

# Stomatal plugs of *Drimys winteri* (Winteraceae) protect leaves from mist but not drought

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**ABSTRACT** Two outstanding features of the flowering plant family Winteraceae are the occlusion of their stomatal pores by cutin plugs and the absence of water-conducting xylem vessels. An adaptive relationship between these two unusual features has been suggested whereby stomatal plugs restrict gas exchange to compensate for the presumed poor conductivity of their vesselless wood. This hypothesized connection fueled evolutionary arguments that the vesselless condition is ancestral in angiosperms. Here we show that in *Drimys winteri*, a tree common to wet forests, these stomatal occlusions pose only a small fixed resistance to water loss. In addition, they modify the humidity response of guard cells such that under high evaporative demand, leaves with plugs lose water at a faster rate than leaves from which the plugs have been experimentally removed. Instead of being adaptations for drought, we present evidence that these cuticular structures function to maintain photosynthetic activity under conditions of excess water on the leaf surface. Stomatal plugs decrease leaf wettability by preventing the formation of a continuous water film that would impede diffusion of CO<sub>2</sub> into the leaf. Misting of leaves had no effect on photosynthetic rate of leaves with plugs, but resulted in a marked decrease ( $\approx 40\%$ ) in leaves from which the plugs had been removed. These findings do not support a functional association between stomatal plugs and hydraulic competence and provide a new perspective on debates surrounding the evolution of vessels in angiosperms.

Botanists have long speculated on the morphology of the first flowering plants and on how particular angiosperm features might have influenced their rise to dominance (1–3). Among these features, the evolution of xylem vessels has been considered a key adaptation allowing the evolution of large, undissected leaves and radiation from wet forests into seasonally arid or disturbed environments (3–5). The traditional view of early angiosperm morphology is that their wood lacked vessels (i.e., contained only tracheids for water conduction) and that this confined them to wet environments (6–10). Winteraceae members (6 genera,  $\approx 65$  species), which are vesselless and grow only in wet habitats, have been frequently cited in support of this viewpoint (6, 10).

The genus *Drimys* (Winteraceae) consists of six species of shrubs and small trees that are common to wet forested areas of tropical and temperate Central and South America ranging from montane cloud forests to maritime forests (11, 12). In these wet regions, *Drimys* plants are often enveloped by clouds and frequent rainfall such that daytime atmospheric humidity exceeds 90% and annual rainfall may be upwards of 4,000 mm (12–15). Despite their wet environments, *Drimys* leaves exhibit a number of seemingly xeromorphic features. Their leaves have a thick cuticle, and the stomata are recessed into the abaxial

leaf surface with each stomatal pore capped by a cutinaceous “plug” (refs. 16–18; Fig. 1). In cross section, stomatal plugs consist of a porous, granular material that fills the cavity above the guard cells (17, 18). The abaxial epidermis of *Drimys*, associated with the cuticular plugs, also is covered by a dense mat of crystalline, wax protuberances (Fig. 1). Assumptions have been made that these peculiar structures markedly reduce rates of water loss under natural conditions and that such transpiration-reducing structures play an essential role in offsetting the hydraulic constraints associated with vesselless wood (7–9, 17). Indeed, some biologists have viewed stomatal plugs as an adaptation that might have played an important role in the survival of the Winteraceae by allowing them to tolerate drought, evolve larger leaves, and thus compete with plants that have vessels (7–10). However, the functional consequences of these structures on gas exchange have not been explored.

Plants with vessels generally have higher hydraulic conductivities than plants that rely solely on tracheids for water transport. The greater resistance to flow in tracheids arises from their smaller diameters and the absence of specialized perforation plates (19). However, the structural distinction between vessels and tracheids is less pronounced in the early angiosperm lineages (9). Whether vesselless angiosperms actually have a lower hydraulic transport capacity than co-occurring taxa with vessels has not been examined. Here we present experimental data on how stomatal plugs influence gas exchange rates in *Drimys winteri* var. *chilensis* (DC) A. Gray. Our data suggest that stomatal plugs are more important for promoting photosynthetic activity than for preventing water loss. We believe that the evolution of stomatal plugs is more likely to be related to the occurrence of *Drimys* in areas that are generally wet (e.g., rainforests and cloud forests) than to the absence of vessels in their xylem.

## MATERIALS AND METHODS

**Plant Material, Treatment to Remove Stomatal Plugs, and Leaf Anatomical Observations.** Twelve plants of *Drimys winteri* var. *chilensis* were grown from cuttings under greenhouse conditions (day/night temperatures of 27/22°C, 400–450  $\mu\text{mol}$  of photons  $\text{m}^{-2}\text{s}^{-1}$  irradiance for 10 hr, relative humidity 70–80%). Stomatal plugs were removed by pressing a nontoxic, sticky putty (Blu-Tack, Bostick, Australia) onto leaf surfaces. This method removes 80–90% of the stomatal plugs but does not damage the cuticle as determined with both light and scanning electron microscopy and contact-angle measurements (see below). Physiological measurements were made on leaves at least 2 days after plug removal. For scanning electron microscopy, leaves were air-dried, sputtered with Au/Pd, and photographed.

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Abbreviations: VPD, vapor-pressure difference; PS, photosystem; ETR, electron transport rate.

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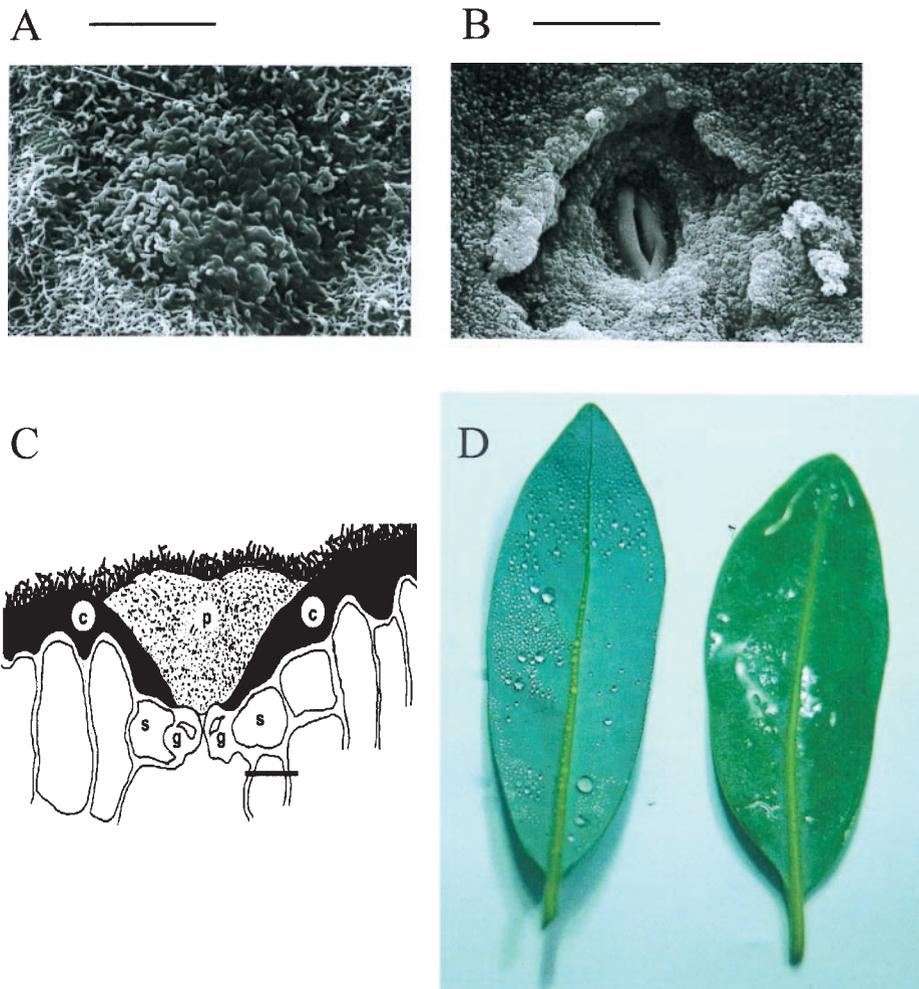


FIG. 1. Scanning electron micrograph (A) and line drawing (C) of a *Drimys winteri* stoma occluded by a cutin plug showing the locations of the plug, cuticle, epicuticular wax, guard cells, and subsidiary cells, and a scanning electron micrograph (B) of a leaf with the plug experimentally removed. (D) Comparison of the leaf-surface wettability of a leaf with (Left) and without (Right) stomatal plugs. p, stomatal plug; c, cuticle; g, guard cell; s, subsidiary cells. (Bars, 20  $\mu\text{m}$ .)

**Water Loss Rates.** Transpiration rates and conductance to water vapor of *D. winteri* leaves were measured with a Li-Cor 6400 Photosynthesis System (Lincoln, NE) at constant light (500  $\mu\text{mol}$  of photons  $\text{m}^{-2}\text{s}^{-1}$ ) and leaf temperature (25°C). For these measurements, six terminal, recently expanded leaves were selected from six plants. Evaporative conditions (vapor-pressure difference; VPD) were chosen to represent the range of conditions that may be expected in the humid forests that *Drimys* would likely encounter. The low evaporative-demand measurements (VPD = 0.5 kPa) represented the most humid conditions (approximately 85% relative humidity) that could be maintained within the gas exchange system without condensation. The high evaporative condition (VPD = 1.8 kPa) was chosen to represent midday conditions, when cloud cover is absent (relative humidity of 40%; refs. 12, 14). Evaporative conditions were modified by altering the flow rate of dry air into the cuvette. Steady-state responses of transpiration and stomatal conductance to decreasing VPD (step changes of  $\approx 0.25$  kPa were made after leaf-gas exchange reached a steady state) were measured on well watered plants. To study the effects of stomatal plug removal on the water-loss properties of *Drimys* leaves, an additional set of six leaves from six plants were selected and treated with Blu-Tack.

Minimum conductances were determined by measuring water-loss rates from detached leaves. We refer to these as epidermal conductance to emphasize that the guard cells are maximally closed. The calculated conductance thus includes

water loss across the cuticle as well as through the closed stomatal complex. Water-loss rates from detached leaves were calculated from the decrease in leaf mass measured for 60 min under constant evaporative conditions (20).

**Photosynthetic Response to Surface Wetness.** Chlorophyll fluorescence emission was measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). We calculated photosystem (PS) II quantum efficiency ( $\phi_{\text{PSII}}$ ) from fluorescence as  $(F'_m - F_s)/F'_m$ , where  $F'_m$  is the maximal fluorescence yield (under a saturation pulse of 5,000  $\mu\text{mol}$  of photons  $\text{m}^{-2}\text{s}^{-1}$ ) and  $F_s$  is the steady-state fluorescence yield under actinic illumination (21). This equation relates the efficiency at which an absorbed photon reaches PSII and drives photochemistry to the proportion of PSII centers that are open in the light (21). Multiplying  $\phi_{\text{PSII}}$  by the absorbed quantum flux (leaf absorbance of *Drimys* leaf was assumed to be 0.78) and assuming this energy flux is equally distributed between PSII and PSI, gross electron transport rate (ETR) through PSII was calculated as  $\text{ETR} = \phi_{\text{PSII}} \times \alpha \times 0.5 \times \text{PAR}$ , where  $\alpha$  is the whole-leaf absorbance and PAR is the incident photosynthetically active light flux (400–700 nm; ref. 21). Mist (droplets 5–20  $\mu\text{m}$  in diameter; total volume  $\approx 1$  ml) was applied to the leaf surface at 5-min intervals with a hand-held water atomizer during fluorescence measurements. To eliminate possible distortion of the leaf fluorescence signal by application of mist to the abaxial leaf surface, fluorescence measurements were made from the adaxial leaf surface.

**Leaf Wettability.** Contact angles were determined visually at  $\times 60$  magnification as the tangent line through a 5-mm<sup>3</sup> droplet pipetted onto a horizontal leaf surface (22).

## RESULTS

Stomatal conductances when evaporative demand was low (VPD < 1 kPa) of both plugged and unplugged *D. winteri* leaves were low (<120 mmol H<sub>2</sub>O m<sup>-2</sup>s<sup>-1</sup>) but fell within the range of values reported for a wide range of cloud-forest tree species (23, 24). When the VPD was <1 kPa, experimentally removing the plugs and associated epicuticular waxes resulted in a significant increase in stomatal conductance (Fig. 2; mean with plugs = 116  $\pm$  3 (SD) mmol H<sub>2</sub>O m<sup>-2</sup>s<sup>-1</sup>; mean without plugs = 106  $\pm$  6 (SD) mmol H<sub>2</sub>O m<sup>-2</sup>s<sup>-1</sup>; Student's *t* test, *t* = 3.241, *P* = 0.009; *n* = 6 for both groups). If we assume that the guard cells of plugged and unplugged leaves are open to an equal extent when the greatest conductances were measured, then the presence of the plugs resulted in only an 8% increase in the resistance to water-vapor transfer through the stomatal pore.

In contrast, as leaves were exposed to progressively greater evaporative demand, the conductance responses of *Drimys* leaves with plugs compared with ones without plugs were markedly different. Leaves without plugs showed a 70% decrease in conductance to water vapor as VPD increased from 0.5 kPa to 1.8 kPa (Fig. 2). This degree of stomatal closure in response to increasing VPD is similar to that reported for a wide variety of plants (25). In contrast, leaves with their stomata plugged exhibited only a 20% decline in stomatal conductance for the same change in evaporative conditions (Fig. 2). Under conditions of water stress, however, leaves with plugs can shut their stomata. Epidermal (minimum) conductance of leaves with plugs averaged 11  $\pm$  3 (SD) mmol H<sub>2</sub>O m<sup>-2</sup>s<sup>-1</sup>; *n* = 3). Because stomatal plugs markedly decrease the capacity of *D. winteri* leaves to regulate water-loss rates with increasing evaporative demand, it is difficult to view them as an adaptation to drought or as playing a compensatory role in relation to a less capable hydraulic system. Only at low VPDs, when water-loss rates are themselves so low as to be unlikely to lead to water stress, did the presence of stomatal plugs result in decreased stomatal conductances.

Stomatal plugs and the associated epicuticular waxes of *Drimys* strongly affect the wettability of leaf surfaces. Water beaded when misted onto leaves with plugs, whereas misting resulted in a uniform water film on leaves from which these features were removed (Fig. 1D). Contact angles for water droplets were significantly greater on leaves with plugs [mean = 123°  $\pm$  6° (SD); *n* = 5] than on leaves without plugs [mean = 35°  $\pm$  5° (SD), *n* = 5; Student's *t* test, *t* = 28.7, *P* =

0.001]. Contact angles for the adaxial side of *Drimys* leaves treated with Blu-Tack [mean = 59°  $\pm$  19° (SD), *n* = 8] were not significantly different from untreated leaf surfaces [mean = 63°  $\pm$  9° (SD), *n* = 8; Mann-Whitney *U* test, *P* = 0.959]. This leads to the conclusion that the treatment used does not markedly change the wettability properties of the epidermis by tearing the cuticle or by leaving particles of Blu-Tack behind that may affect droplet spreading.

To determine the consequences of wettability of *Drimys* leaves on leaf carbon gain, we capitalized on the relationship between chlorophyll fluorescence and photosynthetic electron transport (21). This allowed us to monitor the photosynthetic activity of wet leaves, where traditional gas-exchange measurements encounter substantial technical difficulties. Photosynthetic ETRs decreased approximately 40% following misting of *Drimys* leaves that lacked stomatal plugs (Fig. 3). In contrast, photosynthetic ETR of leaves with plugs was unaffected by misting (Fig. 3). We suggest that the formation of a continuous water film on the leaf surface restricts the availability of CO<sub>2</sub> as an acceptor for photosynthetic electron transport, resulting in an increase in the amount of absorbed light dissipated as heat (21).

## DISCUSSION

Our observations contradict the view (7–10) that in *D. winteri*, stomatal plugs function to restrict transpiration rates. Under conditions of increasing evaporative demand, they markedly reduce the leaf's ability to control water-loss rates (Fig. 2). The exact mechanism by which stomatal plugs inhibit stomatal closure when leaves are challenged with high VPD is unknown. It is possible that they prevent stomatal closure by keeping the humidity high at the guard cells. It is clear, however, that plugs in *Drimys* cannot be considered adaptations for drought because rates of water loss are higher when plugs are present. A major conceptual problem with the idea of regulating water loss by placing a large, fixed resistance in the stomatal pore is that CO<sub>2</sub> uptake would be reduced as well. Minimum conductances measured on leaves with plugs are in the range reported for mesophytic trees and crop species (20), further suggesting that these structures do not play a special role in maintaining leaf water balance.

Stomatal plugs appear to be one part of an elaborate epidermal system resulting in a water-repellent surface that allows unperturbed photosynthetic activity even when leaves are exposed to mist. Because CO<sub>2</sub> diffuses through water 10,000 times slower than in air (22), water films create a barrier

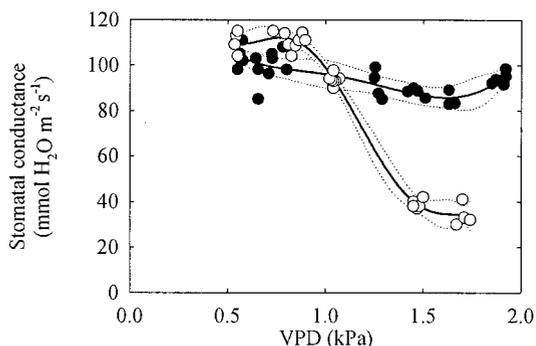


FIG. 2. Effects of stomatal plug presence (●) and absence (○) on stomatal conductance (mmol H<sub>2</sub>O m<sup>-2</sup>s<sup>-1</sup>) with evaporative demand from low (0.5) to high (1.8) leaf-to-air VPDs (in kPa) in *Drimys winteri* leaves (*n* = 4 leaves per group from 4 plants, 95% confidence intervals indicated by dotted lines).

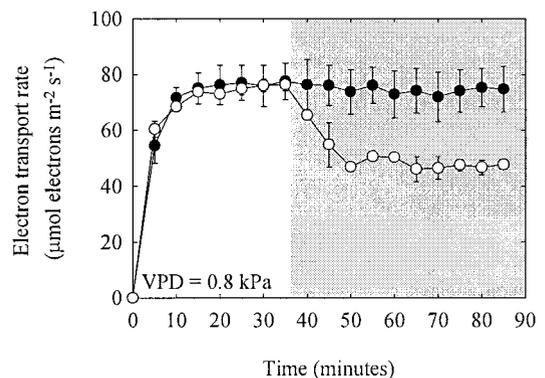


FIG. 3. Effects of misting on photosynthetic ETR ( $\mu\text{mol}$  of electrons  $\text{m}^{-2}\text{s}^{-1}$ ) for *Drimys winteri* leaves with (●) and without (○) stomatal plugs (mean  $\pm$  SEM, *n* = 3 leaves per group from 3 plants). *D. winteri* leaves were misted after 30 min (mist application indicated by the shaded box) under a light intensity of 500  $\mu\text{mol}$  of photons  $\text{m}^{-2}\text{s}^{-1}$ .

for diffusion of CO<sub>2</sub> into leaves. Therefore, avoidance of leaf-surface wetness may be especially relevant for *Drimys* trees that grow in wet environments (e.g., cloud forests or temperate rain forests) because their leaves frequently encounter mist and rain. At the coarse scale of our contact-angle measurements (5- $\mu$ l droplets were used), it is clear that the untreated abaxial epidermis of *Drimys* is much more hydrophobic than that of treated leaves. At this scale, it is difficult to separate the contribution of epicuticular waxes from stomatal plugs on surface wettability. However, chlorophyll fluorescence measurements suggest that at the scale of individual stomata, similar interactions between mist droplets and the cutin composing the plug function to sustain photosynthetic activity (Figs. 1D and 2).

Some vesselless angiosperms (e.g., *Amborella*, *Tetracentron*, and even *Takhtajania perrieri* of Winteraceae) lack stomatal plugs or other cuticular ornamentations associated with the stomatal apparatus (refs. 18 and 26; T.S.F., unpublished observations); this is evidence that vesselless wood does not mandate an extrastomatal mechanism to restrict transpiration. Other angiosperms with vessels (e.g., some of the Myristicaceae) plug their stomata and grow in wet environments (26). The stomata of conifers typically have stomatal plugs or other waxy occlusions that conceal their stomatal pores (9, 16, 27–29). Many lineages with plugged stomata (e.g., Podocarpaceae, Araucariaceae, and Pinaceae) inhabit mesic forests where stomatal occlusions could also function in shedding water from leaf surfaces (9, 28). In contrast, the stomata of several conifers from the driest environments that could benefit from transpiration restriction are unplugged (e.g., *Callitris* and *Widdringtonia*; ref. 28).

Our results bear on debates concerning the evolution of vessels in angiosperms (6, 7, 10, 30, 31). Under the traditional view, the first angiosperms lacked vessels, and this condition was retained in four extant lineages (7, 9, 10): Winteraceae ( $\approx$ 65 species), *Amborella* (1 species), Nymphaeales ( $\approx$ 60 species), and Trochodendrales (*Trochodendron*, 1 species; *Tetracentron*, 1 species). Phylogenetic analyses have favored the alternative view that the first angiosperms had vessels, which were then lost in these lineages (32–35). Although *Amborella* and Nymphaeales may be basal branches within angiosperms and could be primitively vesselless (34), Winteraceae, and especially Trochodendrales, are nested within clades containing plants with vessels (Winteraceae related to Canellaceae; Trochodendrales within eudicots). However, phylogenetic parsimony arguments have, in this case, seemed unsatisfactory because vessel loss is thought to entail a shift from a more effective to a less effective water-conducting system (7, 10). Loss of vessels has seemed all the more unlikely when stomatal plugs are viewed as compensating for an inferior hydraulic system (9, 10).

Our finding that stomatal plugs in *Drimys* do not protect leaves from drought but instead serve to protect leaves from excess water undermines the idea that plugs in *Drimys* evolved to compensate for an inferior water-transport system [including the idea that they allowed the evolution of larger leaves (9)] or that they are a retained adaptation to earlier xeric conditions (27). Determining whether vessels were actually lost in Winteraceae will require further resolution of phylogenetic relationships and tests of possible physiological and mechanical consequences of vesselless wood. The presence of stomatal

plugs in Winteraceae, however, no longer seems to bear on this issue.

Rather than being the mark of a primitive and inefficient hydraulic system, stomatal plugs appear to be an elegant adaptation to life in cloud-forest environments. This finding underscores the need to conduct critical functional tests of alternative adaptive hypotheses. In the absence of such tests, it is all too easy to perpetuate and entrench an explanation that entirely misses a more likely selective basis for the evolution of the trait.

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