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Mesophyll-Driven Blade Expansion in *Pisum sativum* var. *argenteum* Leaves

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Abstract

The growth rate of any multilayered plant organ is limited by the cell layer with the least extensibility. The dicot leaf blade has two epidermal layers covering the mesophyll layers, in which the vascular network is embedded. There has been a lingering uncertainty about which layer limits the rate of blade expansion in dicot leaves. The current study made use of leaf strips cut from the *argenteum* variety of *Pisum sativum* L., in which the epidermal layers can easily be removed with minimal damage. After this procedure, the mesophyll showed accelerated growth in short-term and long-term experiments and light and darkness. Extension of both layers is strongly promoted by acidic solutions. Isolated mesophyll layers expand in response to light. This effect depends on turgor pressure, photosynthesis, and the plasma membrane H^+-ATPase. The data allow concluding that expanding leaf blades share with axial stem organs a similar arrangement of tissue tension: an expanding core tissue pushing against a restrictive epidermal envelope. In complete leaves, partial removal of the epidermis from only one side of the blade causes a strong epinastic or hyponastic response. Removal of matching epidermis strips from both sides of complete blades causes the exposed mesophyll strip to elongate in excess of the neighboring tissue: it buckles.

Keywords

Leaf Expansion, Tissue Tension, Blade Expansion, Epidermal Control, Epinasty, Buckling

1. Introduction

In a single plant cell, the rate of expansion depends on two factors: the extensibility of the cell walls, and the expansive push of turgor pressure [1]. When multicellular plant organs expand, cell expansion depends also on the cell layer with the lowest extensibility since the cells are interconnected by common cell walls. This situa-

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tion has been well studied in dicot hypocotyls and epicotyls where less-extensible outer layers mostly limit elongation rates [2]. When given auxin, however, the situation changes and the wall extensibility of the outer layer increases greatly [3]. These phenomena are known under the name of "tissue tension" and a description of its role in axial organs has been reviewed [4] [5]. The generation and role of tissue tension in flat leaves, however, have remained uncertain [6].

Unlike cylindrically shaped organs, dicot leaves develop a plane, flat and laminate structure of at least four distinguishable cell layers [7]. In addition there is a network of veins within the mesophyll layers. The role of these different cell layers in controlling leaf blade expansion is still controversial. The fact that air spaces form between palisade parenchyma cells as the blade enlarges has been taken as evidence that the mesophyll layers cannot be driving leaf expansion [8]-[10], and has promoted the more common view that the epidermal layer drives leaf blade expansion [11]-[13]. However, it has also been proposed that the continuous "stellate" network of the spongy mesophyll could be driving blade expansion [6].

Since the control of leaf size and shape has been recognized to extend beyond the action of individual genes and cells [14], there have been developmental studies that focus on the role of layers by constructing plants and leaves with a genetically unique cell layer [15]. Two studies involving graft chimeras in which the L1 and L2 layers in the leaf had different genotypes have provided strong evidence that the L2 layer (which gives rise to most of the mesophyll) determines both the size and the shape of the leaf blade [16] [17]. A related study with chimeric tobacco plants found that a reduced epidermis decreased not only the final size but also the rate of cell divisions in mesophyll cells [15]. Similarly, there have been genetic approaches in transgenic *Arabidopsis* plants that address this question. Two studies (one using lack of brassinosteroids and the other—the expression of the cyclin-dependent kinase inhibitor KRP1) showed that the resulting reduction in the size of epidermal cells resulted also in a smaller leaf size [18] [19]. As promising as this approach might be, the outcome does not tell whether the epidermis achieved the effect on the final leaf size because it is driving the blade expansion or varies the strength of restriction.

Therefore the question remains as to which of the cell layers restricts the growth rate in the dark and in the light. To approach this question we investigated the contribution of epidermis, mesophyll, and midvein in blade expansion of the *argenteum* variety of *Pisum sativum*. In *argenteum* blades, epidermal layers are only loosely attached to the mesophyll layers [20] and can be removed with minimal damage to either layer [12] [13] [20]-[23]. The functionality of the isolated epidermis layers has been demonstrated in studies of stomatal function [24], sugar uptake [25], membrane potentials and proton excretion in response to light [12] [13] [22]. In spite of this, the general morphology, cell size, cell differentiation and cell layering patterns in the *argenteum* mutant are indistinguishable from normal pea leaflets [23]. It exhibits similarly sized leaves and comparable leaf growth as normal wildtype peas [26].

Using leaf strips with and without the epidermal layers, results of the following experiments show that the leaf epidermis is the layer with the least extensibility and that it reduces the rate of leaf expansion in both light and darkness. Epidermal expansion shows less plastic and more elastic extensibility than the mesophyll. Although isolated epidermis layers show a smaller growth response to light, they gain the same expansion rate as the mesophyll under an acidic pH of 4.5. It will be shown that this concept is valid also for intact leaf blades, where the unilateral removal of an epidermis causes strong epinastic and hyponastic responses of the blades whereas bilateral removal leads to buckling of the accelerating mesophyll strip inside the complete blade.

2. Materials and Methods

2.1. Experimental Plant Material

Pea plants of the a*rgenteum* mutant of *Pisum sativum* L. (Marx 1978) were grown for 3 weeks in either a growth chamber (Environmental Growth Chambers, E15 Chagrin Falls, OH, USA) at 21˚C and a 12 h photoperiod providing a mixed fluorescent and incandescent light of about 150 µmoles $m^{-2} \cdot s^{-1}$ photons of white light or in a greenhouse at 22˚C day/18˚C night with a natural photoperiod. When measuring light effects, handling and preincubation proceeded under dim white fluorescent light with a fluency rate of less than 5 µmole $m^{-2} \cdot s^{-1}$, herein referred to as darkness. Experiments were carried out with young, partially unfolded leaflets having reached less than 60% of their final length. Unless specified, leaflets were taken exclusively from the 6^{th} to 8^{th} leaves that develop flat blades whereas the 1^{st} to 4^{th} leaves develop blades with the buckled blades (see **Figure 2**). Segments of leaf tissues (15 mm long by 5 mm wide) were cut parallel to the midvein from partially folded, growing leaf-

lets. Except when noted (**Figure 3**), the main vein was not included in the segment. The secondary veins derive at an angle of about 60˚ from the midvein and the longitudinal direction in which the segments are measured. Fine forceps were used to remove one or both epidermal layers as a large sheet. With the exception of **Figure 3**, the peeling was performed before the segment was cut.

2.2. Growth Measurements

Segments, after excision, where mounted between a fixed and a mobile clamp under a load of 1 g which kept the segments in a straight and flat position and prevented them from rolling up after removal of just one epidermal layer. The segments were floating on the incubation medium containing 10 mM KCl and 1.0 mM $CaCl_2$. Expansion of the segments was recorded with a rotary position transducer (Schaevitz RD30) attached to the mobile clamp. Upon mounting between the clamps under a pull of 1 g, segments showed initially a rapid expansion which after 20 converted into a steady growth rate. For collecting the data in **Figure 3** we removed the measured segments for short intervals of less than 30 sec from the recording set in order to remove either a midvein or an epidermis. Illumination, where required, was applied from a projector (Techni-Quip Corp., Hollywood, CA, USA) equipped with a 150 W bulb and a fiberglass light guide that provided 150 µmoles $m^{-2}{\cdot}s^{-1}$ photons of white incandescent light. The effect of acidic conditions on segment extension was determined by replacement of the pH 6.0 incubating solution with a 50 mM Na-acetate buffer solution adjusted to pH 4.5.

For 24 hours-long observation of growth, segments of midvein-free blade tissue, isolated mesophyll or epidermis were placed in Petri dishes which were either kept in the dark or illuminated with 150 µmole $m^{-2}{\cdot}sec^{-1}$ photons of fluorescent white light. The Petri dishes contained three layers of filter paper soaked in incubating solution (10 mM KCl, and 1.0 mM $CaCl_2$ without or with the addition of 5×10^{-4} M sodium orthovanadate or 5×10^{-5} M DCMU). Section length was measured under a dissecting scope with a stage and ocular micrometer. The epidermis segments were placed into the Petri dishes with the cuticle up and the mesophyll segments were placed with the palisade layer up.

2.3. Viability Staining

Some of the epidermis layers were tested for viability. After floating the layers for 10 min on fluorescein diacetate (FDA) solution (Molecular Probes, Portland, Oregon, USA, 1 drop or 0.1 ml of 0.5% FDA acetone stock solution was diluted in 20 ml incubating solution) the share of viable cells was judged by FDA uptake and intracellular conversion into fluorescein. Observations were made under UV excitation using an epifluorescence microscope (Labophot-2, Nikon Corporation Tokyo, Japan) under a 50× magnification. The number of brightly fluorescing was always higher than 80%.

2.4. Techniques for Imaging Cell Layers

Pictures of epidermal and mesophyll layers (**Figure 1**) were taken after staining the isolated layers with either carboxyfluorescein (Molecular Probes) in the case of the epidermis or with fluorescein diacetate (also Molecular Probes) in the case of the mesophyll. The solutions were prepared according to standard procedures [27]. Pictures were taken under UV excitation with various camera bodies connected to an epifluorescence microscope (Labophot-2, Nikon Corporation Tokyo, Japan) under a magnification of 100×.

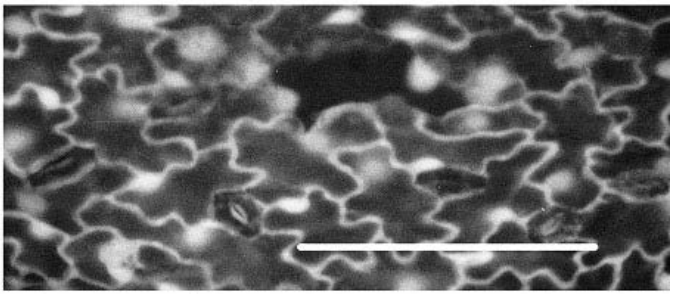
2.5. Presentation of Data

Short-term experiments were repeated at least eight times and various examples are shown in the **Figure 2** and **Figure 4**. Long-term experiments averaged at least five repetitions with 5 segments from 5 different leaves or 25 independent measurements each. Related figures provide the average plus the standard error for easy evaluation of significant differences.

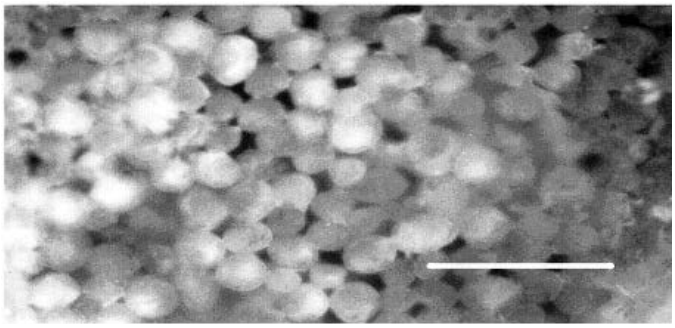
3. Results

3.1. Leaf Layering Characteristics and Particularities in the *Argenteum* Pea

The *argenteum* pea leaflet is a composite of four layers: the adaxial and abaxial epidermis, and the palisade and spongy mesophyll. Paradermal sections through the adaxial epidermal, palisade parenchyma and spongy meso-



(a)



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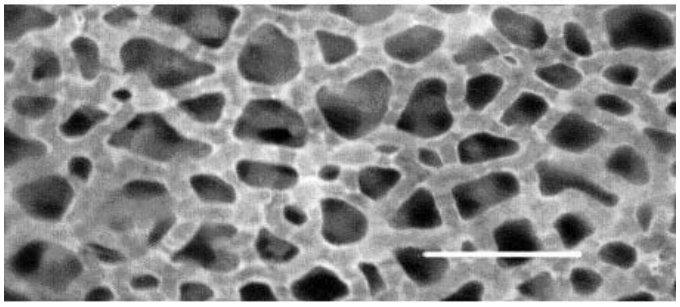


(c)

Figure 1. The pea leaf consists of three distinct layers. Paradermal light microscope pictures of the adaxial epidermis (a), palisade parenchyma (b) and spongy mesophyll (c) from *argenteum* leaflet blades demonstrate the visible differences between the three layers. The bars represent a length of 100 µm.

phyll layers are shown in **Figure 1**. Note the abundant air spaces between palisade mesophyll cells that are typical for the *argenteum* phenotype [20], and the continuous stellate network formed by the spongy parenchyma cells. The blade also contains a network of secondary and tertiary veins, which do not appear in these pictures. Alternatively, good pictures of transverse sections of *argenteum* pea blades are available from previous publications [20] [21] [23].

During the growth of *argenteum* plants, the first four leaves produce buckled surfaces, while the subsequent leaves produce smooth and flat surfaces and are of similar shape and size as normal pea leaves (**Figures 2(a)-(b)**) [28]. The buckling is confined to the upper surface while the lower surface remains flat with the epidermis stretched between the "mountain peaks" of the undulating mesophyll layer. Cross sections of the leaflets demonstrate what is causing this type of buckling (**Figure 2(c)**). The abaxial epidermis is partially detached forming "bridges" over the buckling mesophyll. This scenario is caused by the superior expansion rate of the mesophyll producing sufficient shear stress to disrupt the weak connections between the epidermis and spongy mesophyll layers (see also [20]). The partial detachment reduces the restraint of this epidermis by fitting the excess size of a faster expanding mesophyll into the bulges that form between the remaining areas of attachment to the abaxial

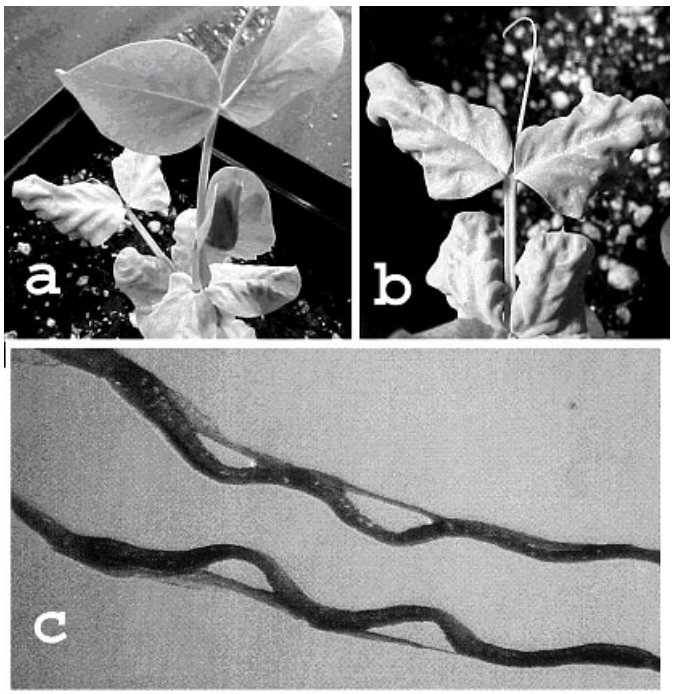


Figure 2. (a)-(c) The phenotype of *argenteum* peas. (a) The first four leaves have a buckled shape while the subsequent leaves develop smooth blades; (b) Close-up of a buckled upper surface and a common epinastic tendency found in the earlier leaves; (c) Cross section through two different leaves (upper one is photographed upside down) shows that the lower or abaxial epidermis gets separated discontinuously from the mesophyll over several 2-mm-long stretches. The adaxial epidermis stays attached and follows the buckled shape of the mesophyll layer suggesting a stronger attachment. Buckling appears as the combined result of the mesophyll exceeding epidermis growth plus a patchy attachment of the abaxial epidermis layer. The loose attachment of the abaxial epidermis allows the longer mesophyll layer to fit its extra length into the epidermal envelope.

epidermis. Note that this behavior does not apply to the adaxial epidermis, which stays attached and buckles together with the mesophyll (**Figure 2(c)**). The figure also shows that the epidermis is stretched under tension by the mesophyll. These observations led to tests of the hypothesis that blade expansion is driven by pressure exerted from the mesophyll, and limited by extensibility of the epidermal layer.

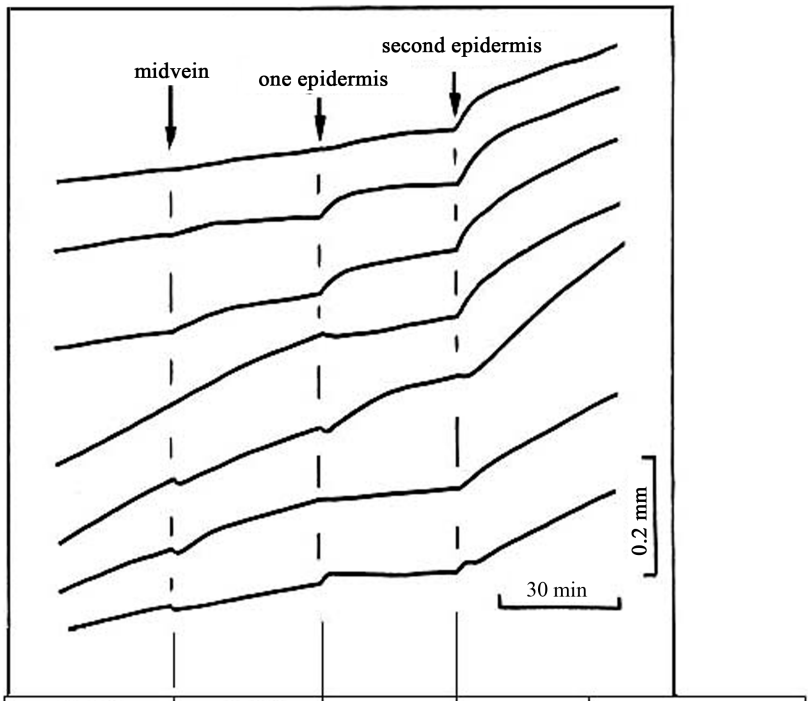
These pictures and those of others as well [20] show that the attachment between epidermis and mesophyll is either inhomogeneous (patchy) or weaker than in normal leaves. However, this no longer applies to the later appearing leaves, which have flat and straight blades. These leaflets are still silvery in appearance from both sides, a feature that was related to the presence of more subepidermal airspaces than in normal blades [20]. However, there are no abnormalities in the shape of the epidermal layer and the mesophyll itself [23] and leaflet size and growth resembles normal pea leaves [26]. For all the following studies we use the later appearing leaves with smooth and flat surfaces as found in normal peas.

3.2. Leaf Expansion Is Driven by the Mesophyll and Restrained by the Epidermis

This is not the first study to elucidate the role of the epidermis on cell and organ growth in argenteum leaves. Wilson and Bruck [23] showed that the removal of the epidermis layers did not alter the leaf morphology, mesophyll cell size, and differentiation or layering patterns in developing argenteum blades. They concluded that the epidermis or its removal exerted no detectable effect on the expansion of the leaflets or on the cell layers in it. While this study compared the morphology of the expanded leaves we set out to determine the role of the com-

ponent leaf tissues on the rate of leaf expansion. This role was first evaluated by measuring the short-time expansion of leaf segments as affected by sequential, successive removal of components (**Figure 3**). Six-mm segments were cut so as to contain the main vein, oriented in the direction of the growth measurement, placed under 1g tension, and the extension was measured continuously in the light. This is done because leaf strips lack the stiff compactness of stems to push the transducer [26]. Excision of the main vein reduced the width of the segments to 5 mm but did not alter the rate of extension significantly, indicating that the vein did not limit the rate of extension of the leaf strip. We then removed one of the epidermis layers (abaxial alternating with adaxial) finding that the removal of one epidermis does not increase the growth rate either, although it gives the mesophyll tissue direct contact and access to the incubation solution. The growth rate only changes when the second epidermis layer is removed, no matter whether it is the adaxial or abaxial one. After a transient relaxation response, the mesophyll strips establish a steady rate that doubles the preceding one (see table insert in **Figure 3**). These data show 1) that the mesophyll expands more rapidly when the epidermis is completely removed, and 2) that the presence of either one of the epidermal layers is sufficient to restrain the expansion of the mesophyll layers.

To test this conclusion further, growth measurements were made of leaf segments over 24 hours, in the dark or in the light and without any external tension (**Figure 4**). In the dark, mesophyll segments (the two epidermal layers were removed) showed on average a slightly (20%) larger expansion than intact segments. Light pro-



1.34 ±0.52	1.14 ±0.46	0.75 ±0.36	2.43 ±0.70	Growth Rate mm/h

Figure 3. The effect of the excision of the midvein (first arrow), removal of either one epidermis (second arrow) and of both epidermis layers (third arrow) on the growth of illuminated segments of *argenteum* pea leaflets. 15 mm-long segments were cut parallel to and including the midvein (6 mm wide), floated on incubating solution with their ends clamped under a load of 1 g and illuminated with 150 μmoles $m^{-2} \cdot s^{-1}$ white light. At the time indicated by the arrows we removed the measured segments for intervals of less than 30 sec from the set to remove either the midvein or an epidermis. A substantial, consistent increase in growth rate occurred only after both epidermal layers had been removed. Shown are 7 examples with varying initial growth rates. The inserted table shows the corresponding steady state growth rates before and after the treatments together with the standard deviation for n = 10 repetitions.

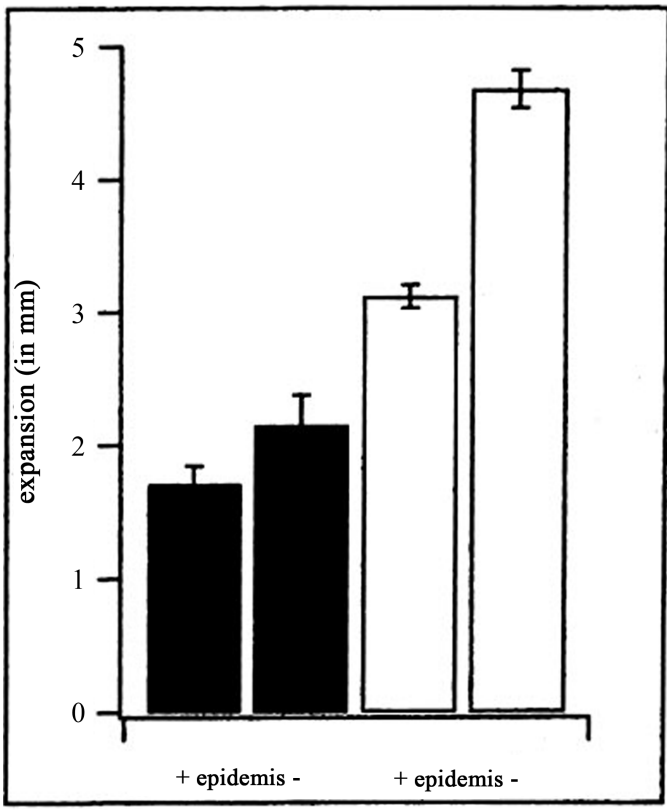


Figure 4. Comparison of the expansion of complete leaflet segments (+epidermis) and isolated mesophyll segments stripped of both epidermis layers (−epidermis) from *argenteum* blades during 24 h incubation in 10 mM KCl + 1 mM $CaCl_2$ solution in the dark (filled columns) and in the light (empty columns). Unlike **Figure 3** there is no tension on the leaf and tissue strips. The removal of the epidermis layers has a small effect in the dark (+23%) but increases the light-induced mesophyll expansion by 50%. The columns represent the arithmetic means of 25 measured segments each; the bars indicate the standard error.

moted the expansion of intact segments by 80%, and the expansion of pure mesophyll segments by 120% showing that removal of both epidermal layers enhanced long-term light-induced leaf growth by 40%. These long-term studies confirm the results from the short-term studies in **Figure 3**. Epidermal layers, both in the light and dark; with and without added tension restrain the expanding mesophyll cells, which are driving the expansion of the intact strips.

3.3. Response of Isolated Epidermal and Mesophyll Layers to Varying Tension

The restrictive role of the epidermis layer could be caused by either insufficient turgor pressure or particularities of its cell wall extensibility [1] [3]. To examine the response of isolated epidermal and mesophyll strips to applied tension, segments were incubated in the light and in unbuffered solution of pH 6, initially under 1 g tension. Using isolated epidermis and mesophyll strips from the same blade we exposed them to relaxation tests applying increased and decreased tension (**Figure 5**). Under increased tension epidermal strips undergo a rapid but transient extension; mesophyll strips show in addition a plastic extension which results in a permanently increased rate of extension. Removal of the increased tension (return to 1 g) leads to shrinking in the epidermis that far exceeds the small and transient shrinking of the mesophyll strips. These data show that epidermal strips show mainly elastic extension while mesophyll strips are able to undergo plastic expansion as well. The data confirm that the epidermis can be elastically stretched under the influence of a faster expanding mesophyll.

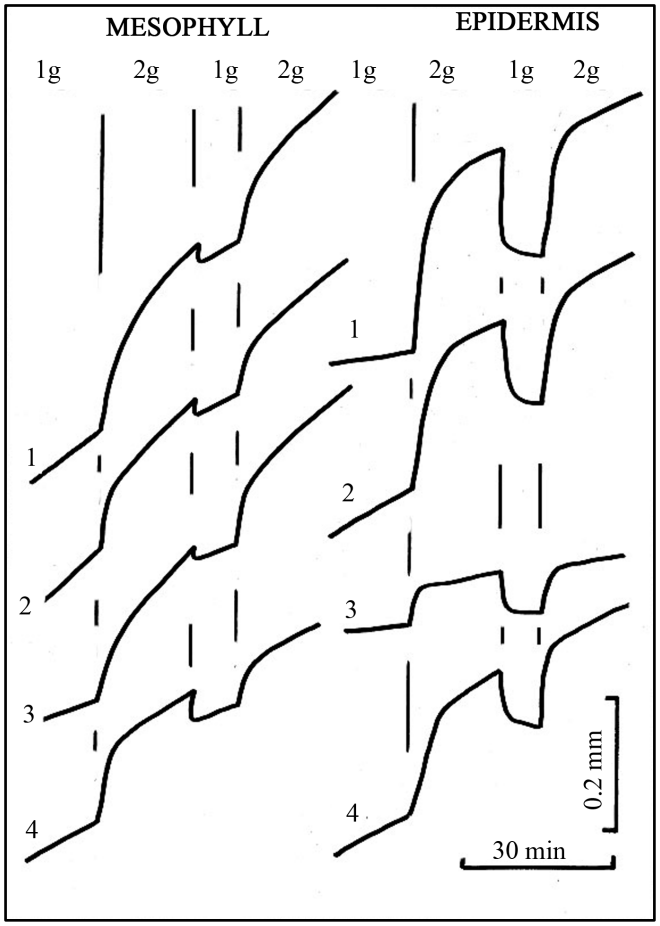


Figure 5. Relaxation experiments with pairs of segment of mesophyll and abaxial epidermis layers isolated from the same *argenteum* leaflets. 4 × 15 mm-segments were cut from the isolated mesophyll and epidermis layers of the same leaflets (numbered 1 - 4) floated on incubating solution with their ends clamped and exposed to a tension of 1 g. After a steady growth rate was established under light the tension was doubled (weight on the pulley increased from 1 g to 2 g), then relaxed (weight reduced from 2 g to 1 g) and reapplied. The experiments show that isolated epidermis strips have a larger elastic relaxation response but a smaller plastic extensibility than mesophyll strips. Shown are 4 pairs out of 7 similar runs.

3.4. Response of Isolated Epidermal and Mesophyll Layers Acidic Cell Wall pH and Photosynthetic and H^+-ATPase Inhibitors

Previous studies with a*rgenteum* pea leaves indicated that leaf expansion in this species is mediated by an acid-growth mechanism [22] [26] [29]. The restraining action of the epidermis might be due to a lower capacity for acid-induced wall loosening, as compared with the mesophyll cells. To test this hypothesis, isolated epidermal segments, and isolated mesophyll tissues lacking both epidermis layers were measured for their ability to undergo rapid expansion in response to acidic solutions (**Figure 6**). Segments of mesophyll and epidermis layers were isolated from the same leaf and attached to continuously recording transducers under 1 gram tension. Under these conditions, light induced a stronger expansion in the isolated mesophyll than in the isolated epidermal strips (**Figure 6**, left column, first arrow**)**. Similar to the action of light, a subsequent change in solution from pH 6.0 to 4.5 accelerated the elongation in both tissues (**Figure 6**, right column, downward arrow**)**. We conclude that the restraint by the epidermis is definitely not due to a lack of capacity for acid-induced wall loosening. On the contrary, with acidic wall pH, epidermal expansion equaled or exceeded mesophyll expansion.

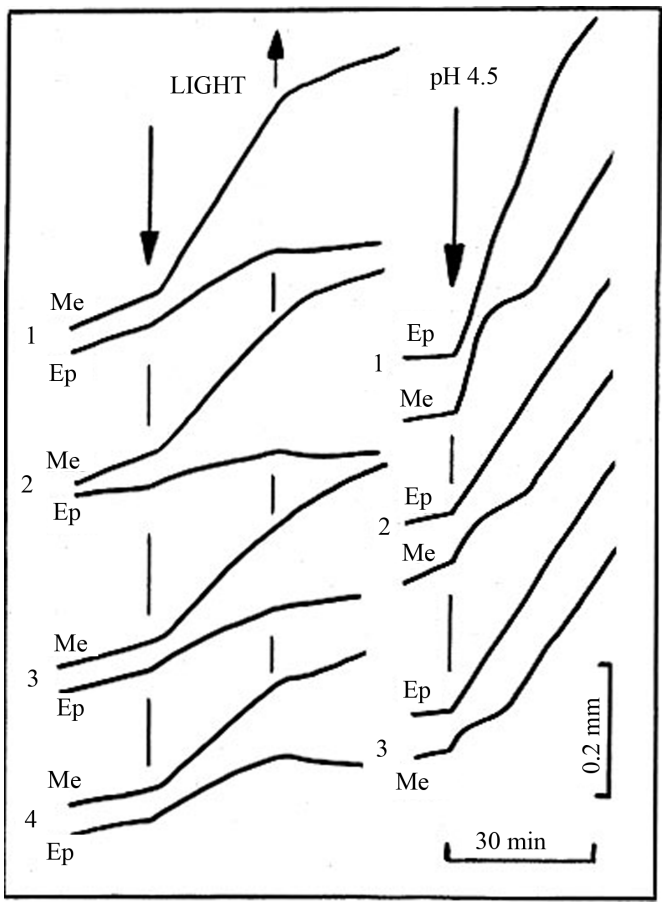


Figure 6. Comparison of the growth responses to light (left curves) and wall acidification (right curves) between segments of mesophyll (Me) and epidermis (Ep) isolated from the same leaflets of *argenteum* peas. 5 × 12 mm-segments were cut from the isolated mesophyll and abaxial epidermis layers of the same leaflets (numbered 1 - 4), floated on incubating solution with their ends clamped under a tension of 1 g. After the segments established a steady growth rate in the dark they were illuminated with 150 μmoles $m^{-2}\cdot s^{-1}$ photons of white light (first arrow) and later returned to darkness (upward arrow). Whereas epidermis strips responds to light with a weak increase in expansion rate, mesophyll strips show strong stimulation. The incubating medium was then replaced by 50 mM sodium acetate buffer at pH 4.5 (third arrow), which leads to rapid acid-induced expansion in both tissue layers. The data show that the weak response of the epidermis to light is not caused by a reduced acid-growth response. Shown are 3 - 4 examples out of 8 similarly responding leaflets.

The following experiments show that the accelerated expansion of the stripped mesophyll is not an artifact but fulfils the characteristics of a growth process. If leaf expansion involves acid-induced cell wall loosening, inhibition of the plasma membrane H^+-ATPase should inhibit leaf expansion in both the dark and the light. **Figure 7** confirms that treatment of isolated mesophyll tissues with the H^+-ATPase inhibitor vanadate eliminates most of the expansion of in both light and dark. The inhibitor of photosynthetic electron transport DCMU has a different action inhibiting the expansion of isolated mesophyll tissues only in the light but not in the dark suggesting that it inhibits the provision of additional ATP from illuminated chloroplasts. The similarity of these results to the behavior of intact leaf strips [26] shows clearly that the expansion of mesophyll strips in both light and dark is a normal growth process that requires increased proton excretion via increased photosynthetic supply of ATP.

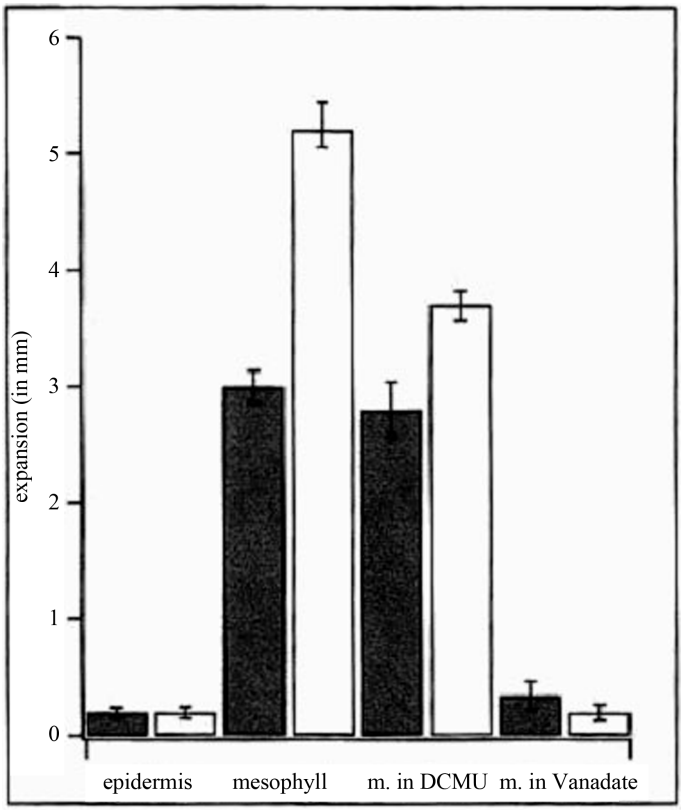


Figure 7. The role of H^+-ATPase and photosynthesis in the expansion of isolated abaxial epidermis and isolated mesophyll layers form argenteum blades. Sections of isolated abaxial epidermis or isolated mesophyll tissues were incubated in the dark (filled columns) and in the light (empty columns) for 24 h in 10 mM KCl + 1 mM $CaCl_2$ solution, without and with the addition of 5 × 10 - 5 M DCMU or 5 × 10 - 4 M sodium ortho-vanadate. While the isolated epidermis layer did not expand under these conditions, the expansion of the isolated mesophyll was almost totally inhibited by vanadate, in both dark and light. Inhibition of photosynthesis with DCMU had no effect in the dark, but inhibited about 60% of the growth stimulation in light. The values represent the arithmetic means of 25 measured segments each. The bars indicate the standard error.

3.5. Induction of Hypo- and Epinastic Blade Responses and Buckling

The preceding experiments with excised leaf, epidermis and mesophyll tissue consistently supported mesophyll-driven blade expansion. Next we wanted to test whether the concept held for intact leaflets attached to an intact plant. Inspired by a previous study [23], we found that *argenteum* pea leaflets on intact plants remain fully turgid even when more than half of the epidermis is removed from one surface (**Figure 8**). Unilateral removal of either one epidermis layer from one half of a young leaflet caused a slow bending of the blade that progressed continuously for 10 h (**Figure 8**). Removal of the adaxial epidermis (a) from one half of the leaflet caused epinastic expansion in the treated portion of the blade (**Figure 8(a)**), removal of the abaxial epidermis caused strong hyponasty with an upward bending blade (**Figure 8(b)**). This result would not be expected if it were caused by the dehydration of the exposed side of the mesophyll. Also, this outcome is not a fast response as one would expect if it were caused by a shrinking epidermis of the untreated side (compare kinetics in **Figure 5**). The nastic bending of the leaf blade is a slow growth response increasing the curvature over several hours to angles exceeding 180˚. **Figure 8** provides direct evidence that the blade curled or rolled because the accelerated growth of the stripped mesophyll exceeds that of the intact side with the attached epidermis. Further support for

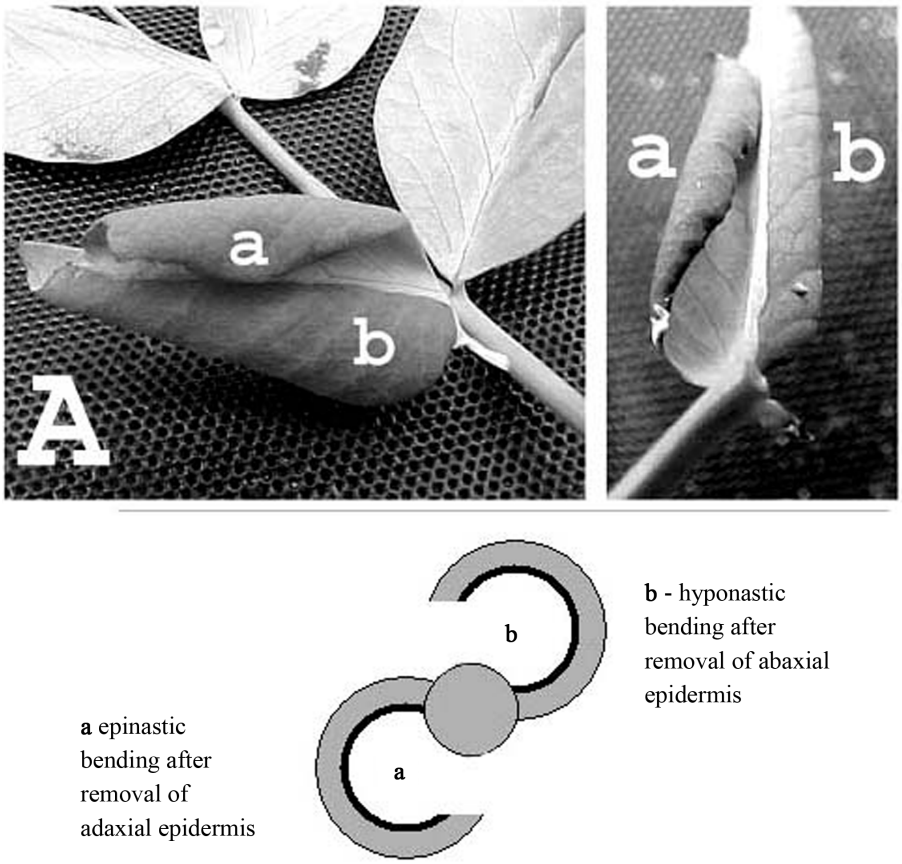


Figure 8. Unilateral removal of epidermal strips causes bending in previously straight blades of intact *argenteum* leaves. Epinastic curving of the blade occurred when the epidermis was removed from one half of the leaflet at the adaxial side (a) in one leaflet of a pair (untreated leaflet serves as a control). Hyponastic curving of the blade occurred on the opposite leaflet half where the epidermis was removed at the abaxial side (b). Note that these responses do not appear immediately but take time to develop and increase. Pictures show two typical experiments out of 10 (A). They were taken from the lower or abaxial side of argenteum leaves 10 h after the epidermal layers were removed from leaflets still attached to intact plants. The bottom part is a cartoon illustrating the removal of the epidermis on opposite sides of each half of the blade.

this interpretation is that the expansion of the intact as well as isolated mesophyll strips is reduced by external mannitol solutions and hence depends on turgor pressure (**Table 1**). The data allow comparison between the reductions in the expansion of mesophyll strips (both epidermis layers removed) and the bending of strips where only one epidermis was removed. Adding 0.3 M mannitol to the solution dropped both values by about a half. This correlation suggests that the epinastic bending of the unilaterally stripped segments is due to the expansion of the mesophyll. It is prevented by either the removal of the remaining epidermis or by increasing osmolarity of the external solution (**Table 1**).

A clear demonstration for the validity of a mesophyll-driven blade expansion in intact plants is given in **Figure 9**. It shows what happens to a complete leaflet after two epidermal strips in matching positions are removed from both the abaxial and adaxial leaf sides. Released from the compression by the epidermal layer this exposed strip of mesophyll tissue accelerates its expansion. The developing excess length of the exposed mesophyll strip causes it to buckle. The mesophyll tissue strip adopts a wavy shape in order to fit into the confines of the intact remainder of the blade, which is restricting. This outcome results from the unequal expansion of mesophyll, complete blade tissue and the midvein. We can also conclude from this experiment that in the absence of epidermal layers, the midvein can become a restrictive element of rapid mesophyll expansion.

Table 1. Light-induced expansion (Δl) of intact leaf strips and isolated mesophyll strips in various mannitol concentrations is compared with the angle of bending which occurs in the same mannitol solutions after the removal of only the abaxial epidermis. Expansion of 10 mm long strips is given in mm, the angle of the bending of the strips towards the remaining epidermis layers in degrees. Values are the arithmetic mean of 7 to 15 measurements and include the standard error. The results were measured after 10 h.

	Mannitol concentration			
	0.0 M	**0.1 M**	**0.3 M**	**0.5 M**
Δl in intact strips (mm)	1.9 ± 0.15	1.3 ± 0.1	1.0 ± 0.1	0.25 ± 0.1
Δl in mesophyll strips (mm)	2.4 ± 0.15	1.6 ± 0.3	0.8 ± 0.1	0.25 ± 0.1
Angle, after removal of one epidermis	370˚ ± 6˚	310˚ ± 13˚	180˚ ± 7˚	32˚ ± 8˚

Figure 9. Removal of two epidermal strips from matching positions at the abaxial and adaxial leaf sides of an *argenteum* leaf blade. The accelerated expansion of the unrestrained mesophyll strip elongates it in excess of the remainder of the blade. The consequence of this unequal expansion is that the longer mesophyll strip buckles to fit the proportions of the neighboring tissue. Bilateral removal of epidermis strips reproduces buckling reliably when it is done next to the midvein. The photograph shows one example out of 10 similar runs and was taken one hour after completion of the procedure.

4. Discussion

4.1. How Does the Mesophyll Drive Leaf Expansion?

A majority of researchers considered that the epidermis is the leaf layer that drives leaf expansion [11]-[13] [18]. The formation of air spaces in the palisade parenchyma during blade expansion suggested that at least palisade cells could not exert the force necessary to drive leaf expansion [8]-[10]. Results presented here show that under the influence of light, isolated mesophyll cell layers show considerable increase in growth rate (**Figure 3**, **Figure 4**, **Figure 6**, **Figure 9**), which involves plastic expansion and requires turgor pressure, photosynthetic electron transport, an active H^+-ATPase and acid-mediated cell wall loosening (**Table 1**; **Figures 5-7**). These data show that the accelerated growth of the isolated mesophyll is not an artifact that is due to a lesser interconnectedness in the isolated mesophyll cells strips. Our data on force relaxation confirm that both the isolated mesophyll and epidermis layers keep their integrity under tension. The expansion of the isolated mesophyll shares the typical dependencies of intact leaf growth: inhibition by vanadate, inhibition of only the light-induced expansion by DCMU, and dependence on the osmolarity of the external medium. The data leave no reasonable doubt that at least in *argenteum* peas it is the mesophyll layer that drives blade expansion together with epinastic and hyponastic blade movements (**Figure 8**).

Concerning the epidermis, our data suggest that it is an elastic layer that is kept under tension by a faster expanding mesophyll layer and that this external tension might be necessary to exceed the yield threshold for growth (**Figures 2-5**, **Figure 7**) [1] [30]. However, under acid cell wall pH, the epidermis can reach expansion rates that perfectly match that of the mesophyll (**Figure 6**).

The concept of the mesophyll as a driving force of leaf blade expansion that we introduce in this paper derived from direct measurements of the growth rates of rapidly expanding leaves. This approach is different from the majority of studies of leaf expansion, which are mostly focused on the final size of the leaves comparing cell numbers, cell sizes and intercellular space. Such studies can easily miss changes that a direct and continuous measurement of the growth of entire leaf strips picks up with ease since they measure the dimensional changes not of one but many cells combined. A 10% increase in length is easier measured in 15 mm leaf strips than rec-

ognized in dimensional changes of single cells.

4.2. Tissue Tension from Different Expansion of the Layers in a Leaf Blade

When the multi-layered, laminate structure of a leaf blade expands as a flat structure, all cell layers must grow to the same extent and rate, since neighboring cells are tied together by shared cell walls. Therefore, the expansion of the entire leaf may be restricted or even arrested by the slower or lack of expansion of one cell layer—a phenomenon called tissue tension. Until now the contribution and role of the different cell layers to tissue tension and growth have been studied in hypocotyls and epicotyls [4] [5]. The generation and role of tissue tension have remained unknown for leaves due to the lack of data for isolated epidermal and mesophyll layers [6].

These data to resolve this situation are now available and all of them support the conclusion that the mesophyll acts as the driving force of blade expansion against the resistance of the epidermal envelop. This situation in leaves resembles the tissue tension found in most growing stems where a tension-stressed, restrictive layer is pushed by the pressure of an internal core [2] [4] [5] [31] [32]. With a centrally positioned mesophyll core pushing, a flat or uncurved plane of blade expansion can only be expected if similar extensibilities of the abaxial and adaxial epidermis walls exist. Unequal extensibilities will cause epinastic or hyponastic movements of the blade. These responses are induced by the removal of the abaxial or adaxial epidermis layers from intact *argenteum* leaves (**Figure 8**) and their angle of bending depends on the expansion of the mesophyll (**Table 1**).

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