the apoplastic water content decreases during P–V measurement, the decrease is taken as the decrease in the cell volume (Fanjul and Rosher 1984). This could induce an apparent decrease of ε. In practice, these problems have not been solved sufficiently due to technical limitations.

We suggest that the most straightforward approach to clarify the significance of ε at a leaf level is to compare ε and the value exactly expressing the elastic properties of cell walls. The mechanical properties of cell walls are accurately evaluated by the stretch technique using an extensometer (Instron technique; Olson et al. 1965, Cleland 1967, Cleland 1984). In this stretch test, the leaf specimen is stretched twice and a load–extension curve is obtained for every stretch. The slope of the second curve near the maximum load gives the elastic (reversible) extensibility of cell walls, because almost the same value appears in successive stretches (Cleland 1967). This reversible extensibility is inversely proportional to Young’s modulus. The turgor pressure in the mesophyll cells estimated by pressure-chamber agrees with the turgor of the single cells measured by pressure-probe (Murphy and Smith 1994). Also, ε is measured in a decreasing process of turgor in P–V technique. Thus, we hypothesized that ε has a linear relationship to Young’s modulus of the cell walls.

The aim of this study is to clarify the relationship between ε and the mechanical properties of cell walls. We compared the ε obtained by the P–V technique and Young’s modulus obtained by the stretch technique. We used young leaves at various stages of leaf development, because the apparent strength of the leaf lamina increased drastically during leaf development. We compared the leaves of *Quercus glauca* and *Q. serrata*, an evergreen and a deciduous species, respectively, because the leaves of *Q. glauca* are apparently stronger than those of *Q. serrata*. We also calculated ε based on a physical model of a single spherical shell using Young’s modulus obtained by stretch tests. These approaches will elucidate the elastic properties of cell walls as the critical determinant of ε.

**Results**

Development of leaf morphology

The leaf area attained 99% of its final size on 24 April in *Q. glauca* (Fig. 1A) and on 26 April in *Q. serrata* (Fig. 1B). We designated these dates as the dates of full leaf expansion (FLE) for these species. Leaf ages in the following text preceded by a – or + sign denote the number of days before or after FLE, respectively (Miyazawa and Terashima 2001).
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Leaf mass per area (LMA) and the leaf density (DW/V_{leaf}) changed during leaf development. Before FLE, LMA decreased to 36.5 and 23.8 g m\(^{-2}\) in *Q. glauca* and *Q. serrata*, respectively (Fig. 2A). LMA subsequently increased to reach approximately 90 and 55 g m\(^{-2}\) by +20 d in *Q. glauca* and *Q. serrata*, respectively. The value for the 1-year-old leaves of *Q. glauca* was 114.5 g m\(^{-2}\). The LMA for *Q. glauca* were approximately twice that of *Q. serrata* at every developmental stage throughout leaf development. In contrast, the leaf density was similar between the species at every developmental stage (Fig. 2B).

The values decreased to <0.25 g cm\(^{-3}\) just before FLE, then increased to around 0.45 g cm\(^{-3}\) after +20 d. The leaf density of the 1-year-old leaves of *Q. glauca* was 0.55 g cm\(^{-3}\). The dry weight per unit cell volume (DW/V_{cell}) increased from about 0.3 g cm\(^{-3}\) just before FLE to around 0.6 g cm\(^{-3}\) on around +25 d in both species.

Leaf water relations

Changes in the parameters obtained from the P–V curves are shown in Fig. 3 and 4. \(\varepsilon\) was around 5 MPa before –6 d (Fig. 3), increased until +15 d and attained the constant level of 20 MPa. \(\varepsilon\) did not differ markedly between the two species at any developmental stage. The symplasmic water content (\(V_{sym}/V\)) remained roughly constant, at around 0.55 in *Q. glauca* and 0.65 in *Q. serrata*, with some fluctuations (Fig. 4A). The ratio of leaf dry weight to leaf fresh weight at water saturation (DW/SW) decreased to <0.2 just before FLE, then increased to >0.4 after +25 d (Fig. 4B). DW/SW was very similar between the species at every developmental stage. The pattern of the changes in DW/SW resembled that of leaf density (Fig. 2B). The value for the 1-year-old leaves in *Q. glauca* was 0.51.

The extensibilities of the leaf strips

The total extensibility decreased from approximately 8.4 to 0.6 \(\mu\)m g•f\(^{-1}\) in *Q. glauca* (Fig. 5A) and from 9.6 to 1.5 \(\mu\)m g•f\(^{-1}\) in *Q. serrata* (Fig. 5B) during leaf expansion. The reversible extensibility was <43% of the total extensibility at the beginning of leaf expansion in both species. The reversible extensibility after +10 d was >83% of the total extensibility in *Q. glauca*, while it was >70% in *Q. serrata*. The reversible extensibility of *Q. glauca* was approximately half that of *Q. serrata* after +15 d. Preliminary experiments showed that the methanol fixation generally did not significantly influence the extensibilities of our freeze–thaw leaf specimens, although Cleland (1967) reported artifacts in the fixation in *Avena coleoptile*.

There were significant linear relationships between Young's modulus and \(\varepsilon\) \((P < 0.01;\) Fig. 6A). The coefficients of
determinations ($R^2$) were 0.78 and 0.84 in *Q. glauca* and *Q. serrata*, respectively. The $y$-intercepts were 1.21 and $-1.42$ MPa in *Q. glauca* and *Q. serrata*, respectively. The high $R^2$ and the small $y$-intercept indicated a strong relationship between Young’s modulus and $\varepsilon$. Neither the slopes nor the intercepts of the two regression lines differed significantly ($P > 0.05$).

The values of total and reversible compliances (DT and DE) obtained by the stretch test were corrected using the ratio of the cross-sectional area of cell walls to that of the leaf. In practice, we estimated the ratio from the leaf thickness under pressure (8.4 and 16.8 MPa in the leaves before and after FLE, respectively) divided by the original leaf thickness. The ratios were $0.26 \pm 0.03$, $0.28 \pm 0.05$, $0.36 \pm 0.02$ and $0.35 \pm 0.02$ on $-6$, $-1$, $34$ and $53$ d in *Q. glauca*. The ratios were $0.36 \pm 0.04$, $0.30 \pm 0.04$, $0.27 \pm 0.04$ and $0.29 \pm 0.03$ on $-5$, $0$, $31$ and $50$ d in *Q. serrata*. Thus, we roughly assumed that the ratios were 27 and 35% before and after FLE in *Q. glauca*, and were 33 and 28% in *Q. serrata*. The corrected Young’s modulus was 2.9- to 3.7-fold larger than the original values.

When $\varepsilon$ is plotted against the corrected Young’s modulus (Fig. 6B), the difference between the slopes of both species was not significant ($P > 0.05$), but that between the intercepts was significant ($P < 0.01$). In addition, when $\varepsilon$ was plotted against the corrected $1/\text{DT}$ (Fig. 6C), intercepts of approximately 5 MPa appeared in both species. Neither the slopes nor the intercepts of the two regression lines differed significantly ($P > 0.05$).

**Leaf anatomy**

Light micrographs of leaf transverse and paradermal sections in expanding and mature leaves are shown in Fig. 7. From the transverse sections, the leaf volume per unit leaf area ($V_{\text{leaf}}/\text{LA}$, i.e. leaf thickness) was stable at around 200 and 100 \(\mu\text{m}^3\)/\(\mu\text{m}^2\) in *Q. glauca* (Fig. 7A, C) and *Q. serrata* (Fig. 7B, D), respectively, throughout leaf development. The mean

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**Fig. 4** Changes in the symplasmic water content relative to total water content of the leaf ($V_0/V_t$) (A) and the ratio of dry weight to fresh weight at water saturation of the leaf (DW/SW) (B). Filled circles are data from *Q. glauca* and open circles are from *Q. serrata*. The circles in parentheses were data from the 1-year-old leaves of *Q. glauca*. Vertical bars represent the standard deviations. Leaf age is expressed in terms of the number of days before and after FLE.

**Fig. 5** Changes in the total (filled circles) and reversible extensibility (open circles) of leaf strips measured by the stretch technique in *Q. glauca* (A) and *Q. serrata* (B). The circles in parentheses were data from the 1-year-old leaves of *Q. glauca*. Vertical bars represent the standard deviations. Leaf age is expressed in terms of the number of days before and after FLE.
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Volume of a palisade tissue cell ($V_{pal}/N_{pal}$) was about 1,200 $\mu$m$^3$ on around –7 d in these species, then increased to about 3,000 and 2,000 $\mu$m$^3$ by 9 d in Q. glauca and Q. serrata, respectively. From the paradermal sections (Fig. 7E, F, G, H), the number of cells per unit area ($N_{pal}/LA$) decreased before FLE, then remained stable at around 15,000 mm$^{-2}$ in both species. The mean diameter of a palisade tissue cell increased before FLE, but the increase was <28%. The cell diameter was around 8 $\mu$m after FLE in both species (Fig. 8A).

Cell expansion, but not cell division, mainly occurred during the period of leaf expansion examined in this study. The number of cells in the first layer of the palisade tissue per leaf was around 3.3x10$^7$ after –8 d in both species. The increases in cell volume and intercellular air space (data not shown) explained the decreases in LMA (Fig. 2A) and in the leaf density (Fig. 2B) that were observed before FLE.

Representative electron micrographs of the paradermal sections of the first cell layer of the palisade tissue in mature leaves are shown in Fig. 7I and J. Cell wall thickness ($T_{cw}$) was approximately 0.1 $\mu$m at the beginning of the observation in both species (Fig. 8B). $T_{cw}$ increased to approximately 0.4 $\mu$m by +10 d in Q. glauca, while it remained at around 0.2 $\mu$m after FLE in Q. serrata. The difference in $T_{cw}$ between the species agreed with neither the similarity in the dry weight per unit leaf volume (DW/V$_{leaf}$, leaf density) (Fig. 2B), that per unit cell volume (DW/V$_{cell}$), nor DW/SW (Fig. 4B) between the species.

**Discussion**

The results support our hypothesis that the $\varepsilon$ obtained by the P–V technique is closely related to the reversible properties of cell walls. The relationships between Young’s modulus

![Bulk elastic modulus graph](https://academic.oup.com/pcp/article-abstract/47/6/715/1826324)

![Corrected Young's modulus graph](https://academic.oup.com/pcp/article-abstract/47/6/715/1826324)
Fig. 7 Representative light micrographs of the leaf transverse sections (A–D) and the leaf para-dermal sections in the first cell layer of palisade tissue (E–H) observed at a magnification of ×200 and ×400. Electron micrographs of the leaf para-dermal sections in the first cell layer of palisade tissue (I, J) observed at a magnification of ×2,000. Micrographs A, C, E, G, I were obtained from Q. glauca and B, D, F, H, J are from Q. serrata. Leaf age is expressed in terms of the number of days before (+) and after (−) FLE.
and ε were significant and the y-intercepts of the regression lines were small in the two *Quercus* species (Fig. 6A, B).

ε increased from around 5 MPa before -6 d to the constant level of 20 MPa up to +15 d in both species (Fig. 3). This increase of ε did not correlate with the change of some characteristics which could have an influence on ε. The value of ε increased without consistent changes in $V_p/V_s$ (Fig. 4A). This is in contrast to the consideration that apoplastic water in cell walls could serve as a capacitor against water loss from protoplasts (Fanjul and Rosher 1984), and the result that a decrease in ε occurred with an increase in apoplastic water content (Joly and Zaerr 1987). ε increased even after FLE without changes in the mean volume or diameter of a palisade tissue cell (Fig. 8A). This is in contrast to the theoretical prediction that ε will increase with a decrease in the cell size (Tyree and Jarvis 1982). Thus, we suggest that the increase in ε during leaf development was not accompanied either by changes in the apoplastic water stored in the leaf or by the cell size.

Another important finding is that there was only a small difference in ε between *Q. glauca* and *Q. serrata* throughout leaf development (Fig. 3). In contrast to this similarity, there was a large difference in some leaf characteristics between the two species. The apparent mechanical strength of the leaf lamina was clearly greater in *Q. glauca* than in *Q. serrata*, as indicated by the lower total extensibility of leaf specimens of the former species especially after FLE (Fig. 5). The leaf anatomical characteristics such as the total number and volume of cells in a leaf and the mean volume of a palisade tissue cell differed between these species (Fig. 7). Moreover, the wall thickness of mesophyll cells in *Q. glauca* was approximately twice that of *Q. serrata* at the same developmental stage (Fig. 7J, 8B). Thus, we suggest that ε relates to neither the species differences in the apparent mechanical strength of leaf lamina, cell number and cell volume, nor the wall thickness of the leaf.

We examined whether the values obtained by the stretch test relate to the mechanical properties of cell walls. When we apply a stress to the cell wall which has a polymeric structure, the wall undergoes deformation. This deformation will partly revert after removal of the stress. Thus, the cell wall is considered as a viscoelastic material which has the intermediate properties of elastic solids and plastic liquids (Cosgrove 1993). In Instron experiments, the viscoelastic properties of cell walls were separated into elastic and plastic components by means of stretching the isolated cell walls twice. Comparing the two stress–strain curves, the compliance (the ratio of strain to stress) in the first stretch is larger than that in the second stretch. The compliance in the second stretch (DE) represents the elastic component of the mechanical properties of the cell walls, because similar stress–strain curves appeared in the third and subsequent stretches (Cleland 1967). The difference in the compliance between the first and second stretch indicates the plastic component (DP) which represents the wall properties which do not revert after the first stretch. Thus, the compliance of the first stretch (DT) is the sum of both the elastic and plastic component of the mechanical properties of the cell walls.

In our results, both the corrected Young’s modulus (1/DE) and the corrected 1/DT have a close relationship with ε, but only the regression lines in the latter had evident y-intercepts in both species (Fig. 6B, C). These intercepts indicate that DT included a marginal component to explain the value of ε, especially in expanding leaves. This component was DP because the intercepts in the corrected Young’s modulus (1/DE) versus ε were negligible. Thus, we suggest that ε does not relate to the plastic properties of the cell walls. This poor connection of ε to the plastic component can be explained because ε is obtained in the process of decreasing turgor in P–V measurement. The irreversible properties of the walls do not apply pressure to protoplasts during a decrease in tension in the walls (Wenkert et al. 1978). Therefore, we concluded that the ε obtained by the P–V technique has a close relationship to the elastic properties of cell walls.

**Fig. 8** Changes in the diameter of a palisade tissue cell (A) and the cell wall thickness ($T_{cw}$) (B). Filled circles are data from *Q. glauca* and open circles are from *Q. serrata*. The circles in parentheses were data from the 1-year-old leaves of *Q. glauca*. Vertical bars represent the standard deviations. Leaf age is expressed in terms of the number of days before and after FLE.
We estimated the bulk elastic modulus ($E_{\text{stretch}}$; MPa) from Young’s modulus ($E$) obtained by the stretch technique based on the following equation (see Appendix):

$$E_{\text{stretch}} = \frac{2Et}{3r(1-\nu)} \tag{1}$$

This equation is derived from a single spherical shell which has a radius $r$, a wall thickness $t$, and Poisson’s ratio of $\nu$. We adopted 0.25 for $\nu$ based on the assumption that the specimen was isotropic (Niklas 1992). We estimated the average value of $E_{\text{stretch}}$ for mature current year leaves after $+25$ d. We used an $r$ value of 4 $\mu$m for both species (Fig. 8A) and used $t$ values of 0.43 $\mu$m in $Q$. glauca and 0.21 $\mu$m in $Q$. serrata, respectively. The $E$ was estimated from the corrected Young’s modulus (Fig. 6B). The result is that $E_{\text{stretch}}$ was 43.6 and 29.3 MPa in $Q$. glauca and $Q$. serrata, respectively. $E_{\text{stretch}}$ in $Q$. glauca was 1.5-fold larger than that in $Q$. serrata.

In reality, the average $\varepsilon$ obtained by the P–V technique during this period was 20.1 and 21.1 MPa in $Q$. glauca and $Q$. serrata, respectively (Fig. 3). Thus, $E_{\text{stretch}}$ was larger than $\varepsilon$ by 217 and 139% in $Q$. glauca and $Q$. serrata, respectively. The discrepancy between $E_{\text{stretch}}$ and $\varepsilon$ indicates that some parameter(s) used in equation 1 was not adequate. The difference in $E_{\text{stretch}}$ between the species was mainly due to the difference in $t$. Microscopic observation indicates that the cell wall thickness ($T_{cw}$) in $Q$. glauca was twice that in $Q$. serrata (Fig. 7I, J, 8B), although the effect of the larger $t$ on $E_{\text{stretch}}$ was counterbalanced by the smaller $E$ in $Q$. glauca to some extent. If $E_{\text{stretch}}$ is approximately equal to $\varepsilon$, the effective thickness will be roughly 0.20 and 0.16 $\mu$m in $Q$. glauca and $Q$. serrata, respectively. Thus, we suggest that the mechanically ‘effective’ thickness of cell walls for $\varepsilon$ is smaller than the observed wall thickness in mesophyll cells. The cell walls in $Q$. serrata may be stronger than those in $Q$. glauca in unit cross-sectional area. This consideration would agree with the similar leaf density (Fig. 2B) despite a marked difference in cell wall thickness (Fig. 8B) between the two species.

We should discuss some problems in the measurement of Young’s modulus which is an average value among the leaf cells. First, the accurate estimation of the cross-sectional area of cell walls in the leaf strip is difficult. We attempted to eliminate the spaces other than cell walls in the cross-sectional area of the strip by applying vertical pressure at 16.8 MPa to the specimen. However, the area bearing tensile stress during the stretching was not known. This relates to the second problem that Young’s modulus increases with an increase in the maximum force of the extension probably due to an increase in the cell wall area bearing the tension (Cleland 1967). In theory, the stress applied on the cell walls by the extensometer is smaller than that exerted in vivo by turgor pressure. Young’s modulus in our results may be an underestimation. Thirdly, tertiary veins and epidermis would contribute to Young’s modulus, although we did not clamp both ends of any one tertiary vein by the two clips. Fourthly, the time dependency of deformation of cell walls was not taken into consideration. The rate of stretching (20 mm min$^{-1}$) was considerably faster than that of the cell volume change in vivo. Consequently, DE would evaluate only the instantaneous elastic properties, although $\varepsilon$ from the P–V technique would include both the instantaneous and the delayed elastic properties. However, we consider that the latter have a marginal effect on the total elastic properties when the cell walls are under high tensile stress. This consideration is supported by the results of Kamiya et al. (1963) and Cleland (1967).

Concerning the discrepancy between $E_{\text{stretch}}$ and $\varepsilon$, several problems should be pointed out. First, the assumption of isotropy of the specimen would cause the overestimation of $E_{\text{stretch}}$ (Niklas 1992). Secondly, we used the cell volume at full turgor for $V$ in equation 2 for $\varepsilon$, whereas we used the length of the segment before stretching for $l_0$ in equation 4 for $E_{\text{stretch}}$. When we use the cell volume at turgor loss for $V$, $\varepsilon$ is approximately 90% of the present values. Thirdly, we simplified a leaf structure to use equation 1 and attributed the discrepancy between $E_{\text{stretch}}$ and $\varepsilon$ to the effective thickness of cell walls which actually bear the stress. However, the discrepancy may also include some characteristics from multicellularity of the leaf. Further studies are required.

In conclusion, we clarified that $\varepsilon$ is closely related to the reversible properties of cell walls. $\varepsilon$ increased during leaf development irrespective of the constant apoplastic water content and cell size. The $\varepsilon$ values of the two species were always similar irrespective of the differences in the apparent mechanical strength and leaf structure. The marked $y$-intercepts in the regression lines of $1/DT$ versus $\varepsilon$ indicated that $\varepsilon$ was not related to the irreversible properties. The estimation of $\varepsilon$ using a physical model indicated that the effective wall thickness for $\varepsilon$ is smaller than the observed wall thickness in mesophyll cells.

Materials and Methods

Plant materials

We used $Q$. glauca Thunb. ex Murray and $Q$. serrata Thunb. ex Murray (Fagaceae). Both are common species in warm temperate forests in Japan. $Quercus glauca$ is an evergreen species. Leaf longevity ranges from 1 to 3 years. Most of the leaves from the previous year are shed when current year leaves develop in spring, especially in open places. $Quercus serrata$ is a deciduous species. Leaves emerge in April and fall in December. The study site was in a secondary forest on the Toyonaka Campus, Osaka University (34°N, 135°E, 55 m above sea level). The mean annual temperature was 16.3°C with the highest mean monthly temperature of 28.9°C in July and the lowest of 4.1°C in January. The annual precipitation was 975 mm in 2001 (Toyonaka Meteorological Observatory).

One healthy mature tree of $Q$. glauca (1,754 cm$^2$ in basal area and 10.8 m in height) and one of $Q$. serrata (734 cm$^2$ in basal area and 16.8 m in height) were selected. Several branches of these trees were used for the following experiments. The photosynthetically active photon flux density on these branches relative to that in an open site (rPPFD) was approximately 25%.
Leaf area and leaf dry weight

The length and width of the leaf lamina were measured throughout leaf development. Fourteen or 15 leaves on three branches of the *Q. glauca* or *Q. serrata* tree were measured continuously. LMA (g m⁻²) was measured from five leaf disks (1.24 cm² in total area) from several leaves. The disks were cut from the middle part of the leaf and did not include any primary and secondary veins.

Pressure–volume measurements

From the same trees, shoots about 300 mm long were cut. The bases of the shoots were cut again under water. Several leaves on this sample shoot were used for the P–V measurement and the remaining leaves were used for other experiments. Each of the shoots was put in a flask and covered with a polyethylene bag to ensure the leaves were absorbing water fully. Next day, a sample leaf was cut from the shoot. Immediately, the leaf weight was measured with a balance, and the leaf water potential was measured with a pressure chamber (Soilmoisture Equipment, Santa Barbara, CA, USA). The sample leaf dried naturally on the laboratory bench. In parallel with this drying, we measured decreases in the leaf weight and leaf water potential continuously. We put the data in the original spreadsheet software and constructed the P–V curves of the leaf (Scholander et al. 1965, Tyree and Hammel 1972). We obtained >12 (at least seven) points before the turgor loss point, and another 4–6 points thereafter.

We calculated ε as follows (Fanjul and Rosher 1984, Maruyama et al. 1988):

\[ \varepsilon = V \cdot \frac{\Delta \Psi \varepsilon}{\Delta V} \]  

where \( V \) is the volume of symplasmic water in the fully turgid leaf and \( \Psi \varepsilon (=\Pi) \) is the pressure potential. In practice, we selected more than four data points which make the linear part with the largest slope in the \( P \) versus FWC curve. The slope of the regression line to these points was designated as \( \varepsilon \) (Tyree and Jarvis 1982). We minimized the effect of heterogeneity in turgor changes among the leaf cells by choosing the largest slope in the curve, because the heterogeneity would always cause a decrease in \( \varepsilon \).

We also obtained the following parameters according to Maruyama and Morikawa (1983): the water potential at the turgor loss point \( (\Psi \varepsilon_{\text{tlp}}; \text{MPa}) \), the osmotic potential at water saturation \( (\Psi \varepsilon_{\text{sat}}; \text{MPa}) \), the relative water content at the turgor loss point \( (\text{RWC}_{\text{tlp}}; \% ) \), the free water content at the turgor loss point \( (\text{FCWC}_{\text{tlp}}; \% ) \), the molar concentration of solutes in the leaf cells at water saturation \( (N_s; \text{mol m}^{-3}) \), the ratio of symplasmic water content to total water content \( (V_{l}/V) \), and the ratio of leaf dry weight to leaf fresh weight at water saturation \( (DW/\text{SW}) \). We measured 3–5 leaves for each developmental stage for each species.

Stretch test of leaf strips

The leaves on the sample shoot were stored in a freezer (−80°C) until use. Leaf strips (2×15 mm) were cut from the middle part of the leaves. The long axes of the strips were parallel to the primary veins. The strips were fixed in boiling methanol for 5 min and stored in fresh methanol at room temperature for 1 d. The specimens were rinsed for 2 h in de-ionized water just before the stretch test. We obtained four specimens from each leaf, and measured 12 specimens in total at each developmental stage.

We conducted stretch tests with an extensometer (Tensilon RTM-25, Toyo Baldwin, Tokyo, Japan). The specimen was clamped using two clips separated by 3.7 mm. Secondary veins were excluded from the part between the clips, and tertiary veins were almost perpendicular to the direction of stretching. The extensometer carried out the first extension, and when the tensile force in the specimen reached the maximum value set in advance, the extensometer stopped and kept that position for 1 min. After returning to the original position, the extensometer started the second stretch. The rate of stretching was 20 mm min⁻¹. The force applied to and the extension of the specimen were detected using a force transducer and a micrometer (±10 μm), respectively. Data were recorded on a PC using the original software at intervals of 2 ms (Hoson et al. 2002, Soga et al. 2002). We applied stress of approximately 1.4 MPa maximum to the specimens of expanding leaves in both species. We increased the maximum stress to 1.7 MPa with maturation of the leaves.

We calculated the extensibility (μm g⁻¹) from the slope of the regression line, which was applied to the extension as a function of the applied force for the range between 80 and 100% of the maximum force. The extensibility calculated from the first stretch was the total extensibility, and the value from the second stretch was the reversible extensibility. The total (DT; MPa⁻¹) and reversible (DE; MPa⁻¹) compliances were calculated as follows:

\[ DT = \frac{A_{\text{tvp}}}{l_0} \]  

and

\[ DE = \frac{A_{\text{trip}}}{l_0} \]

where \( l_0 \) is the original length of the strip before the first extension (3.7 mm) and \( A_{\text{tvp}} \) is the cross-sectional area of the leaf strip. The reciprocal of DE was Young’s modulus \( (E ; \text{MPa}) \) (Cleland 1967). \( A_{\text{trip}} \) was estimated from the width of the strips (2.0 mm) multiplied by the thickness of the strip. The thickness was measured with an outside micrometer which applies weak constant pressure (approximately 0.7 MPa) to the strips for the measurement.

We also estimated the cross-sectional area of the cell walls in the leaf strips by measuring the leaf thickness under pressure. First, we prepared similar leaf specimens (2×5 mm in width and length) from the sample leaves and measured the leaf thickness with the outside micrometer. The specimen was then divided into four pieces. Each of the pieces was placed on each corner of a rectangular acrylic plate (170×230×4.9 mm) and another similar acrylic plate (3.0 mm in thickness) was placed on the pieces. We applied pressure at 16.8 MPa (8.4 MPa in expanding leaves) to the leaf pieces sandwiched by the plates held together by two paper clips. Then, we again measured the leaf thickness under pressure, and calculated the ratio of the value to the original thickness. We multiplied this ratio by \( A_{\text{tvp}} \) measured just after the stretch test, and used this value as the cross-sectional area of cell walls in the leaf strip. The leaves used were at −6, −1, 34 and 53 d of FLE in *Q. glauca*, and at −5, 0, 31 and 50 d in *Q. serrata*.

Leaf anatomy

We collected the leaves from the sample shoots. We cut leaf segments (approximately 1×1 mm) from the middle part of the leaves. The samples were fixed by 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at 4°C and evacuated with a vacuum pump. The samples were rinsed in ice-cold distilled water and post-fixed by 2% osmium tetroxide for 3 h at 4°C. The samples were dehydrated by an acetone and propylene oxide series and embedded in Spurr’s epoxy resin (Spurr 1969). Semi-thin sections of 0.8 µm thick were cut from the samples with a glass knife using an ultramicrotome (Reichert Ultracut S, Leica, Vienna, Austria). We prepared a transverse section of the leaf and a paradermal section of the first cell layer of the palisade tissue for each leaf. The sections were stained with 0.5% toluidine blue O on a hot plate. We observed these sections at a magnification of ×200 or ×400 under a light microscope (BX50, Olympus, Tokyo, Japan) and took micrographs with a digital camera.
Leaf cells on the light micrographs of the transverse sections were traced (Yano and Terashima 2004) with image editing software (Photoshop 6.0, Adobe systems, San Jose, CA, USA). The leaf volume per unit leaf area \((V_{\text{leaf}}/\text{LA})\) was calculated as follows (Syvertsen et al. 1995):

\[
\frac{V_{\text{leaf}}}{\text{LA}} = \frac{A_{\text{leaf}}}{W}
\]

(5)

where \(A_{\text{leaf}}\) is the area of the tissues including all cell types and intercellular air spaces in the transverse section. \(A_{\text{leaf}}\) was measured using image analysis software (Scion image, Scion Corporation, Frederick, MD, USA). \(W\) is the width of the section. \(W\) was always >730 \(\mu\)m of the transverse section that did not include the vascular tissue. We also calculated the volume of the whole cells \((V_{\text{cell}}/\text{LA})\) and that of palisade cells \((V_{\text{pal}}/\text{LA})\) per unit leaf area.

The dry weight per unit of leaf volume (\(\text{DW}/V_{\text{leaf}}\), equal to the leaf density) was calculated as follows:

\[
\frac{\text{DW}}{V_{\text{leaf}}} = \frac{\text{DW}}{\text{LA}} \times \frac{\text{LA}}{V_{\text{leaf}}}
\]

(6)

where \(\text{DW}/\text{LA}\) is the LMA. The dry weight per unit of cells (\(\text{DW}/V_{\text{cell}}\)) was also obtained from \(\text{LA}/V_{\text{cell}}\). We calculated the number of palisade tissue cells per unit leaf area \((N_{\text{pal}}/\text{LA})\) and that per leaf. For each point of \(N_{\text{pal}}/\text{LA}\), we counted >2,700 cells in the paradermal sections. The cell numbers were divided by the area which was occupied by the cells and intercellular spaces, surrounded by the bundle sheath extensions.

We calculated the mean volume \((V_{\text{pal}}/N_{\text{pal}})\) and the diameter of a palisade tissue cell as follows:

\[
\frac{V_{\text{pal}}}{N_{\text{pal}}} = \frac{V_{\text{pal}}}{\text{LA}} \times \frac{\text{LA}}{N_{\text{pal}}}
\]

(7)

and

\[
\text{Diameter} = 2 \times \frac{\sqrt{A_{\text{pal}}}}{\pi}
\]

(8)

where \(A_{\text{pal}}\) is the transverse area of a palisade tissue cell in the paradermal sections. At least 450 cells were measured for each point. We obtained an average of three leaves (two leaves in a few instances) for each developmental stage.

We also cut the ultra-thin paradermal sections of 80 nm from the first cell layer of the palisade tissue. The sections were stained with lead citrate for 10 min and then with uranyl acetate for 10 min. We took micrographs of the sections at a magnification of \(\times2,000\), or \(\times10,000\) for the very young leaves under an electron microscope (JEM-1200EX, JEOL datum, Tokyo, Japan). We measured the cross-sectional area of the cell walls \((A_{cw})\) and the perimeter length at the center of the cell walls \((L_{cw})\) for single cells on the electron micrograph. The thickness of the cell wall \((T_{cw})\) was calculated as \(A_{cw}/L_{cw}\). Ten cells from a representative leaf were examined for each developmental stage.

Statistical analyses

Regression lines were calculated by the least squares method. The statistical significance of the regression coefficients was detected by the \(t\)-test. Differences in the regression coefficients and in the intercepts were detected using analysis of covariance (ANCOVA). All statistical tests were conducted according to Sokal and Rohlf (1995).

Appendix

We derived a physical equation for the bulk elastic modulus of a single cell which has a spherical shell structure. We improved the equation originally proposed by Tyree and Jarvis (1982) and discussed in Wu et al. (1985), concerning the strain of the wall in the thin spherical shell.

According to Hooke’s law, the strain of an isotropic rectangular body under unidirectional stress is:

\[
\frac{l - l_0}{l_0} = \frac{\sigma}{E}
\]

(9)

where \((l - l_0)/l_0\) and \(\sigma\) are the strain and stress, and \(E\) is Young’s modulus. When the body is under three-dimensional stress, the strain in the \(X\) direction is written using Poison’s ratio \((\nu)\) as follows:

\[
\frac{l - l_0}{l_0} = \frac{1}{E} \sigma_x (1 - \nu(\sigma_y + \sigma_z))
\]

(10)

where \(\sigma_x\), \(\sigma_y\), and \(\sigma_z\) are the stresses of the \(X\), \(Y\), \(Z\) direction, respectively. When a thin plate is under stress parallel to the plane \((\sigma_x = \sigma_y, \sigma_z = 0)\), the strain parallel to the plane is, in the differential form:

\[
\frac{dl}{l} = \frac{1 - \nu}{E} \frac{d\sigma}{\sigma}
\]

(11)

The stress–strain relationship in a spherical shell under an internal pressure \(P\) is given by equation 11.

The stress in the wall of the shell under \(P\) is (Nobel 2004):

\[
\sigma = \frac{E t}{2l} \frac{P}{r} (\nu + 1)
\]

(12)

where \(r\) is the radius of the spherical shell and is a function of \(P\). The small change in \(\sigma\) induced by the small change in \(P\) is thus:

\[
\frac{d\sigma}{dP} = \frac{P}{2l} \frac{dr}{dP} + \frac{r}{2l}
\]

(13)

then,

\[
\frac{d\sigma}{dP} = \frac{r \cdot dP}{2l}
\]

(14)

Because \(d\sigma/dP\) is equal to the change in the radius \((dr/r)\), equation 11 can be rewritten using equation 14 as:

\[
\frac{dr}{r} = \frac{1 - \nu}{E} \left( \frac{P}{2l} + \frac{r}{2l} \right)
\]

(15)

From equation 15, the change in \(r\) with the change in \(P\) is, in the reciprocal form:

\[
\frac{dP}{dr} = \frac{2Et}{r^2(1 - \nu)} - \frac{P}{r}
\]

(16)

The bulk modulus of elasticity \((\epsilon)\) is:

\[
\epsilon = \frac{V \cdot dP}{dV}
\]

(17)

\(\epsilon\) of the spherical shell is written using \(dV/dr = 4\pi r^2\) as:

\[
\epsilon = \frac{\pi \cdot \frac{dP}{dr}}{3}
\]

(18)

From equations 16 and 18, the bulk elastic modulus of a spherical shell is:

\[
\epsilon = \frac{2Et}{3r(1 - \nu)} - \frac{P}{3}
\]

(19)

The second term in the right hand side is usually negligible in comparison with the first term.
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