The development of stomata and other epidermal cells on the rice leaves

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Abstract

In the leaves of rice (*Oryza sativa*), stomatal initials arose from two asymmetric cell divisions and a symmetric division. Guard mother cells (GMCs) and long cells in stomatal files (LCSs) were formed through the first asymmetric division of the precursor cell of GMCs. Subsidiary cells (SCs) were produced by the second asymmetric division of subsidiary mother cells or LCSs. Following SC formation, GMCs divided once symmetrically to generate guard cells and then differentiated terminally to form mature stomata. The developmental patterns of long cells, prickle hairs and short cells (phellem cells and silica cells) were also examined. Interestingly, we found that the different developmental stages of stomata and epidermal cells occurred in the similar location of immature leaves of the same phyllotaxis. In addition, two spacing patterns ("one stoma, one long cell" and "one short cell row") probably exist in rice leaves.

Additional key words: long cells, *Oryza sativa*, prickle hairs, short cells, stomatal density.

Introduction

Stomata are cell complexes specialized for gas exchange between plants and their environments. They are essential for plant productivity and adaptation to external surroundings (Yang and Sack 1995). The epidermis acts as a barrier, prevents water loss, perceives stimuli, transmits signals to the rest of the plant body and secrets different compounds (Guimil and Dunand 2006). Stomata and other epidermal cells represent accessible model systems for studying cell patterns and specification in plants (Nadeau and Sack 2002, 2003). Rice (*Oryza sativa*) is a classic model of monocotyledons and an important crop. Therefore, studies on the stomata and epidermal cells of rice are significant to science and agriculture application. Stomatal development in a model plant *Arabidopsis* includes several asymmetric and symmetric divisions (Yang and Sack 1995, Zhao and Sack 1999). The "one-cell spacing pattern" rule has been well described, ensuring that stomata do not directly contact each other (Sachs 1991, Geisler *et al*. 2000). Several important genes related to stomatal morphogenesis have been found (Bergmann and Sack 2007). The developmental processes of stomata and subsidiary cells (SCs) in maize have also been summarized (Cerioli *et al*. 1994, Gallagher and Smith 1999, 2000, Cartwright *et al*. 2009, Sack and Chen 2009). Although the basal stages of stomatal morphogenesis of rice have been proposed (Kaufman 1959, Hoshikawa 1989, Kamiya *et al*. 2003, Peterson *et al*. 2010), there have been no studies on the development of long and short cells in rice. In this study, we investigated the developmental processes of SCs in detail and identified the morphogenic courses of long cells, short cells, and prickle hairs on the leaves of rice.

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Abbreviations: GCs - guard cells; GMCs - guard mother cells; LCSs - long cells in stomatal files; LC1s - ordinary long cells; LC2s - flanking stomatal row long cells; LC3s - flanking short cell row long cells; SCs - subsidiary cells; SMCs - subsidiary mother cells; TBO - Toluidine Blue O.
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Materials and methods

The rice (*Oryza sativa* L. var. *japonica* cv. Zhonghua 11) seeds were germinated in sterilized water and cultivated either in pots in the Lanzhou Valley under natural summer conditions, or in vessels in a phytotron with a 12-h photoperiod, photon flux density of 350 - 400 μmol m^{-2} s^{-1}, 65 - 80 % relative humidity, and day/night temperature of 32/22 °C. Each vessel contained 16 plants growing in alluvial loam soil. Plant management adhered to normal agricultural practices.

The dental resin impression method was used (Kagan *et al*. 1992, Geisler *et al*. 2000) with the vinyl polysiloxane as an impression material (*Badia*, Polesine, Italy). The impressions on glass slides were observed under a *Motic B1* light microscope and photographed with a digital camera (*Olympus C-7070*, Tokyo, Japan). All impressions from the same immature leaf blade exhibited the consecutive developmental processes of the epidermal cells.

For paraffin sections, blades of mature 8th leaves were fixed in mixture of 5 % formaldehyde, 5 % glacial acetic acid and 63 % ethanol and dehydrated in a graded ethanol series (Komorisono *et al*. 2005). Later, the samples were embedded in paraffin, and sectioned at 5 μm by a microtome (*Leica RM 2235*, Germany). Sections were stained for 1 - 2 min in an aqueous 0.1 % Toluidine Blue O (TBO) solution and then were photographed.

Thirty plants in the same growth periods were used and in each plant, the impressions were obtained from the mature 3rd, 5th, 7th and flag leaves. These impressions were observed by light microscopy, and stomatal density and the number of stomatal rows were investigated in a field of 0.0324 mm^2 with seven replicates for each impression. The distance of long cells in stomatal files (LCSs) and between two stomatal rows were measured in six randomly selected visual fields of each impression. The stages of epidermal development were measured on sequential impressions away from the leaf blade base in five different leaf blades. All of the measurement data were analyzed using the *Microsoft Excel* program. Values were expressed as means ± SD and calculated from at least three independent experiments. Student's *t*-test was performed, and $P < 0.05$ indicated statistical significance.

Results

Stomatal complex of rice leaf blade was composed of two dumbbell-shaped guard cells (GCs) which were flanked by two ellipsoid-shaped SCs. Stomata were usually arranged in a single file in low phyllotaxis leaves (Fig. 1*A*), and two or more adjacent stomatal rows were common in high phyllotaxis leaves (Fig. 1*B*). Long cells and short cells were also distributed in linear rows (Fig. 1*A*). According to the distribution and position of long cells, they were divided into four types: ordinary long cells (LC1s), flanking stomatal row long cells (LC2s), flanking short cell row long cells (LC3s), and long cells in stomata files (LCSs) (Fig. 1*A*). Short cells included phellem cells and silica cells, and their alternating arrangement formed a row (Fig. 1*A,C*).

To further understand whether two asymmetric divisions and one symmetric division occurred in rice stomatal morphogenesis, the whole stomatal development was observed in detail. We found that the first asymmetric division occurred in the precursor cell of guard mother cells (GMCs). It was located in the base of the immature rice leaf blade, and produced a GMC (small daughter cell) and a square young LCS (Fig. 2*A-C*). With the LCSs elongating and broadening, the young LCSs produced lobes to surround their adjacent GMCs (Fig. 2*D-F*), and the papillae emerged on the LCSs (Fig. 2*G*). At maturity, the LCSs encircled their adjacent stomatal complexes. Meanwhile, they interlocked with other long cells *via* the serrated lobes of their sides (Fig. 2*J,K*). During the first asymmetric division, the

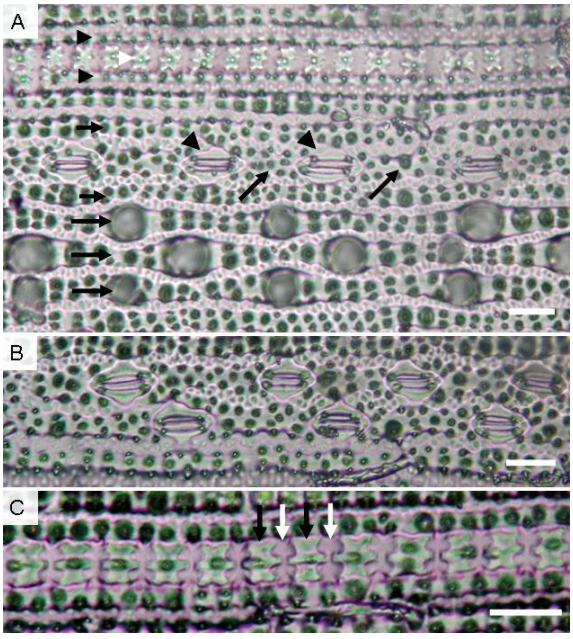

Fig. 1. Stomata, long cells and short cells on the abaxial leaf blade of rice. *A* - Stomata (*black diagonal arrowheads*), long cells (LC1s, *long black horizontal arrows*; LC2s, *short black horizontal arrows*; LC3s, *black horizontal arrowheads*; LCSs, *long black diagonal arrows*), and short cells (*white horizontal arrowhead*) were distributed in linear rows; *B* - Two adjacent stomatal rows; *C* - Magnification of the short cell row in panel (*A*), which consisted of phellem cells (*black arrows*) and silica cells (*white arrows*). *Bars* = 20 μm.

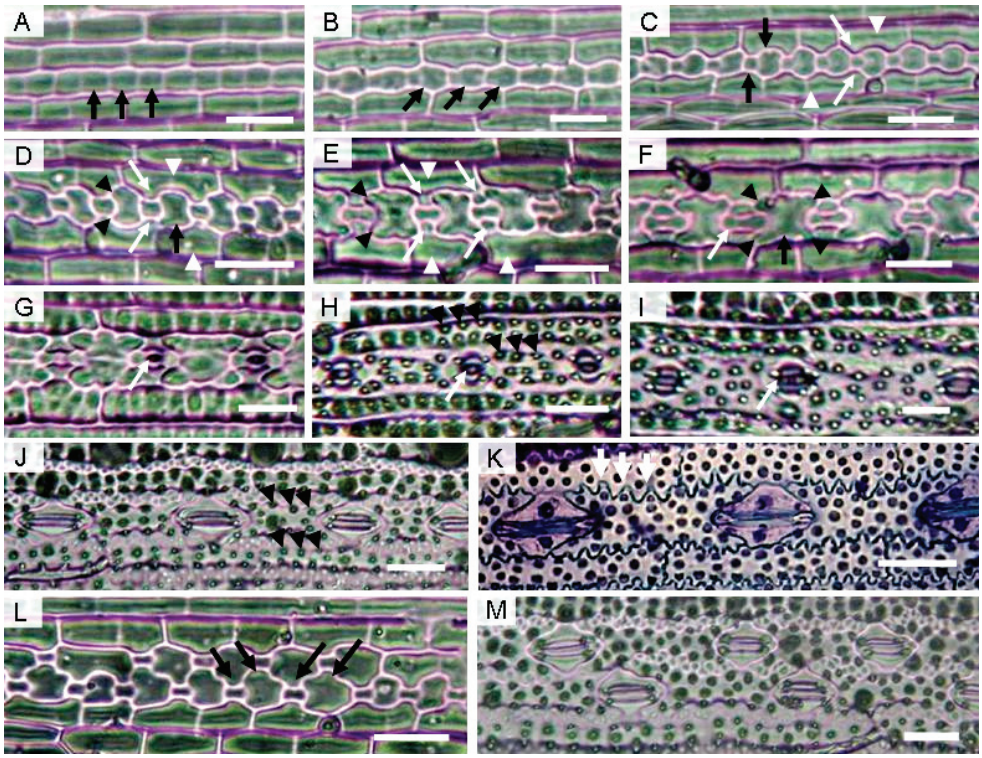

Fig. 2. Morphogenic course of LCSs and SCs in the abaxial leaf blade of rice. *A* - Precursor cells of GMCs and LCSs appeared (*black arrows*); *B* - These square cells divided asymmetrically (*black arrows*); *C* - GMCs (*vertical upward black arrows*), young LCSs (*vertical downward black arrows*), and young SMCs (*white arrowheads*) with protuberances (*white arrows*); *D* - Protuberances (*white arrows*) of SMCs further extruded, and lobes (*black arrowheads*) of young LCSs (*black arrow*) and immature LC2s (*white arrowheads*) were present; *E* - Small SCs (*white arrows*) and LC2s (*white arrowheads*); *F* - Developing LCSs (*black arrow*) surrounded their adjacent "three-cell complexes" (*white arrow*) gradually; *G* - GMCs divided symmetrically (*white arrow*), and papillae appeared; *H* - The division spread to the two ends of GMCs (*white arrow*), *black arrowheads* indicate the papillae; *I* - Two young GCs; *J* - Serrated lobes of the sides of LCSs and LC2s appeared (*black arrowheads*); *K* - Mature serrated lobes (*white arrows*); *L* - The SCs at the adjoining side of adjacent stomatal rows (*black arrows*); *M* - Mature adjacent stomatal rows (*A-J, L-M* - dental resin impressions of the epidermis of the rice leaf, *K* -mature epidermal longitudinal sections of the rice leaf stained with TBO, *bar* = 20 μm).

subsidiary mother cells (SMCs) produced protuberances toward their adjoining GMCs (Fig. 2*B,C*). These protuberances separated later and formed small SCs flanking their adjacent GMCs and LC2s in the second asymmetric division (Fig. 2*E*). These young SCs differentiated gradually and at last surrounded their adjoining GMCs. When SCs formed, the GMCs divided once symmetrically to generate GCs (Fig. 2*F-I*). When the symmetrical divisions ended, two mature ellipsoid-shaped SCs encircled two dumbbell-shaped GCs to constitute a stomatal complex (Fig. 2*J,K*). The morphogenic course of stomata in two or more adjacent stomatal rows was similar to the developmental process of a single file. However, the SCs at their adjoining sides were produced not by SMCs but by the asymmetrical division of their corresponding adjacent young LCSs, (Fig. 2*L*).

It is very interesting to see that the entire consecutive developmental course of stomata and other epidermal cells could be all observed in different positions on an immature rice leaf blade. In the same phyllotaxis leaves from different plants, the distance from the immature leaf base to the place where different stomatal morphogenic stages occurred was approximately equal. The position where GMCs were produced by the asymmetric division of their precursors was approximately 0.61 cm away from the leaf base, and the place where the small SCs were separated from their relevant SMCs was 1.23 cm away from the base. The symmetric division of GMCs occurred at a site approximately 2.11 cm away from the base, and the place where stomata began to mature completely was approximately 3.20 cm away from the base.

The LC1s were distributed widely in the leaves of rice. Firstly they originated from a number of small rectangular cells that emerged in the base of immature rice leaf blades (Fig. 3*A*). Then, some small rectangular cells were divided into two young LC1s with different lengths, and the undivided cells were also young LC1s (Fig. 3*B*). Subsequently, the main morphological differences of these growing LC1s were their elongating and broadening tendencies (Fig. 3*C*). Meanwhile, papillae appeared on the surfaces of these LC1s (Fig. 3*D*). Finally,

the serrated lobes formed at the sides of LC1s, interlocking them with each other (Fig. 3*E,F*). The uneven division of these small rectangular cells occurred 0.06 cm away from the rice leaf base, and the first papillae emerged on all long cells approximately 1.23 cm away from the base. Furthermore, approximately 1.85 cm away from the base, the surfaces of long cells were covered with sufficient great numbers of papillae. Moreover, the LC1s matured approximately 3.05 cm away from the base. LC2s were large daughter cells, and they were produced by the asymmetric division of SMCs (Fig. 2*A-F*). The morphogenic processes of LC3s were similar to LC1s (data was not shown), and LC3s were narrower than LC1s at maturity (Fig. 1*A*).

Short cells included phellem cells and silica cells, and prickle hairs were located in these cell rows (Fig. 1*C*). Firstly, young phellem cells with tight arrangement appeared at the base of immature rice leaf blades. Afterward, cell protuberance emerged on this young phellem cell row (Fig. 3*G*). Ultimately, it further extruded and developed into a prickle hair (Fig. 3*H-J*). However, there were no obvious morphological changes in these young phellem cells during the development of prickle hairs. When GMC divided, the profiles of growing phellem cells became noticeable, and papillae emerged on their surfaces (Fig. 3*K*). Later, silica cells formed between the two phellem cells, and their growth caused these phellem cells to separate gradually (Fig. 3*L,M*). According to our observation, the small silica cells were possibly produced by the divisions of these developing phellem cells. However, the true precursors of silica cells remained to be elucidated. The sides of phellem cells eventually formed "3" or "Ɛ" shapes, and silica cells formed "8" shapes. They arranged alternately and constituted a short cell row terminally (Fig. 3*N*). Through the development of prickle hairs and short cells, prickle hairs appeared approximately 0.25 cm away from the base of immature leaf blades and matured approximately 1.74 cm away from the base. The obvious morphological changes of young phellem cells occurred at a position approximately 2.16 cm away from the base, and phellem cells and silica cells matured approximately 5.39 cm away from the base. The results above indicated that certain developmental stages of stomata, long cells, prickle hairs, and short cells were probably fixed in the determined fate of immature rice leaves in the same

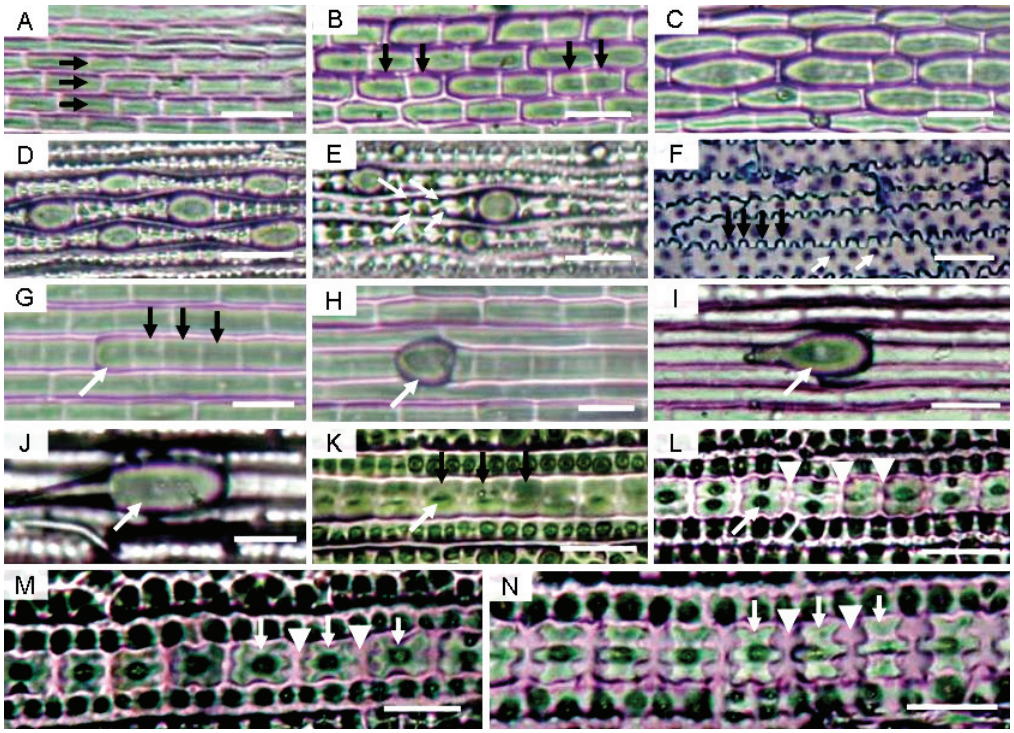

Fig. 3. Morphological development of LC1s (*A* - *F*), prickle hairs, and short cells (*G* - *N*) on the abaxial leaf blade. *A* - Small rectangular cells (*black arrows*); *B* - Two young LC1s divided unevenly (*black arrows*); *C* - Growing LC1s; *D* - The surfaces of the young LC1s were covered sufficiently with papillae; *E,F* - Mature papillae (*white arrows*) and serrated lobes (*black arrows*); *(G-N)* - Morphological developmental course of prickle hairs and short cells; *G* - Young phellem cells (*black arrows*) and cell protuberance emerged on this young phellem cell row (*white arrow*); *H* - Protuberance (*white arrow*) further extruded; *I* - Protuberance developed into a thorn-shaped tuber (*white arrow*); *J* - Mature prickle hair (*white arrow*); *K* - Developing phellem cells (*black arrows*) became clear and papillae (*white arrow*) appeared on their surfaces; *L* -Silica cells appeared (*white arrowheads*); *M* - Phellem cells formed "3" or "Ɛ" shapes (*white arrows*), and silica cells became "8"-shaped (*white arrowheads*); *N* - Mature phellem cells (*white arrows*) and silica cells (*white arrowheads*); *(A-E,G-N* - impressions of the epidermis, *F* - longitudinal sections of the mature rice leaf stained with TBO, *bar* = 20 μm).

Table. 1. LCSs length, stomatal density, number of stomatal files and distance between stomatal files on the rice leaf blades from plants with different phyllotaxis. Means ± SD. Means were calculated from three independent experiments. * indicated that the means were significantly different at $P < 0.05$ according to an unpaired *t*-test.

Types	3rd leaf	5th leaf	7th leaf	Flag leaf
Length of LCSs [μm]	49.49 ± 1.69*	42.90 ± 1.84*	31.57 ± 1.97*	23.18 ± 1.26*
Stomatal density [mm^{-2}]	175.93 ± 7.08*	254.12 ± 8.34*	410.49 ±14.91*	627.57 ± 25.4*
Numbers of stomatal files [mm^{-1}]	13.33 ± 0.58*	16.85 ± 0.33*	19.81 ± 0.75*	26.85 ± 1.09*
Distance between two stomatal files [μm]	68.52 ± 1.49*	56.43 ± 1.47*	59.55 ± 1.69	60.26 ± 2.45

phyllotaxis.

With phyllotaxis enhanced, the length of LCSs in leaf blades decreased, and the number of stomatal files increased in the unit area. These two factors contributed to the increase of the stomatal density (Table 1). The space between two non-adjacent stomatal rows on the 5th leaf was shorter than on the 3rd leaf, resulting in increased number of stomatal files per unit area of the 5th leaf (Table 1). While the space between two non-adjoining stomatal rows in the 5th leaf was similar to the 7th leaf and the flag leaf (Table 1). On the 7th leaf blade, most stomatal files were distributed in single rows, whereas two adjoining rows were usually observed in the 7th leaf blade. In addition, the frequency of adjacent stomatal rows was elevated in higher phyllotaxis leaves. Furthermore, in the flag leaf blade, once some stomatal rows arranged in three or more adjacent rows, most of them were arranged in two adjacent rows (data was not shown). These findings suggest that the appearances of adjacent stomatal rows might be the primary reason for the increased number of stomatal files per unit area which was observed in upper leaves.

Discussion

The stomatal patterning of rice was consistent with *Poaceae* family. In particular the distribution of stomata and long cells was similar to maize. Although the stomata pattering of *Poaceae* was different from the *Arabidopsis* species, they may have some homology of morphological development.

In this work, we mainly focused on the development of LCSs and SCs on the abaxial surface of rice leaves. Both of LCSs and GMCs originated from the precursor cells of GMCs in the base of immature rice leaves. LCSs were large daughter cells, and they were produced from these precursor cells by the first asymmetric division. In the single stomatal file, SCs were daughters and they were generated from SMCs by the second asymmetric division. However, SCs originated from SMCs and LCSs which are adjacent to GMCs in two or more adjacent stomatal rows. The developmental courses of stomata, LCSs and SCs on the adaxial leaves of rice were the same as those on the abaxial leaves. *SCARECROW* (*SCR*) gene, first reported in *Arabidopsis*, affected the asymmetric divisions (Di Laurenzio *et al.* 1996). Similarly, *OsSCR* was expressed in developing stomata of rice leaves, which hinted that *OsSCR* might be correlated with stomatal development program (Kamiya *et al*. 2003, Itoh *et al*. 2005) In maize, the mechanism of asymmetric division in SMCs was elucidated. For example, SCs in *brk1* mutants were abnormal, and SMCs in *dcd1* and *pan1* mutants also showed disrupted divisions (Gallagher and Smith 1999, 2000). Proteins as BASL and EPF2 in *Arabidopsis* and PAN1 in maize, regulating independently asymmetric divisions, were associated with the formation of the stomatal complex (Cartwright *et al*. 2009, Dong *et al*. 2009, Hunt and Gray 2010). We speculated that relevant genes and proteins should have homology in rice. Nevertheless, the further detailed investigations of the molecular mechanism of stomatal and epidermal cells development in rice are still needed.

Furthermore, in abaxial and adaxial leaf sides of rice, the ratio of stomata to LCSs was nearly 1:1 in leaves of different phyllotaxis. The directly adjacent stomata in one stomatal file were rarely found, indicated that the "one stoma, one long cell" spacing pattern might exist in rice. Moreover, the pattern was likely more regular than the “one-cell spacing pattern” in *Arabidopsis* (Sachs 1991, Geisler *et al*. 2000). Recently, the roles of the *Arabidopsis* SPCH, MUTE, and FAMA orthologs in rice and in maize stomatal development have been reported (Liu *et al*. 2009). Research also showed that there were similarities between the rice and maize, allowing the investigation of the genes critical in stomatal patterning and differentiation (Liu *et al*. 2009, Peterson *et al*. 2010). Therefore, there could be analogous genes and signal pathways, which were relevant to stomatal development in *Arabidopsis*, maize and rice.

The decreased lengths of LCSs and the increased numbers of adjacent stomatal files per area unit area might be the main reason for the increased stomatal density with phyllotaxis. In addition the adjoining stomatal rows could also increase the stomatal density. However, it was interesting to see that the adjoining

stomatal rows that appeared in high phyllotaxis did not disorder the entire distribution of epidermis in the rice leaf. Therefore, we speculate that the adjacent stomatal rows are involved in improving the efficiencies of gas exchange.

The received definition of long cells has not so far been unified, some authors called them general pavement cells (Park *et al*. 2010, Peterson *et al*. 2010). Long cells were divided into four types (LC1s, LC2s, LC3s, and LCSs) according to their distribution. The development of LC1s could be divided into four stages which were as follows: *1* - small rectangular LC1s appeared; *2* - some LC1s divided unevenly; *3* - LC1s were elongated and broadened gradually; and *4* - papillae appeared. At maturity, the surfaces of adult LC1s were covered with papillae, and the serrated lobes at the sides of LC1s caused them to interlock with each other. The basal developmental course of LC2s was similar to LC1s, except that the young LC2s were large daughter cells of SMCs asymmetric divisions that differentiated as long cells. The morphogenesis of LC3s was the same as LC1s.

In the development of rice leaves, the epidermal precursors only appeared at the base of immature leaf blades, which leaded to the formation of new stomata. Other epidermal cells were restricted in certain areas near the leaf base. In addition, different developmental stages of stomata and other epidermal cells occurred at predetermined length intervals of the leaves, and their precursors were not observed in mature leaves of rice. The development courses of stomata and epidermal cells in rice and in maize are very similar (Kamiya *et al*. 2003, Sack and Chen 2009). There was a certain discrepancy in development processes of stomata and other epidermal cells between *Arabidopsis* and maize (Bergmann and Sack 2007, Sack and Chen 2009). But the development of lobes in pavement cells of *Arabidopsis* and long cells of maize were both affected by the *brick-1* gene. Epidermal cells of *brk1* mutant lacked lateral marginal serrated lobes in *Arabidopsis* and maize (Frank and Smith 2002, Djakovic *et al*. 2006), suggesting that some similar genes might function in the epidermal cell morphogenesis of both dicotyledons and monocotyledons.

No detailed description about the morphogenesis of prickle hairs and short cells has been reported previously, hence, in this article, we investigated the developmental patterns of prickle hairs and short cells. Prickle hairs were located on the short cell file, and they originated from the cell protuberances that emerged on the young phellem cell rows in the base of the immature rice leaf blades. Ultimately, protuberances extruded toward the leaf tip and formed the thorn-shaped prickle hairs. Prickle hairs were present in the epidermis of rice and maize leaves, and they were normal, sharply pointed in wild-type. Macrohairs were long and pointed in maize epidermis, but they have not been observed in rice. The pattern of prickle hairs and macrohairs were controlled by the *brk-1* gene, and they were shorter and blunter in *brk-1* mutant of maize than in wild-type (Frank and Smith 2002).

A short cell row was composed of phellem cells and silica cells alternatively arranged in lines. The phellem cells and silica cells originated later than stomata and long cells in rice. There were four stages in their formation. The first three were: *1* - young phellem cells appeared, *2* - profiles of young phellem cells became clear gradually and *3* - papillae emerged. Silica cells were formed, and their growth gradually separated young phellem cells from each other. These silica cells might be produced by the division of the growing phellem cells. Finally, *4* - the sides of phellem cells became "3"- or "Ɛ"-shaped, and silica cells became "8"- shaped and they arranged alternately to constitute a short cell file. However, adjoining short cell rows were never observed in both of abaxial and adaxial leaf blades in rice according to this study. Thus the "one-short cell row spacing pattern," in which short cell rows were separated by at least one intervening long cell file, might exist in the epidermis of rice leaf. In other familiar model of maize, wheat and barley, there were not short cell rows and dense papillae in epidermis. In mature leaves of maize, short cells were composed of pairs of siliceous-suberous cells which nested randomly among the long cells. It was tough to specify the precursors of silica cells explicitly, because they were not found in our morphological study and the genes that affected the development of phellem cells and silica cells in rice are not known. Further studies on short cells at the genetic and molecular levels will be vital.

Rice is considered as a representative model plant of monocots and model crop for evolutionary and agronomical studies of cereals (Wang *et al*. 2008). Stomata and other epidermal cells are essential for plant productivity and survival (Schroeder *et al*. 2001, Raven 2002). Accordingly, this study has explicitly described the developmental processes of stomata, long cells (LCSs, LC1s, LC2s and LC3s) and short cells (phellem cells, silica cells) in rice leaves. Further detailed studies on the stomata and epidermal cells of rice are not merely significant for the theory and the molecular basis of the plants evolution, but also they can help to improve crop yield and viability in different environments.

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