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Research article

Micromorphology and development of the epicuticular structure on the epidermal cell of ginseng leaves

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ABSTRACT

Background: A leaf cuticle has different structures and functions as a barrier to water loss and as protection from various environmental stressors.
Methods: Leaves of *Panax ginseng* were examined by scanning electron microscopy and transmission electron microscopy to investigate the characteristics and development of the epicuticular structure.
Results: Along the epidermal wall surface, the uniformly protuberant fine structure was on the adaxial surface of the cuticle. This epicuticular structure was highly wrinkled and radially extended to the marginal region of epidermal cells. The cuticle at the protuberant positions maintained the same thickness. The density of the wall matrix under the structures was also similar to that of the other wall region. By contrast, none of this structure was distributed on the abaxial surface, except in the region of the stoma. During the early developmental phase of the epicuticular structure, small vesicles appeared on wall–cuticle interface in the peripheral wall of epidermal cells. Some electron-opaque vesicles adjacent to the cuticle were fused and formed the cuticle layer, whereas electron-translucent vesicles contacted each other and progressively increased in size within the epidermal wall.
Conclusion: The outwardly projected cuticle and epidermal cell wall (i.e., an epicuticular wrinkle) acts as a major barrier to block out sunlight in ginseng leaves. The small vesicles in the peripheral region of epidermal cells may suppress the cuticle and parts of epidermal wall, push it upward, and consequently contribute to the formation of the epicuticular structure.

1. Introduction

The cuticle is an extracellular hydrophobic layer consisting of cutin and wax. It is deposited on the outer surface of epidermal cells of all land plants and has a primary role in water regulation, self-cleaning behavior, light reflection at the cuticle interface, and protection from biotic and abiotic stressors such as herbivores, pathogens, UV-B radiation, and high temperature [1–6]. A possible role for the cuticle as a suppressor of organ fusion early in organogenesis, and a connection between the cuticle and fertility have been described [7]. Various studies of the cuticle with diverse morphological structures and chemical compositions have been reported in different species [8–14]. Onoda et al [15] revealed that the leaf cuticle varies for > 100 times across species, and that a thicker cuticle is more resistant to tearing. They concluded that the thickness of the cuticle probably affects increasing mechanical resistance, and subsequently may confer a longer leaf lifespan among evergreen species. The cutin composition and fine structure of the cuticles were substantially changed during the growth and development of plant organs [16].

Epicuticular wax, which is the outermost layer of the cuticle, may be amorphous or may possess a crystalline structure or platelets. Modification or partial removal of epicuticular waxes by γ-irradiation reduced the barrier properties of the cuticle. Therefore, epicuticular waxes may be a major determinant of cuticle permeability [17,18]. Through anatomical and physiological

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changes, leaves generally acclimate to sun and shade conditions for photosynthesis [19]. Because most ginseng species are shade plants, their leaves may easily experience serious leaf burn under direct sunlight because of the simple structure of the epidermis. However, a few cultivars of ginseng have leaves that are healthier under sunlight [20]. The relationship between the epidermal structure of the leaf and light sensibility is largely unknown. The objectives of this study were to determine the characteristics and development of epicuticular structure and to correlate fine structure with leaf burning in ginseng leaves.

2. Materials and methods

2.1. Scanning electron microscopy

The five cultivars of *Panax ginseng*—Gopoong, Geumpoong, Sunpoong, Yunpoong, and Chunpoong—were provided by KT & G Central Research Institute (Daejeon, Korea). For scanning electron microscopy (SEM), 4-year-old ginseng leaves were fixed in 2% glutaraldehyde in 25mM phosphate buffer (pH 7.1) for 2 hours and dehydrated in a graded ethanol series, and placed in isoamylacetate. Critical point drying (Bioradical E3000 dryer; Nakahara, Tokyo, Japan) was performed using liquid carbon dioxide at 1000 psi. The dried specimens were mounted on the stubs, and a 180-nm coating of gold was applied with a sputter coater (JFC 1110E; JEOL Ltd., Tokyo, Japan). The results were observed with a scanning electron microscope (Hitachi S 3500N; Instruments, Hitachi, Ltd., Tokyo, Japan) operated at 20 kV.

2.2. Transmission electron microscopy

The ginseng leaves were also fixed in 4% (v/v) glutaraldehyde in 25mM phosphate buffer (pH 7.1) for 2 hours, and postfixed in 2% (w/v) osmium tetroxide solution in same buffer for 1 hour. Dehydration was accomplished in a graded ethanol series. The samples were placed in propylene oxide prior to further treatment, and then embedded in a Spurr mixture. Semithin sections were cut with a glass knife and ultramicrotome (Reichert Ultracut S, Vienna, Austria). They were then stained with toluidine blue and basic fuchin for preliminary screening with a light microscope. Ultrathin sections approximately 70 nm thick were cut with a diamond knife, and then stained on copper grids with 1% (w/v) uranyl acetate and

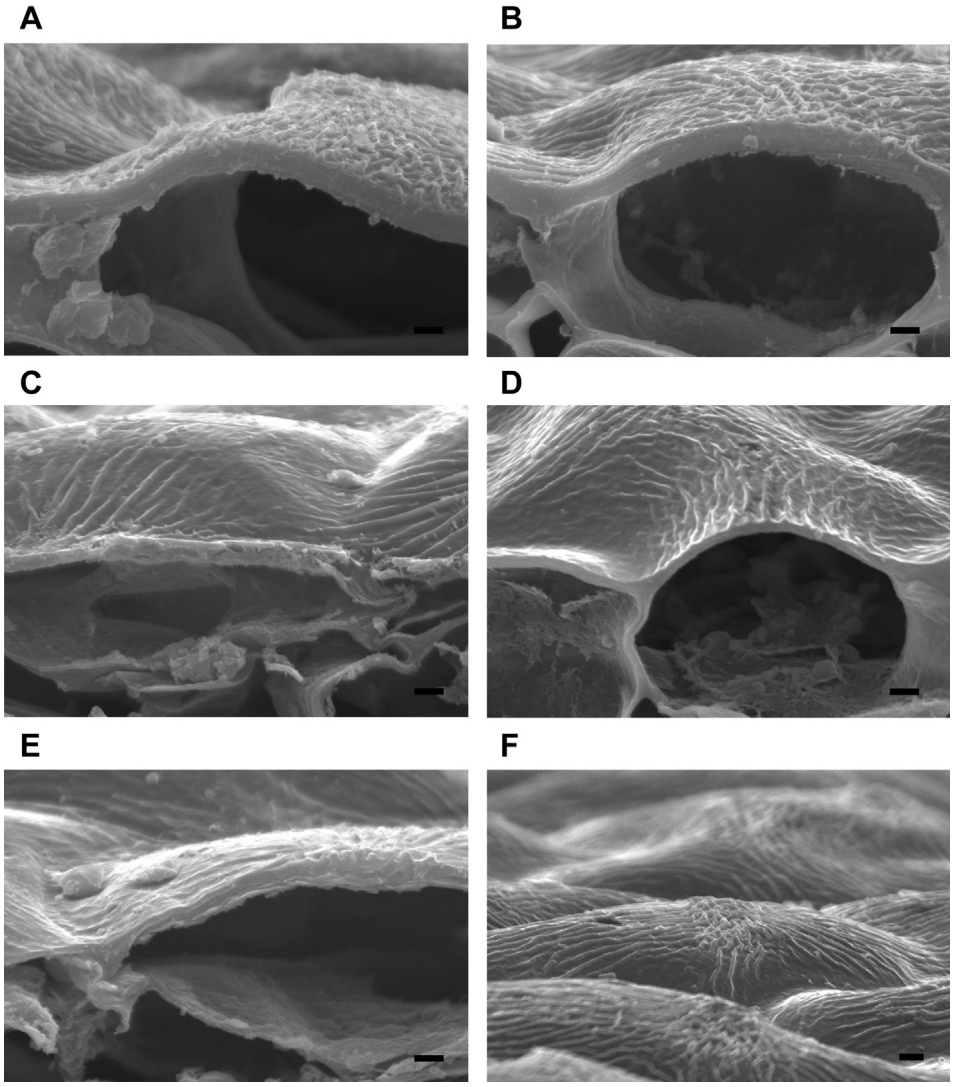

Fig. 1. Scanning electron microscopy (SEM) micrographs of epidermal cells on the adaxial leaf surfaces of ginseng cultivars. A longitudinally sectioned epidermal cells show the wrinkled epicuticular structure on the adaxial surface and epidermal wall of (A) Gopoong, (B) Geumpoong, (C) Sunpoong, (D) Yunpoong, and (E) Chunpoong. (F) The epidermal cell surface shows a radial arrangement of the epicuticular structures of Sunpoong. Bars, 2 μm (A–E) and 1 μm (F).

lead citrate [21]. They were examined with a transmission electron microscope (JEM 2000 EX II; JEOL Ltd.) operated at 80 kV.

2.3. Statistical analysis

For statistical measurement of the thickness of the epidermal cell wall and cuticular layers, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Micromorphology of the epicuticular structure

The adaxial surface of the leaves of the five ginseng cultivars appeared flat and entirely lacked trichomes and stomata when examined by SEM. The thickness of the epidermal cell wall on the adaxial surfaces was 1.1 ± 0.1 μm in Gopoong, 1.4 ± 0.1 μm in Geumpoong, 2.6 ± 0.2 μm in Sunpoong, 1.9 ± 0.1 μm in Yunpoong, and 2.7 ± 0.1 μm in Chunpoong. By contrast, the thickness of the epidermal cell wall on the abaxial surfaces was 1.4 ± 0.2 μm in Gopoong, 1.3 ± 0.1 μm in Geumpoong, 0.9 ± 0.1 μm in Sunpoong, 1.4 ± 0.2 μm in Yunpoong, and 1.4 ± 0.1 μm in Chunpoong (Fig. 1A–1E). The epicuticular structure, which is situated high on the central region of epidermal cells, uniformly appeared on the adaxial surface of the cell, and radially extended to the marginal region of the epidermal cell. The epicuticular structure often occurred on the abaxial surface of flanking subsidiary cells in all cultivars. The epicuticular structure of all ginseng cultivars showed mostly the same pattern. The thickness of the cuticle on the adaxial surface was 134.3 ± 2.6 nm in Gopoong, 134 ± 3.5 nm in Geumpoong, 143.5 ± 2.0 nm in Sunpoong, 115 ± 1.8 nm in Yunpoong, and 133.6 ± 2.3 nm in Chunpoong (Fig. 1A–1E). At higher magnification, the epicuticular structure was observed on the adaxial surface, but not on abaxial surface, except around the stomata (Fig. 1F). The epidermal cells had distinctively thickened periclinal walls with a smooth cuticle; their longitudinal thickness distinguished them from the inner mesophyll cells. The upper and lower epidermises differed in the thickness of their cuticles and epidermal cell walls. The thickness of the outer periclinal wall of epidermal cell was approximately 2.6 ± 0.3 μm, whereas the thickness of the inner periclinal wall of adaxial surface was 0.9 ± 0.1 μm (Fig. 2A). The thickness was significantly different at $p < 0.05$. It was estimated as nearly three-fold thicker than the thickness of other mesophyll cells. The numerous chloroplasts in the mesophyll cells obtained plastoglobuli and starch grains and had a pronounced stacking of thylakoids (Fig. 2B, 2C). The adaxial epidermal wall frequently showed a characteristic polylamellate structure. These wall layers, which have a stratiform appearance, had different densities (Fig. 2D). Slightly thick cuticles covered both leaf surfaces, and were characteristically more prominent on the abaxial surface. At some positions along the epidermal wall surface, protuberances occurred in peak-like formations. Longitudinal sections of transmission electron microscopy (TEM) images revealed that the cuticle thickness at these positions remained the same and the epidermal wall

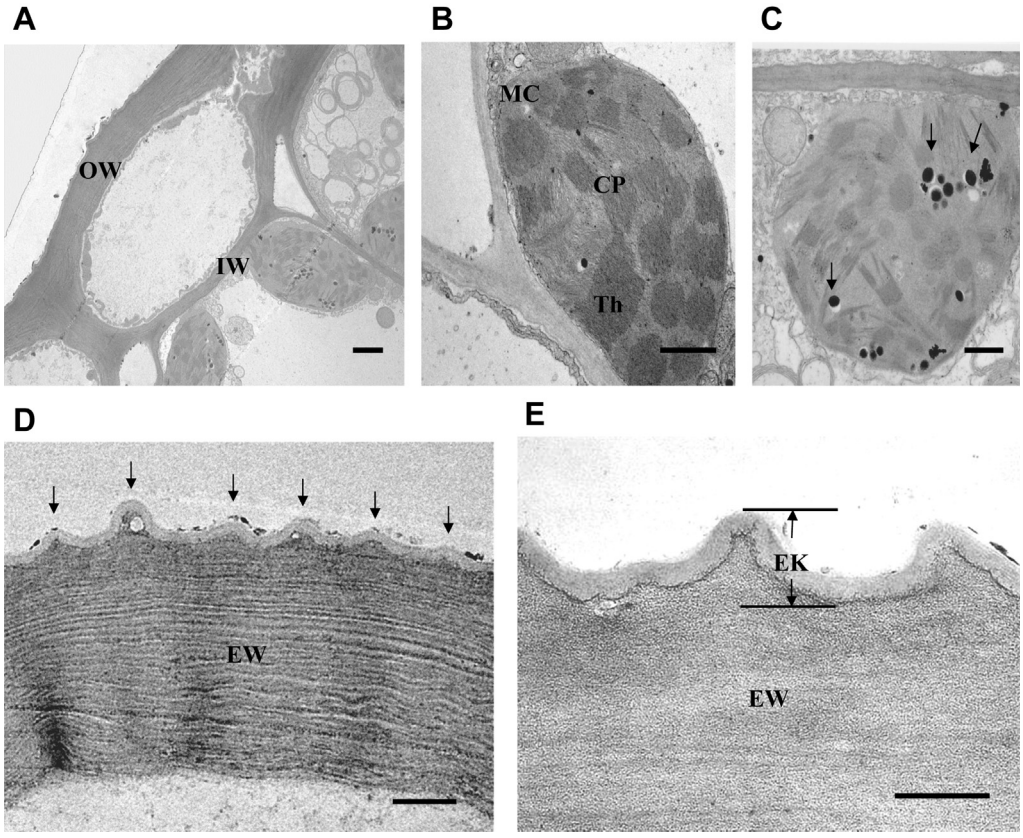

Fig. 2. Transmission electron microscopy micrographs of an epidermal cell and mesophyll cell, which show the cuticle layer and the epidermal wall. (A) The epidermal cell has a remarkably thick wall with the cuticle on the periclinal wall. The outer periclinal wall (OW) covered with cuticle is much thicker than the inner periclinal wall (IW). (B–C) The mesophyll cell (MC) shows a chloroplast (CP) with pronounced stacking thylakoids (Th) and some osmiophilic plastoglobulei (arrows). (D) A longitudinal section of the epidermal wall shows the characteristic polylamellate structure. The arrows indicate the epicuticular structures on the epidermal cell. (E) A higher magnification image of the epidermal wall (EW) shows epicuticular wrinkles (EK). Bars, 2 μm (A); 1 μm (B–D); and 0.5 μm (E).

matrix under the protuberances was very similar to that of other regions. This fine structure, termed “epicuticular wrinkle” in this study, measured 1.1 ± 0.2 μm in height and 0.7 ± 0.1 μm in diameter under the TEM level (Fig. 2E).

3.2. Formation of the epicuticular structure during early development

Two types of vesicles with different sizes and densities were associated with the formation of the epicuticular structure. At the early developmental phase of cuticle formation, the outer region of the epidermal cell had no vesicles under the cuticle (Fig. 3A). Small vesicles possessing electron-opaque substances were often detectable in the region between the cuticle layer and the epidermal wall (Fig. 3B). These vesicles, which contained electron-opaque contents of similar density with that of the cuticle, appeared to be coalesced to the cuticle (Fig. 3C). At the late developmental phase of the epicuticular structure, relatively large and electron-translucent vesicles were aggregated under the cuticle (Fig. 3D–3F). They were often fused to each other and increased in volume, which compressed the cuticle layer (Fig. 3E). These vacuoles contributed to lifting the cuticle layer, and consequently projecting parts of the surface area outward (Fig. 3F).

4. Discussion

Jeffree [22] report that the plant cuticle is a complex structure composed of two basic layers: an outermost epicuticular wax layer and an underlying cuticle membrane layer. Using TEM, the cuticle membrane in most plants possesses two layers: an outer translucent cuticle proper and an inner opaque cuticular layer, defined by the presence of osmiophilic cellulose microfibrils. Our results on the characteristics of the cuticle in ginseng leaves differed slightly from the descriptions in previous reports. The surface of the cuticle appeared as an electron-translucent continuous layer. Furthermore, the residual matrix of the inner surface was not clearly distinct from that of other species [23]. Yeats et al [24] found that a tomato mutant with deficiencies in cutin had an extremely thin cuticle and increased sensitivity to water loss and pathogen susceptibility. In general, the water loss rate is correlated significantly with the total cutin composition, but it was not significantly correlated with the total surface wax amount [12]. In *Clivia* in which cutin is not a homogeneous polyester, the monomeric composition of the cuticle similarly changes constantly during leaf development [13].

Because most ginseng species are shade plants, their leaves usually have thin cuticles and an epidermal cell wall < 3 μm thick. However, some subtropical plants have exceptionally thick cuticles

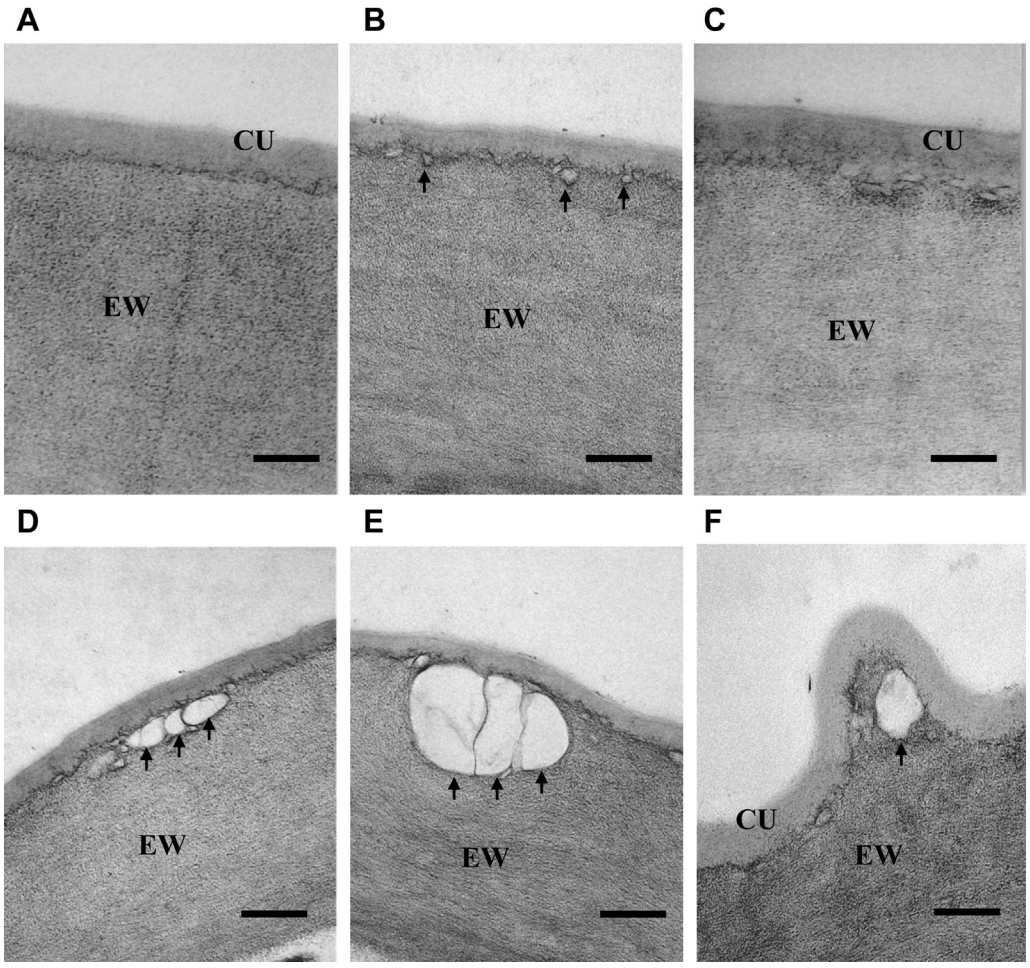

Fig. 3. Transmission electron microscopy micrographs of epidermal cells show the cuticle layer of different stages in the development of epicuticular structures. (A) At the early developmental stage, the epidermal wall (EW) with the cuticle (CU) shows no vesicles. (B) The irregular outline of the small vesicles (arrows) with a density similar to that of the cuticle, appear fused to the cuticle. (C) New cuticle material is deposited at the wall–cuticle interface and results in an irregular CU surface. (D) Vesicles (arrows) containing electron-translucent substances are aggregated between the cuticle layer and the EW. They often become partially flattened. (E) Electron-translucent vesicles (arrows) beneath the wall are in contact with each other and coalesce. The wall is somewhat compressed where membranes are appressed to the cuticle. (F) An epicuticular structure shows one vesicle (arrow) contributing to a projection of the wall matrix toward the cuticle. Bar, 200 nm.

up to 30 μm thick [25]. Lee et al [20] reported that the cuticle density of the epidermal surface was the major determinant for decreasing leaf burning in ginseng leaves. Yunpoong, a high temperature injury-resistant ginseng cultivar, has a higher cuticle density on the epidermal surface, compared to several other cultivars. It was also anticipated that the thicker cuticle of the leaves would decrease damage from leaf burning. This result was obtained from TEM micrographs by measuring the distribution area of the epicuticular wax-like structure on epidermal surfaces.

In our study, the thickness of the cuticle ranged widely from 115 nm to 170 nm on the adaxial surface of the five cultivars. Epicuticular wax is lipids that are commonly integrated and superimposed on the cuticular surface [4]. However, the epicuticular wax-like structure of ginseng leaves differed distinctively from other species' typical epicuticular wax. As Fig. 2 shows, the wrinkled structure was simply the outwardly projected surface area of the cuticle without any attached components. In addition, the cuticle of the wrinkled structure has the same thickness as that of other epidermal wall regions. Based on this result, the wrinkled structure of ginseng leaves may be termed "epicuticular wrinkle," as a different type of cuticle structure. This study also suggests that the epicuticular wrinkles on the cuticle surface, instead of epicuticular wax, function as a barrier to block out sunlight. In ginseng leaves, the adaxial epidermal wall frequently showed a characteristic polylamellate structure. These wall layers, which have a stratiform appearance, represent different densities. These results indicate that somewhat different compositions of wall materials are progressively added to the periclinal wall in cuticle development. This study's results are supported by previous reports that the outer periclinal wall of the epidermis tends to be thicker than the inner periclinal wall, in addition to having more cellulose microfibrils arranged differently from those of the inner wall [26].

For the development of the cuticular layer and epicuticular structure, vesicles of different sizes and densities were aggregated in the outer epidermal wall. The surface feature of many small vesicles was irregular where they contacted each other. With respect to this subject, Mahlberg and Kim [27] proposed that cuticle development involves two phases: (1) a structural phase in which precursors solidify rapidly as deposits thicken the cuticle and (2) an amorphous phase in which precursors permeate the cuticle's exterior surface to increase its girth or surface area. It has been proposed that waxes are exuded to the outer cuticular surface through the lamellate regions in the cuticle, through volatile fluid, or through molecular diffusion [28,29]. Thickening of the cuticle was accomplished by interposition of a cuticular layer between the cuticle proper and the cell wall [13]. In *Nepenthes* species, epicuticular wax crystals primarily consisted of triacontane (C_{30} aldehydes), or dotriacontane (C_{32} aldehydes) [30]. In *Cannabis,* some vesicles penetrate the subcuticular wall to contact the cuticle whereupon their contents contribute to thickening the inner cuticular surface [31]. Vesicles with different electron densities and features were present between the cuticle and epidermal wall of the ginseng leaves. Vesicles with contents of similar densities as that of the cuticle appeared to be coalesced with the irregular cuticular surface. This indicates that they may differ in composition and contribute different components to the developing cuticle. This result is consistent with the report that cuticular waxes are produced in the epidermal cells prior to being exported to the extracellular surface [29].

The electron-translucent vacuoles, which may contribute to lifting the cuticle and projecting parts of the epidermal wall outward, are suggestive of a relevant structure for the formation of an epicuticular wrinkle.

Our most significant conclusion is that the characteristics of the cuticle such as density of the epicuticular structure and thickness of the cuticle may markedly prevent some shade leaves from leaf burning under direct sunlight. However, the outermost structure of the cuticle such as the epicuticular structure may be more important than the thickness of the cuticle, as demonstrated in Yunpoong. It has a thin cuticle of 115 nm, compared to the cuticle of other cultivars (ranging from 133 nm to 143 nm), but Yunpoong has the densest epicuticular wrinkle. However, Yunpoong is the cultivar most resistant to leaf burning. By contrast, Chunpoong has a thick cuticle of 133.6 nm, but it is a cultivar representatively susceptible to leaf burning. Based on a previous report in which the density of the epicuticular wrinkle was incorrectly described as the cuticle density, the densities of Yunpoong and Chunpoong were 53.0% and 17.9% respectively [20]. This finding corroborates that the density of epicuticular wrinkle is more effective against leaf burning, compared to the thickness of the cuticle. Because of its characteristic morphology, epicuticular wax or the epicuticular wrinkle of epidermal surfaces can be useful as a taxonomic key of plant classification in the near future. They are also significant for researchers who have been studying the cuticle for the relationship between plants and external environmental stressors.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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