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ORIGINAL ARTICLE

Role of the outer stomatal ledges in the mechanics of guard cell movements

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Abstract

Key Message **The modelling showed that outer ledges prevent wide opening of the stomatal pore and its lifting above leaf epidermis. This stomatal mechanics is combined with xeromorphic features of leaf epidermis.**

Abstract Methods of light, scanning, and transmission electron microscopy were used to study the stomata of the leaf epidermis in evergreen *Acokanthera oblongifolia* (Apocynaceae), *A. oppositifolia* (Apocynaceae), *Carissa spectabilis* (Apocynaceae), *Exbucklandia populnea* (Hamamelidaceae), and *Trochodendron aralioides* (Trochodendraceae). The stomata of their leaf epidermis are located on subsidiary cells, have large outer ledges, and lack inner ledges. To elucidate the role of the ledges, we applied dynamic modelling using the finite-element method. The application of dynamic modelling has shown that outer ledges prevent wide opening of the stomatal pore and their rising above the surface of leaf epidermis. The results of the modelling are supported by the observed deformations in the guard cells of the real stomata. This stomatal mechanics is combined with such stomatal xeromorphic features as thick cuticle, stomatal cavities, and waxy plugs (in *A. oblongifolia*). All studied species show similar leaf anatomy. It has much in common with the leaf anatomy of species connected in their origin with subhumid Tertiary laurophyllous forests.

Introduction

Stomata are structural elements of epidermis. They are composed of two guard cells divided by the stomatal pore, or aperture. The stomata are surrounded by epidermal cells that may or may not differ from the ordinary epidermal cells. In the first case, these cells are called subsidiary cells, in the second—neighbouring. The guard and subsidiary or neighbouring cells together compose stomatal apparatus or stomatal complex (Esau 1960; Wilkinson 1979; Evert 2006). The stomata control transpiration and gas exchange. The guard cells are capable of movements, which cause the pores open or close. The stomatal movements depend on turgor pressure in the guard cells, their shape, and wall structure (Sharpe et al. 1987; Wilmer and Fricker 1996; Niklas and Spatz 2014).

Earlier work (Von Mohl 1856; Schwendener 1881) suggested that shape changes in guard cells and, therefore, in the pore opening depend on the asymmetrical thickness of their walls. The typical guard cells of the dicotyledons are kidney-shaped. Their walls facing away from the pore (dorsal walls) are thinner than the walls bordering the pore (ventral walls). Increased turgor pressure in guard cells forces their thin dorsal walls to lengthen out and to protrude into subsidiary or neighbouring cells. Since the tensility of the thin dorsal and thick ventral walls is different,

the turgid guard cells curve and pore opens. Mechanical modelling was used to test this hypothesis (Jost 1907). The model consisted of two caoutchouc tubes, completely closed; united by their ends, but with the central region free, and having the strengthening layers glued along sides facing each other. Pumped by air or water, tubes became separated from each other in the middle.

In addition to this type of guard cells, which have thin dorsal and thick ventral walls, several types of the stomata that differ by the wall thickening and guard cell movements were described (Schwendener 1881; Haberlandt 1924; Guttenberg 1959).

Deformation of the guard cells leading to the stomatal pore opening may depend also on the geometry of their cross sections. Usually, guard cells are elliptical in section. If they are thin-walled, they assume circular shape in cross section as a result of increasing of the internal turgor pressure. The cell walls adjacent to the pore become less curved and the pore opens (Meidner and Mansfield 1968). Therefore, the elliptical shape of the guard cells plays an important role in stomata functioning as valves (Cooke et al. 2008). This hypothesis was verified by the finite-element shell analysis (Cooke et al. 1976, 2008). The model developed by Cooke et al. (1976) is correct for stomata with thin-walled and elliptical guard cells. Such stomata are characteristic of some plants growing in moist environments, where periods of water stress are unknown or very infrequent (DeMichele and Sharpe 1973).

Ziegenspeck (1938, 1941, 1955), utilizing polarization microscopy, discovered radial micellation of cell walls of guard cells. The micelles of cellulose belt the guard cell perpendicular to its longitudinal curved axis (Sharpe et al. 1987) and fan out radially from the pore to the periphery of the stoma (Raschke 1975) (Fig. 1). They prevent large deformation of the guard cell cross section at higher turgor pressure within it. The guard cells can stretch mainly in the direction of their longitudinal axes (Raschke 1975). However, their ventral walls contain longitudinal micelles
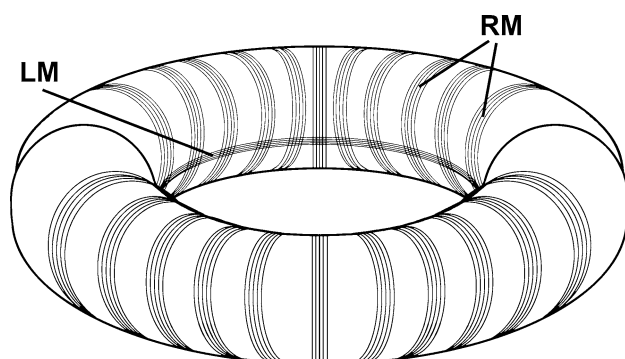
limiting its extension (Fig. 1). The increased turgor pressure forces the guard cells to bend laterally and the pore opens. These mechanisms involved in stomatal opening were illustrated by the experimental simulation using latex balloons (Aylor et al. 1973). Bands of tape on the balloons simulated radial micellation (Aylor et al. 1973). These experiments were supplemented by an illustrative mathematical modelling.

In the opinion of Aylor et al. (1973), the uneven thickening of the walls is not necessary for the opening of the stomata. This assertion dominates now. The point of view about the influence of the wall thickness even is not mentioned in some textbooks (for example, Sitte et al. 2002). There are, however, data showing that thickening of the walls affects stomatal movements and gas exchange (Franks and Farquhar 2007).

All guard cells have ledges formed by cuticle or wall material that is covered with a cuticle (Esau 1960; Wilkinson 1979; Evert 2006). They are located on upper or both the upper and the lower sides of the guard cells not far from the stomatal pore. Von Mohl (1856) suggested that the ledges must influence changes in shape of the turgid guard cell. In the opinion of Vaihinger (1941), the thick ledges prevent excessive opening of the stomatal pore, and may influence the direction of the guard cell movement. According to Raschke (1975), it is questionable whether the cuticular ledges take part in stomatal opening. The modern notions about the mechanics of guard cells movements do not consider the presence of the ledges on these cells. Our study aims to elucidate the role of the guard cell ledges on stomatal mechanics in woody plants which have these structures particularly well developed, as well as to evaluate their biological and evolutionary significance. The research goals are (1) to investigate distinct structural features of stomatal complexes having large outer ledges; (2) to determine the impact of these ledges on stomatal movements using dynamic modelling; (3) to classify the structural type of leaves possessing such stomatal complexes; and (4) to compare the obtained results with the existing views on functions of stomatal ledges.

Materials and methods

Plant material

Methods of light, scanning, and transmission electron microscopy were used to study the stomata of the leaves in evergreen: *Carissa spectabilis* (Sond.) Pichon (Apocynaceae), *Acokanthera oblongifolia* (Hochst.) Codd (Apocynaceae), *A. oppositifolia* (Lam.) Codd (Apocynaceae), *Exbucklandia populnea* (R.Br. ex Griff.) R.W. Brown (Hamamelidaceae), and *Trochodendron aralioides*

Fig. 1 Diagram of arrangement of cellulose microfibrils in the guard cell walls. *RM* radial microfibrils, *LM* longitudinal microfibrils

Sieb. et Zucc. (Trochodendraceae). The choice was determined by the presence of the large stomatal outer ledges in these species (Bailey and Nast 1945; Bondeson 1952; Pautov et al. 2016). All plant material was collected in the Botanical gardens of St. Petersburg State University and the Komarov Botanical Institute (St Petersburg, Russia) and in the botanical garden "Southern Plants" (Adler, Russia).

The studied species are recorded in the floras of South Africa (*A. oblongifolia*, *A. oppositifolia*, and *C. spectabilis*), South and South-East Asia (*E. populnea*), and also in eastern Asia (S. Japan, Taiwan, S. Korea) (*T. aralioides*) (Dyer et al. 1963; Takhtajan 1980; Leeuwenberg et al. 1985; Fu and Endress 2001; Zhang et al. 2003). Their representatives are found in various habitats. *A. oppositifolia* occurs, in particular, on exposed rocky slopes, in scrub forests, along watercourses and in coastal bushes (Dyer et al. 1963). *A. oblongifolia* and *C. spectabilis* are found in sclerophyllous maquis on litoral dunes and woodlands not far from the coast (Dyer et al. 1963; Leeuwenberg et al. 1985). These species are very similar morphologically. Their names are treated as synonyms, by some regional authors (Leeuwenberg et al. 1985). We found that the stomata of *A. oblongifolia* differed from those of *C. spectabilis* by the presence of stomatal plugs (Fig. 2a, c). *E. populnea* inhabits mountain evergreen forests up to 1200 m (Zhang et al. 2003). *T. aralioides* grows in mountain beech, mixed coniferous—broad-leaved and broad-leaved evergreen forests on the heights ranging from 300 to 2700 (3000) m (Takhtajan, 1980; Fu and Endress 2001).

We sampled leaves from three individual plants of *A. oblongifolia* and *E. populnea*, two of *C. spectabilis*, and one of *A. oppositifolia* and *T. aralioides*. All plants except *T. aralioides* were grown in greenhouses. These plants are small trees up to 6 m height. Some of them were grown in pots. The others (*E. populnea* and *A. oblongifolia*) were transplanted from pots in greenhouse soil five to ten years ago. Most of the plants were more than 50 years old. *A. oblongifolia*, *C. spectabilis,* and *T. aralioides* have reached their reproductive period. *A. oblongifolia* and *T. aralioides* had both flowers and fruits, and *C. spectabilis* had flowers only. The leaves were collected in the crown periphery, from its middle part. Three leaves from each tree were taken for anatomical study. For the species presented in collections by one or two trees only, we used leaves from 3- to 5-year-old seedlings to increase the sampling size to 3 individuals per species. Three leaves were also collected from each of the seedlings.

Leaf anatomy

The fragments of the leaf epidermis were obtained using the maceration method (Kerp 1990). The specimens of freshly collected leaves were placed into the mixture of full-strength nitric acid (HNO3) and potassium chlorate (KClO3) for 1–1.5 h, then washed by distilled water. Next, the samples were kept in the mixture of potassium chloride (KOH) and distilled water for 30–60 min, then washed by distilled water. Separation of upper and lower epidermises was done using preparation needle under stereomicroscope Leica EZ4 (Germany). We used safranin for staining cell walls of epidermis. The stomatal complexes were studied using light microscope Leica DM1000 (Germany).

The fragments of freshly-collected leaves were prefixed in modified Karnovsky's mixture of 3 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer, (pH 7.4) (Karnovsky 1965). They were preserved at 4–6 °C for no more than 7 days. Then, they were rinsed in phosphate buffer and fixed in 2 % osmium tetroxide. The material was dehydrated in acetone series and embedded in epon-araldite mixture (Mollenhauer 1964; Kuo 2007). Ultrathin sections were made by Ultracut E ultramicrotome (Reichert-Jung) and stained with Reynolds' lead citrate (Reynolds 1963). The ultrathin sections of leaves were studied and photographed using transmission electron microscopes (TEM): Tesla BS-500 (Czech Republic) and Zeiss Libra 120 (Germany).

For the scanning electron microscopy (SEM) fragments, freshly collected leaves were fixed with 3 % glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4). They were kept at 4–6 °C for no more than 30 days. Then, the material was dehydrated in ethanol and isoamyl acetate series, critical-point dried in liquid CO2 (Sargeant 1983; Pathan et al. 2010), mounted on aluminum stubs, sputter coated with a layer of gold (70–100 nm), and observed with SEM JSM-6390LA (USA).

Modelling of stomatal movements

Partial differential equations (PDEs) arise in mathematical modelling of many physical, chemical, or biological phenomena. Often the equations under consideration are too complex for finding their solutions by analytical means and one has to resort to seeking numerical approximations to the unknown analytical solution. The finite-element method (FEM), sometimes called finite-element analysis (FEA) is a numerical technique, which provides approximate solution for PDEs (Basov 2005; Lawrence 2006; Madenci and Guven 2006). To use FEA framework to study displacements and stresses at each material point caused by a force applied on an object, the geometry of the object is subdivided or discretized into small but finite regions—elements, for which the governing differential equations can be developed. For each element, a proper simple function is used to approximate the true function governing the deformation of the object. These algebraic equations,
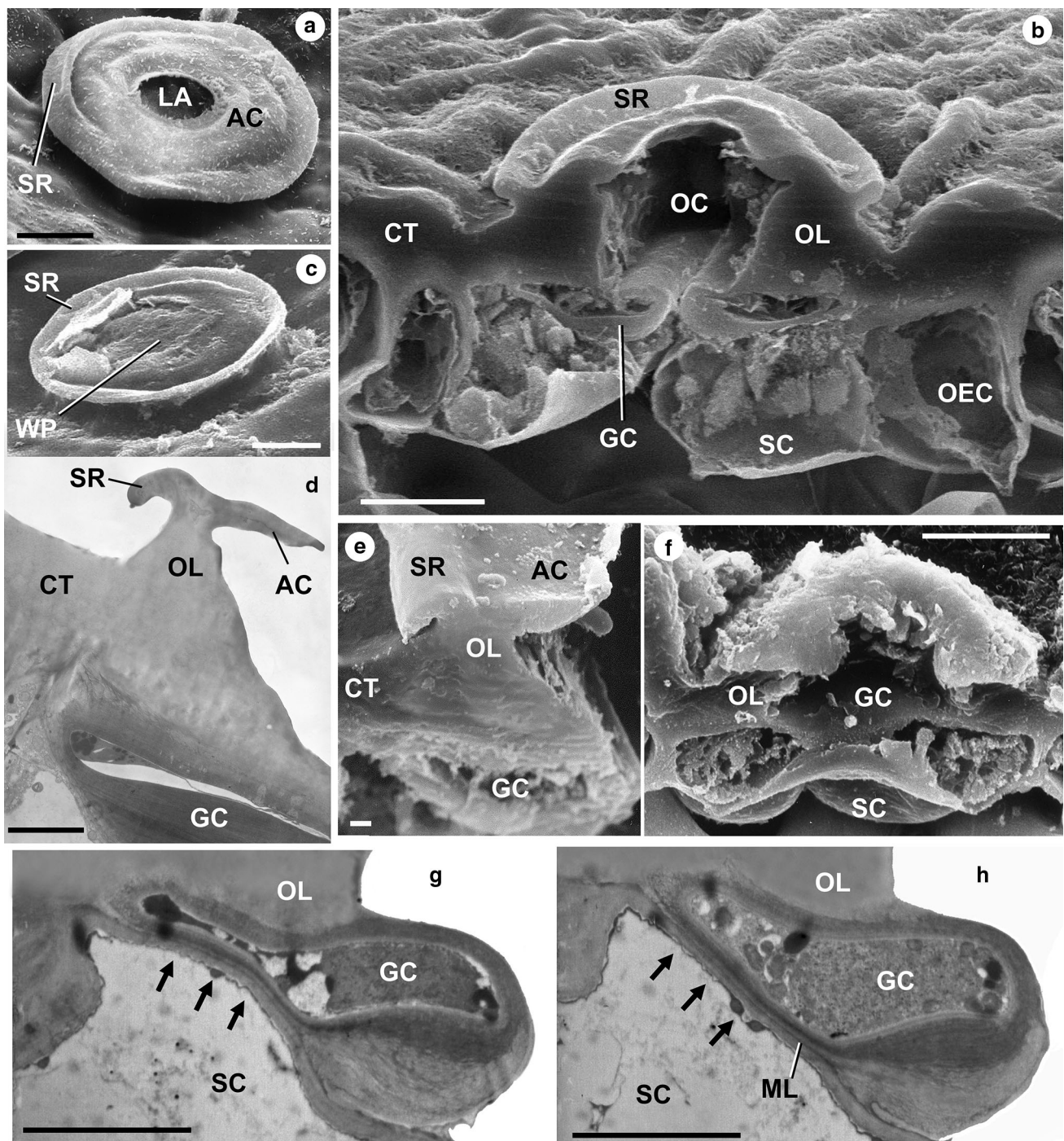
Fig. 2 Stomata of *Carissa spectabilis* (**a**, **e**), *Exbucklandia populnea* (**b**), *Acokanthera oblongifolia* (**c**), *Acokanthera oppositifolia* (**d**), *Trochodendron aralioides* (**f**–**h**). **a**, **c** SEM micrograph of stomata. **b** Cross section of stoma under SEM. **d**, **e** Transverse section of outer stomatal ledges under TEM (**d**) and SEM (**e**). **f** Longitudinal section of guard cell under SEM. **g**, **h** Cross sections of guard cells under TEM (bordering with each other and thin walls of guard and subsidiary cells, where the maximal movements take place, are marked by the *black arrows*). *SR* stomatal rim, *LA* outer stomatal ledge aperture, *AC* arch of the outer cavity, *OL* outer ledge, *OC* outer cavity, *GC* guard cell, *SC* subsidiary cell, *OEC* ordinary epidermal cell, *WP* waxy plug, *CT* cuticle, *ML* middle lamella. *Scale bars* **a**–**c**, **f** = 10 μm; **d**, **g**, **h** = 5 μm; and **e** = 1 μm

representing a finite-element mesh, are numerically solved for the unknowns at nodes. It means that computed quantities include local deformations in response to the applied force. Commercial finite-element packages (ANSYS, Comsol, MSC/NASTRAN, MSC/MARC, ABAQUS, etc.) greatly facilitate the analyses allowing for automated

calculations. The user should only accurately describe the geometry and assign the material properties for all components of the structure. The finite-element shell analysis of stomatal mechanics was implemented using modelling in the ANSYS software complex (ANSYS v.13.0).

The stomata of the studied species have identical structure. They are united by the presence of the well-developed outer ledges and similar wall thickening in the guard cells. In modelling, we took into account the shape of the guard cells, uneven thickness of their walls, positioning, and size of the outer ledges. Obtained geometric data were used as parameters for the construction of the finite-element models.

The biological tissues, including plant cell walls, are usually heterogeneous and anisotropic (Wu and Sharpe 1979; Sharpe et al. 1987). The mechanical parameters which characterize the degree of heterogeneity and anisotropy are difficult to measure. In accordance with the traditional approach to the studying of the stoma mechanics (DeMichele and Sharpe 1973; Sharpe et al. 1987), we assumed that the cell material is isotropic, homogenous, and obeys the Hook's law. Since the linear law of elasticity is adopted, the cells deformations are proportional to p/E value, where p is the normal pressure, E—modulus of cell wall elasticity. In the computations, it was taken that Poisson's ratio equals 0.48 (Sharova 2004). When a material is compressed in one direction, it usually tends to expand in the other two directions perpendicular to the direction of compression. Poisson's ratio is a measure of this effect. The turgor pressure was simulated by creating the load distributed on the inner surface of the guard cells. Due to the bilateral symmetry of the stoma, only one half of the guard cell is modelled (Fig. 3a).

Modelling steps:

1. *Construction of 3D model* The shape of the guard cells, uneven thickness of their walls, localization of the outer stomatal ledges on these cells, the relative sizes of guard cells, and ledges established through the analysis of the real stomata (Fig. 2f, g) were exactly incorporated using the ANSYS functions when developing the models (Fig. 3).
   To estimate the effect of the outer ledges of the guard cells on stomatal mechanics, the following three models were constructed:
   Model 1 The guard cells with the walls of uneven thickness without the outer ledges (Fig. 3b).
   Model 2 The guard cells with the walls of uneven thickness and the outer ledges. The cell walls and ledges have equal elastic modulus, i.e., they have similar mechanical characteristics (Fig. 3c).
   Model 3 The guard cells with the walls of uneven thickness and the outer ledges. The elastic modulus of the ledges is larger than modulus of the guard cell walls, i.e., the material of ledges is more rigid (Fig. 3d).

2. *Choice of finite-element types* Element choice in ANSYS library depends on geometry and physical characteristics of an object as well as on the goals of modelling. The following types of finite elements were used: volumetric three-dimensional elements SOLID95, SOLID45, and plane two-dimensional element PLANE42.

3. *Subdivision of the guard cells into finite elements* The FEM solution is approximate, but with increasing the number of elements, its accuracy increases. We selected the number of elements in such a way to provide the highest accuracy. The guard cell walls were subdivided into 3000 elements (Fig. 4). With the increase of the number of elements, the resulting deformations of the guard cell did not change. The points where a set of finite-element meets are called FEM nodes (Fig. 4).

4. *Evaluation of modelling* The deformations of the turgid guard cells were assessed. As the result of analyses the following characteristics of the three models were obtained: deformation patterns of the guard cells, isolines of the total displacement of their walls, and displacement of the segment OB bordering the stomatal pore (Fig. 4). The point "O" of the segment OB corresponds to the center of symmetry of the closed stoma. The positive displacement of the segment OB along X axis indicates that the stomatal pore is opening; along Y—that the pore is moving upwards or downwards with the reference to its initial position where the stoma is closed.

Results

Morphomeric analysis of leaves

The leaves of all studied species are simple. They have, in general, very similar anatomic structure. Blades of *A. oblongifolia*, *A. oppositifolia*, *C. spectabilis*, and *T. aralioides* have an average area of 30 cm^2, in *E. populnea*— 150 cm^2. Leaves are thick. The blade thickness is 300–370 μm in *E. populnea*, *T. aralioides*, *A. oblongifolia*, *C. spectabilis*, and circa 450 μm in *A. oppositifolia*. The number of cell layers in the mesophyll varies from 9–10 in *E. populnea* and *T. aralioides* to 14–15 in *A. oppositifolia*. Leaf blades in all species are dorsiventral. The ratio of palisade tissue thickness to the mesophyll thickness is 20–30 % in *A. oblongifolia*, *A. oppositifolia*, *C. spectabilis*, *E. populnea*, and 47 % in *T. aralioides*. As it was already noted (Bailey and Nast 1945; Golysheva 1976), mesophyll

Fig. 3 3D volumetric reconstructions of the half of the guard cells. **a** General view. **b–d** isolines of the total displacement of guard cell walls after application of the load distributed on the inner surface: **b** Model 1. The guard cells with the walls of uneven thickness without the outer ledges. **c** Model 2. The guard cells with the walls of uneven thickness and the outer ledges. The cell walls and ledges have equal elastic modulus, i.e., they have similar mechanical characteristics. **d** Model 3. The guard cells with the walls of uneven thickness and the outer ledges. The elastic modulus of the ledges is larger than modulus of the guard cell walls, i.e., the material of ledges is more rigid. The state of cells is shown before (*dotted line*) and after their distortion (*solid line*). On the scales, the values of displacements are indicated (μm)
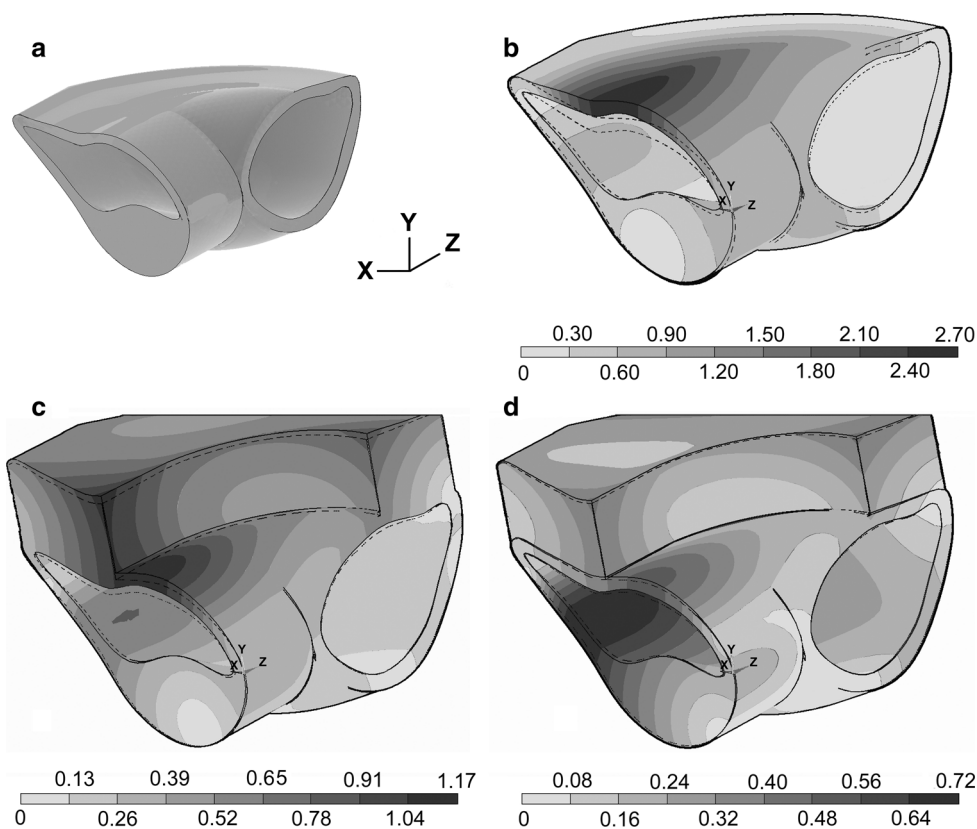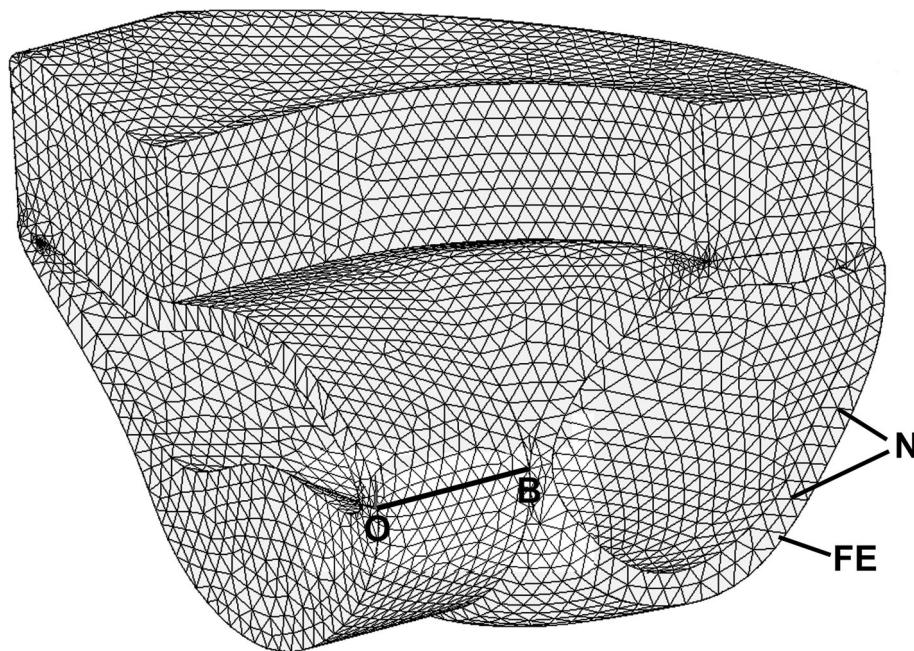

Fig. 4 Subdivision of the guard cell into finite elements (*FE* finite element, *N* finite-element model (FEM) nodes, *OB* segment bordering the stomatal pore). Displacements of the FEM nodes lying on segment OB were used for the evaluation of the stomatal pore movements (Fig. 5)

of *T. aralioides* contains astrosclereids. The leaf blades are covered with thick cuticle (Fig. 2b, d, e).

Leaves are hypostomatic. An average stomatal index in studied leaves is 4–5 % in *A. oblongifolia*, *A. oppositifolia*,

C. spectabilis, *E. populnea,* and 11 % in *T. aralioides*. The average number of stomata per 1 mm² is 100–120 in *A. oblongifolia*, *A. oppositifolia*, *C. spectabilis*, *E. populnea*, and circa 200 in *T. aralioides*.
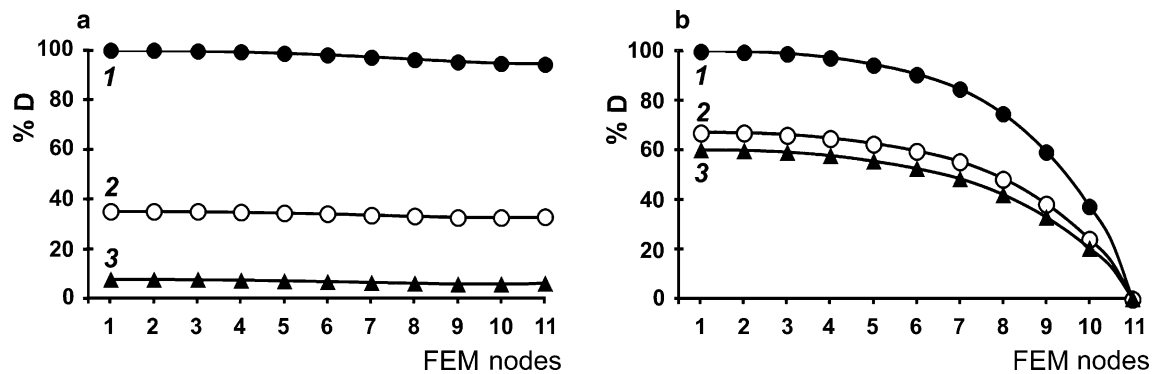
Fig. 5 Displacement of the segment OB bordering the stomatal pore along Y (**a**) and X (**b**). Abscissa displays finite-element model (FEM) nodes of the segment OB bordering the stomatal pore (node 1—"O", node 11—"B" according to Fig. 4). Ordinate is the percentage of displacement (% D) taken from maximum displacement in the Model

1. Numbers along the lines correspond to the model number. Positive displacement of the segment OB along Y axis indicates vertical movement of the opening stomatal pore above the leaf surface as compared with its initial position when stoma is closed; along X—the width of the stomatal pore opening

Morphomeric analysis of stomata

The stomata of *C. spectabilis*, *A. oblongifolia*, *A. oppositifolia,* and *E. populnea* are cyclocytic. They are usually laterocytic in *T. aralioides*. The guard cells of all studied plants are located on subsidiary cells (Fig. 2b, g, h). As a result of such disposition, the stomata are upraised above the epidermis surface (Fig. 2a, c). The number of subsidiary cells is 6–8 in *C. spectabilis*, *A. oblongifolia*, *A. oppositifolia*, *E. populnea,* and 3–5 in *T. aralioides*. There are ordinary epidermal cells on the stoma poles of the last species. The average length of the stomata varies from 34 μm (*E. populnea* and *T. aralioides*) to 41.5 μm (*C. spectabilis*). The stomata have large outer cuticular ledges (Fig. 2b, d, e). The sizes of the cross sections of both ledges and guard cells are comparable. Pairs of stomatal ledges form walls and the arch of the outer cavity (Fig. 2b). The arch is perforated with a narrow outer stomatal ledge aperture (Fig. 2a). The stomatal pore lies on the bottom of the outer cavity. There are outer stomatal rims on the outer ledges in *A. oblongifolia*, *A. oppositifolia*, *C. spectabilis*, and *E. populnea* (Fig. 2a–e). The inner ledges are usually absent. Sometimes, there are their rudiments on the walls of the guard cells. The cavities of *A. oblongifolia* are filled with waxy plugs (Fig. 2c).

The guard cell wall is thickened unevenly. The inner tangential wall bordering with stomatal pore is very thick (Fig. 2g, h). In contrast to the former, the other part of this wall is the thinnest. The thin part of the tangential wall is connected with subsidiary cell walls by middle lamella (Fig. 2h). For example, in *T. aralioides,* the average thickness of the wall part bordering with pore is 3.7 μm (from 45 cells) and the wall part connecting the subsidiary cells is 0.4 μm (from 20 cells). The walls of the subsidiary cells contiguous with thin walls of the guard cells are also very thin. Their average thickness is less than 0.3 μm

(from 20 cells) in *T. aralioides*. For comparison, the thickness of subsidiary cell walls turned towards the intercellular spaces reaches more than 1.5 μm. No differences in the structure of stomata were found between the leaves of mature plants and those of the seedlings of the same species.

Finite-element shell analysis of stomatal mechanics

The geometry of the real stomata (Fig. 2f, g) was used for modelling the mechanics of the guard cells movements (Fig. 3). The models were constructed to estimate effect of the outer ledges on the deformation of the guard cells during opening of the stoma.

Model 1 The guard cells with the walls of uneven thickness without the outer ledges.

When the intracellular pressure increases, the swelling guard cells are bending outwards (Fig. 3b). The opening stomatal pore moves in the same direction above the leaf surface (Fig. 5a). The main movements in the opening stoma are localized in the central parts of the outer walls of the guard cells. In contrast, the weak movements are localized in the inner tangential walls mostly on the stomatal poles as well as in the thickened part bordering with stomatal pore.

Model 2 The guard cells with the walls of uneven thickness and the outer ledges. The cell walls and ledges have equal elastic modulus.

Addition of the outer ledges to the simulations leads to a decrease in the movements of the outer cell walls and increase in the movements of the inner walls (Fig. 3c). Both vertical and lateral movements of the opening stomatal pore are less than in the previous model (Fig. 5a, b).

Model 3 The guard cells with the walls of uneven thickness and the outer ledges. The elastic modulus of the ledges is larger than modulus of the guard cell walls.

The maximal movements take place in the inner walls that border subsidiary cells (Fig. 3d). The stomatal pore has little vertical movements during its opening (Fig. 5a). The width of the open stomatal pore is, in this model, narrower than in the previous models (Fig. 5b).

The modelling showed that outer ledges limit both vertical movements and the width of the stomatal pore. The more the stiffness of the ledges, the less the movements of the pore (Fig. 5). The results of the modelling are supported by the observed deformations in the guard cells of the real stomata. As shown by the models 2 and 3 (Fig. 3c, d), in stomata having large outer ledges, the movements increase in the inner walls of the guard cells (Fig. 2g, h).

Discussion

We have shown that the leaves of *A. oblongifolia*, *A. oppositifolia*, *C. spectabilis*, *E. populnea,* and *T. aralioides* have specific stomatal complexes. Their guard cells are located on subsidiary cells, have large outer ledges, and lack inner ledges. The outer ledges form stomatal cavities. The application of dynamic modelling has established that large outer ledges influence the movements of guard cells. For stomata having no ledges, the modelling has demonstrated that the turgid guard cells, located on subsidiary cells, are bended above the leaf surface. The opening pore also moves above the leaf surface. The outer ledges constrain these movements and cause the sinking of the inner tangential walls of the guard cells into the subsidiary cells. The ledges prevent wide opening of the stomatal pore and its lifting above leaf epidermis. Such stomatal mechanics has biological and evolutionary importance.

All studied species are evergreen plants with leaves having similar anatomy. They are thick and hypostomatic. Their mesophyll is dorsiventral. It consists of many layers of cells. Most of the mesophyll volume are taken up by spongy tissue. This tissue includes from 7–9 (*E. populnea*) to 11–13 (*A. oppositifolia*) cell layes. Thus, the mesophyll of these species has a mesomorphic structure. The formation of their leaves in the evolution was accompanied by the xeromorphis of the epidermis. Several xeromorphic features of this tissue were described (e.g., Haberlandt 1924; Bissing 1982; Gamalei 1988; Jordan et al. 2008) and include: thick cuticle, disposition of the stomata within the cavities, or sinking of the stomata into leaf tissues and stomatal plugs. These characters were found in various taxa in arid habitats. The aforementioned features were recorded in all studied species. Among these adaptations are occurrence of thick cuticle covering the ordinary cells (Fig. 2b), stomatal cavities (Fig. 2a, b), and waxy plugs (*A. oblongifolia*) (Fig. 2c), stomatal mechanics that prevents lifting of the opening stomatal pore above the leaf surface (Fig. 5a).

The same structural features of leaves are observed in the sclerophyllous plants of the Mediterranean climate areas (Walter 1974). The evergreen sclerophyllous taxa are connected in their origin with subhumid tertiary laurophyllous forests (Axelrod 1975). Many of these structural features are likely to have originated, according to Axelrod (1975), in ancestral taxa inhabiting relatively warm and moderately mesic conditions. They proved to be preadaptive to the climates with lower rainfall, shorter rainfall periods, and greater ranges of temperatures. The aridization of climate led to selection for xeromorphic structures of the epidermis in the areas of low precipitation and high evaporation to reduce water loss. The structure of epidermis depends not only on environmental conditions but also on the vascular tissue structure. Xeromorphosis of the epidermis, including the cavities and plugs presence, is typical of dicotyledons, having primitive xylem (Bailey and Nast 1945). Among studied species, *T. aralioides* has such xylem. This species was considered earlier as lacking vessel elements in the secondary xylem (Bailey and Thompson 1918; Bailey and Nast 1945; Smith 1945; Carlquist and Schneider 2002). According to Li et al. (2011), *Trochodendron* has primitive vessel elements. They are very long and have oblique end walls with scalariform perforation plates having many bars. Such secondary xylem conducts the water slowly (Kramer and Kozlowski 1979). It is suggested that structural peculiarities reducing transpiration compensate for slow water conduct to the transpiring leaves.

The role of the stomatal cavities and plugs is debatable. The cavities may be formed in dicotyledons not only by outer ledges, but also by cuticle of the cells surrounding the stoma, by these cells themselves, or may even include deeper lying tissues (Wilkinson 1979; Carr and Carr 1980; Van Wyk et al. 1982; Grace et al. 2009). It is considered that they slow down the water loss (Meidner and Mansfield 1968; Roth-Nebelsick et al. 2013). Some studies demonstrate that the reduction of the water loss by these structures is not always considerable or take place at all. For example, the modelling showed that in leaves of *Banksia ilicifolia* with open or partially closed stomata, crypts reduce transpiration by less than 15 % compared with non-crypted, surface positioned stomata (Roth-Nebelsick et al. 2009). In accordance to Gharun et al. (2015), the water loss of montane eucalypts was not directly related to the coverage of the stomatal complexes by outer cuticular ledges. *Eucalyptus pauciflora* displaying largest ledges and narrow outer stomatal ledge apertures had the greatest measured stomatal conductance. These measurements were, however, conducted during the period of relatively abundant soil water.

There is an opinion that these structures not as much reduce the water loss as maintain the stomatal function in

dry air in the presence of adequate water supply by root systems. It is possible that they prevent stomatal closure by keeping the high humidity at the guard cell surface (Feild et al. 1998). Experimental reduction of the relative humidity from approximately 85–40 % led to 20 % decrease of the stomatal conductance in *Drimys winteri* leaves having plugs. In contrast, leaves without plugs showed a 70 % decrease in conductance of the water vapor.

Both ledges and plugs decrease the wettability of stomatal pores and penetration of water in them (Turrell 1947; Schonherr and Bukovac 1972). This facilitates gas exchange in woody plants during the periods of rainfall as well in wet environments, because their leaves frequently encounter rain and mist. The sclerophylls occupy now diverse environments, including ever-wet tropical rainforests (Axelrod 1975).

Thus, it was suggested by several studies that the large outer ledges can reduce the stomatal transpiration, lower sensitivity of stomata to vapor pressure deficit in the presence of adequate water supply by root systems, and limit the penetration of water into the stomata. The expression of these functions could be species specific. The results of our modelling provide important additional insights into the ledge functions. The modern concepts of the stomatal mechanics do not consider the presence of the ledges on the guard cells. In contrast, our modelling results showed decisive influence of the outer ledges on the guard cell movements. The discovered mechanism takes place in stomatal complexes with guard cells lying on subsidiary cells, i.e., on the leaf surface. The outer ledges limit the opening of stomatal pore and its lifting above the leaf surface (Fig. 5). We consider that determined by the development of outer ledges, stomatal mechanics and outer cavities were formed simultaneously in evolution, supplementing each other. It is possible that during the initial stages of xeromorphosis of the epidermis, the main role belonged to the movement mechanics, at the final stages—to the formed cavities. We found such stomatal mechanics in the families, which according to APG III system (Bremer et al. 2009), belong to the Basal Eudicots: Trochodendrales (*T. aralioides*), Core Eudicots: Saxifragales (*E. populnea*), and Asterids: Gentianales (*C. spectabilis*, *A. oblongifolia*, and *A. oppositifolia*). Consequently, it occurs not in a limited taxonomic group, but in several groups occupying different positions in the flowering plant system. This means that discovered by us, stomatal mechanics either has an ancient origin or has arisen in evolution repeatedly and independently in several taxa.

Author contribution statement A.P. formulated problem, conducted TEM imaging and ultra-structural analyses of stomatal complexes, interpreted results, and produced the draft version of the text. S.B. designed modelling. O.I.

conducted modelling. E.K. conducted measurements and morphometric analysis of stomatal complexes, SEM imaging, and preparation of illustrations. Y.S. conducted measurements and morphometric analysis of leaves. G.G. discussed and formulated the results together with A.P. All co-authors discussed and commented on the text.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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