A cell-type-specific defect in border cell formation in the Acacia mangium root cap developing an extraordinary sheath of sloughed-off cells

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INTRODUCTION

Root elongation is an important plant movement that provides above-ground support and facilitates access to water and nutrients in soils. The root cap protects root apical meristem (RAM) from soil contact during root elongation and from various adverse factors including drought and pathogen attacks (Barlow, 2002; Iijima et al., 2008). The root cap also plays roles in modulating environmental signals, such as mechanical contact, water, light or gravity (Blancaflor et al., 1998; Barlow, 2002; Friml et al., 2002; Swarup et al., 2005; Iijima et al., 2008).

Border cells are readily detachable cells found on the outer peripheral root cap in most plant species, including ferns, gymnosperms and angiosperms (Hawes and Pueppke, 1986; Hawes et al., 1998; Hamamoto et al., 2006). These cells are assumed to play multiple roles in modifying rhizosphere environments, including mechanical contact (Bengough and McKenzie, 1997; Iijima et al., 2003), microbial colonization/repellence (Gochhauer et al., 1990; Hawes et al., 1998; Gunawardena and Hawes, 2002) and toxic metals such as aluminium (Al) (Miyasaka and Hawes, 2001) and iron (Zhang et al., 2011). Additionally, the number of border cells released from the root surface varies greatly among plant species (Hawes and Pueppke, 1986), and this may be partially affected by the state of attachment of border cells to the root surface (Kawata et al., 1979). The root tip of Arabidopsis thaliana, known to release few border cells, produces border-like cells, which are organized in a sheath-like pattern and are associated with microbial colonization (Vicré et al., 2005; Driouich et al., 2007, 2010; Durand et al., 2009). RAMs are largely grouped into three types (i.e. closed, intermediate-open and open), based on the association of tiers of initials for each tissue (Clowes, 2000; Groot et al., 2004; Heimsch and Seago, 2008). Plants with open-type RAM produce more border cells than those with closed-type RAM (Hamamoto et al., 2006); the RAM of arabiudopsis is closed (Wenzel et al., 2001). However, it remains unclear which features of different RAM types regulate the number and association patterns of border cells. Furthermore, the roles of border cells and border-like cells in root growth and development are not well understood, in part because they are easily dispersed into solution as groups of single cells or as cell aggregates (Hawes et al., 1998; Driouich et al., 2010).

Previous studies have revealed that formation of border and border-like cells is regulated by cell wall-modifying enzymes that loosen intercellular connections in a spatio-developmental manner (Hawes and Lin, 1990; Durand et al., 2009; Bennett et al., 2010). For instance, pectin methyl esterases (PMEs) control the methylation status of pectin (Micheli, 2001). In pea roots, PME activity is highest at the outer periphery of the root cap (Stephenson and Hawes, 1994), and antisense repression of the PME gene reduces the release of border cells (Wen et al., 1999). Border cells attached to the root surface provide negative feedback for mitosis in columella root cap initials (Brigham et al., 1998; Iijima et al., 2003;
The columella root cap cells and lateral root cap cells are derived from different initials, and their propagation patterns are well organized at the cell boundaries (Dolan et al., 1993; Wenzel and Rost, 2001; Wenzel et al., 2001). A root spatial expression study revealed distinct differences in gene expression patterns between the columella and lateral cap cells in arabidopsis plants (Birnbaum et al., 2003). In the root cap, several genes involved in cell-wall modification or sugar metabolism are expressed in a spatio-dependent manner (Woo and Hawes, 1997; Woo et al., 1999; del Campillo et al., 2004; Wen et al., 2007; Durand et al., 2009; Bennett et al., 2010). However, it remains to be fully understood whether the different cell types of the root cap affect the formation patterns of border cells.

Some woody plants form unique root caps that differ from those of annual plants in terms of life cycle and growth patterns. The two Ephedra species have a large root cap being 1-2 mm long from the tip to the columella initials (Pillai, 1966; Peterson, 1983). The caps of Abies procera roots have suberized outer layers during the winter dormancy season (Wilcox, 1954), and the roots of Pinus radiata and P. sylvestris release the highest numbers of border cells of 28 crop species examined (Hawes and Pueppke, 1986). In general, root elongation rates in woody plants are much slower than those of annual crops such as maize (Zea mays) and soybean (Glycine max; Brundrett, 1991). In maize varieties, primary roots with longer root caps have slower elongation rates than those with shorter root caps (Pilet, 1986). A recent study, in contrast, revealed that root growth and border cell formation are independently controlled by phytochemicals in root exudates (Curlango-Rivera et al., 2003). The detailed relationship between root cap development and root growth is not fully understood.

Acacia mangium is a leguminous tree that can grow quickly in the acidic soils of tropical regions and have nutritional advantages occurring through symbiosis with nitrogen-fixing bacteria and arbuscular mycorrhizae (Norisada et al., 2005). The seedlings of A. mangium also exhibit strongly Al-tolerant root elongation with small amounts of citrate release (Osawa et al., 2011). To examine the detachment pattern of border-like cells under friction, the roots were excited with a surgical blade at 0.5, 1.0 or 1.5 mm from the tip under a stereomicroscope and then exposed to the solution for 48 h. At each time point, root lengths were measured with a ruler, and root apexes were photographed under a light or stereo-microscope. To determine the area of peeled border-like cells, the projected images of peeled cells and the structural root in the images were measured based on intensity thresholds of the objects using image analysis software (WinRHIZO; Règent Instruments, Québec City, Canada).

To examine the detachment pattern of border-like cells under friction, the root system of each species was placed between two sheets of moist filter papers (No. 2; Advantec Toyo) with a polyurethane-foam block as a damper for the root—filter paper contact in a plastic root box. The root boxes had open faces at the top and bottom. The boxes were vertically placed in the pool of 0.5 mM calcium chloride solution (pH 4.5) with the lower portions of the polyurethane and filter paper immersed. The roots were grown for 48 h in filter papers, and the tip positions were marked on the surface of the plastic box at 24-h intervals.

**MATERIALS AND METHODS**

**Plant culture**

Seeds of Acacia mangium harvested in Brunei were obtained from Sumitomo Forest Co. Ltd (Tokyo, Japan). The seeds were immersed in boiling water (100°C) for 1 min for scarification and then surface-sterilized in a 0-1% (v/v) sodium hypochlorite solution for 20 min. Seeds were sown in moistened vermiculite soil at 25°C. Three- or four-week-old seedlings were transferred to a 7-L plastic box containing one-fifth strength Hoagland solution. The seedlings were grown hydroponically for another 2 months with exchange of solution every 3 or 4 d. The sheaths of border-like cells in newly developed roots (approx. 10 cm in length) in the solution culture were removed with forceps or by scraping on moist filter papers before the start of each treatment. Seeds of the soybean ‘Wase-hakuchou’ were purchased from Takii Co. Ltd (Kyoto, Japan). Six-day-old soybean seedlings were prepared as previously described (Osawa et al., 2011). To observe border-like cells in a minimally stained condition, the seedlings of both species were exposed to 0-5 mM calcium chloride solution (pH 4-5) for 1 d before each treatment. All seedlings were grown in a 14 h/10 h light/dark cycle (at 150 μmol m⁻² s⁻¹ photosynthetic photon flux density) at 28°C (light) and 22°C (dark).

**Root growth conditions**

Before the start of each treatment, the sheath of border-like cells and/or tissue debris at the root apex was gently scraped off using a moist glass-microfibre paper (Advantec Toyo, Tokyo, Japan). For all treatments (for a maximum of 7 d), roots were exposed to 0-5 mM calcium chloride solution (pH 4-5) exchanged every 24 h. For temperature treatments, the roots of A. mangium seedlings were exposed to the solution for 24 h in the growth chamber at 25, 21 or 18°C. For acclimation, the roots were exposed to the solution for 24 h at the corresponding temperatures prior to the start of the treatment. To determine the effects of the removal of the root apex on border-like cell formation, the roots were excited with a surgical blade at 0.5, 1.0 or 1.5 mm from the tip under a stereomicroscope and then exposed to the solution for 48 h. At each time point, root lengths were measured with a ruler, and root apexes were photographed under a light or stereo-microscope. To determine the area of peeled border-like cells, the projected areas of peeled cells and the structural root in the images were measured based on intensity thresholds of the objects using image analysis software (WinRHIZO; Règent Instruments, Québec City, Canada).

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used. The age of the seedlings used for the border-cell collection was 4-d-old for *A. mangium* and 2-d-old for soybean. Border cells and border-like cells were collected by the method described by Brigham et al. (1995) with minor modifications. To reduce clogging of the partially peeled border-like cells on the root surface, two sections (0–1 mm and 1–3 mm) of root apices were placed directly into 50 μL of 0.5 mM calcium chloride solution by excising from the 1- and 3-mm regions with a surgical blade. The recovery solution was incubated for 5 min at 25 °C with occasional vortexing and pipetting, and then 20 μL of the solution was transferred onto a glass slide. To assay mucilage production, 20 μL of the solution containing 2 mM of Indian ink was added to the suspension. The border cells were divided into single cells or aggregated cells based on the connection of more than four cells in a row in light micrograph images. For each root section, the cells were counted from three to six replicates. Due to the partial remnants of clogged cells in the 1- to 3-mm section, cell numbers and projected areas of 20 samples of border-like cells were measured. Then, the total number of cells attached to the root surface area was calculated on the assumption that a single layer of cells was detached from the root surface. The surface area of each root section was measured with WinRHIZO.

**Fluorescein diacetate staining**

The viability of border cells and border-like cells was assayed by hydrolysis of fluorescein diacetate (Vicre` et al., 2005). After the root-growth treatment in different conditions, roots were exposed to a 0.5 mM calcium chloride solution containing 12.5 μg mL⁻¹ fluorescein diacetate for 10 min. After three washes with the calcium chloride solution, roots were observed with a fluorescence microscope using a 470- to 495-nm excitation filter, a 505-nm DCLP mirror and a 510- to 550-nm emission filter.

**Histochemical analysis**

For cell verification, peeled border-like cells floating in the 2- to 5-mm tip region were stained with either 0.1 % (w/v) safranin O in deionized water or lactophenol cotton blue in 22 % (v/v) phenol (Muto Pure Chemicals, Tokyo, Japan) for 10 min. Autofluorescence of the border-like cells was observed with a fluorescence microscope using a 330- to 385-nm excitation filter, a 400-nm DCLP mirror and a 420-nm emission filter. For root structure analysis, longitudinal sections of the 10-mm root tip region were prepared by embedding with glycol-methacrylate, as described previously (Osawa et al., 2011), and observed under a light microscope.

**RESULTS**

**Sheath formation by border-like cells**

A hydroponic culture system was used to examine the development of *A. mangium* roots under minimally disturbed conditions. In solution culture, it was noted that border-like cells, which look like fibrous tissue, developed as sheath-like structures that surrounded the entire root apex up to 5 mm behind the tip (Fig. 1A). The sheath of border-like cells autonomously detached every 1–2 weeks (Fig. 1B). The sheath developed in nutrient solution had a more brownish colour than those in calcium chloride solution with no change in
the structural arrangement (Fig. 1A, B). This colour development appeared to be a reaction with ferrous iron, because it did not occur in the nutrient solution without ferrous sulphate (data not shown). The developmental patterns of sheaths from A. mangium and soybean seedlings were compared, the latter species being a representative legume crop that releases a relatively large number of border cells (Hawes et al., 1998). In A. mangium, border-like cells began to float around the root apex 1 d after the removal of the existing sheath with forceps and then enveloped the root apex up to the 5-mm region behind the tip at day 7 (Fig. 1C). In soybean, only a few border-like cells were evident in the 0- to 1-mm region of the root apex at day 7 (Fig. 1D).

The fibrous tissue floating around the A. mangium root apex in the 2-mm region was observed under a microscope to characterize its cell type. Safranin O staining revealed that the tissue was composed of border-like cell, >100 μm in length, arranged in a brick-wall pattern, and some portions of the sheet of cells were split longitudinally (Fig. 1E). Infection with a pathogen or mycorrhizae was unlikely to be the cause of the formation of these fibrous tissues or subsequent sheath development, because cells in the fibrous tissues were minimally stained with lactophenol cotton blue (see Supplementary Data Fig. S1, available online), and roots growing under sterile conditions developed a sheath (data not shown). To address whether secondary wall components such as lignins contributed to the fibrous tissue structure, the autofluorescence pattern of detached fibrous tissues was observed. When excited at wavelengths between 330 and 385 nm, the shorter side of the intercellular connection of the border-like cells had strong autofluorescence that most likely emanated from lignins in cell walls (Fig. 1F).

Root cap structure

To identify the origin of the border-like cells, the root structure of A. mangium was analysed by histochemistry. The sheath of border-like cells was removed first before exposing the roots to solution (Fig. 2A). After 48 h of root growth, the outer cell layers of the lateral root cap were partially attached to the 0- to 2-mm tip region of the root apex, leaving both ends free from the root surface (Fig. 2B). Meanwhile, there was no apparent connection of the layered cell sheets to the daughter cells at the tip of the columella root cap. Enlarged and transverse images showed a similar distribution pattern of toluidine blue O-reactive substances in the middle lamella between the peeled border-like cells and the undetached lateral root cap cells (see Supplementary Data Fig. S2). Based on these results and the arabidopsis type in which border-like cells are more exclusively distributed and connected to the root tip (Vicré et al., 2005), we propose that the fibrous tissues that detached from the lateral root cap represent a distinct type of border-like cells in A. mangium roots. Next, the structure of the root apex of soybean at a root growth rate comparable to that of A. mangium seedlings was examined. After 12 h of root growth, very few groups of connected cells were present on the root surface of soybean (Fig. 2C). The amount of root elongation in soybean after 12 h (22.3 ± 3.5 mm) was nearly the same as that of A. mangium (17.8 ± 4.0 mm) after 48 h in solution culture.

**Peeling patterns of border-like cells**

To understand the spatial detachment pattern of border-like cells, roots were stained with a cell viability marker to enhance their visibility. After removal of the sheath, the roots of A. mangium or soybean were grown for 0, 6 or 24 h and then stained with 12.5 μg mL⁻¹ fluorescein diacetate for 10 min. In A. mangium roots, acropetal peeling of border-like cells was extensive in the 1- to 3-mm region behind the tip at 6 h (Fig. 3A). Further, the area of peeled border-like cells that emit fluorescence was similar to the total area in bright-field image. The total area of peeled border-like cells also increased with time, though the amount and spatial distribution of peeled cells with fluorescence in the roots were same at 6 h and 24 h. This suggests that the peeled border-like cells may remain viable in the vicinity of the root surface for some time. In soybean, single border cells with fluorescence were visible in 0- to 1-mm tip region at 24 h (Fig. 3A). However, no border-like cells were found in either fluorescent- or bright-field images during the entire experimental period.
The spatial distribution of the terminal ends of the viable border-like cells that peeled toward the acropetal or basipetal direction was determined using threshold images of roots stained with fluorescein diacetate (Fig. 3B). The total number of peeled border-like cells in each 1-mm zone from the tip was counted for three roots at each time period. The

![Fig. 3. Lateral root-cap cells peeled from the root elongation zone. (A) Time course of changes in the root surface after the removal of the sheath in root apices of A. mangium (on the left) and soybean (on the right). Images of the roots stained with fluorescein diacetate under fluorescence (upper panel) and bright-field (lower panel) microscopy are shown. (B) An enlarged threshold image of fluorescence of fluorescein-diacetate-stained root of A. mangium at 6 h. The connecting points of acropetal (downward-pointing arrows) and basipetal (upward-pointing arrows) peelings of border-like cells to the root surface are indicated. Scale bars = 500 μm. (C, D) Spatial distribution of the connecting points of peeled border-like cells in A. mangium root apex 0 h (open columns), 6 h (light grey columns), and 24 h (dark grey columns) after removal of the sheath. The connecting points of the acropetal- (C) or basipetal peeling (D) of border-like cells at each 1-mm interval section were counted using the threshold images as shown in (B). Bars represent the mean ± s.d. (n = 3). No bars at each time point indicates no peelings with a corresponding direction in the interval.](https://academic.oup.com/aob/article-abstract/108/2/279/152596)
number of border-like cells that peeled in the acropetal direction was greater between the 1- and 4-mm tip sections at both 6 h and 24 h (Fig. 3C). However, the number of border-like cells that peeled across the basipetal direction was limited to the 0- to 1-mm tip section (Fig. 3D). The total number of peeled border-like cells was 3 times larger in the acropetal than in the basipetal peelings, and the numbers of peeling border-like cells that is directionally ungrouped were \(<1.0 \pm 0.3\) in each root sections (data not shown). These results suggest that spatial position may be a factor that influences the peeling direction of border-like cells.

**Root elongation drives border-like cell peeling**

The accumulation of border-like cells at low temperatures was examined further to determine if peeling was linked to root elongation. After removal of the sheath, roots of *A. mangium* were exposed to solution at 25, 21 or 18 °C for 24 h. The observation of root apices under a light microscope revealed that a smaller proportion of the root apex was covered by peeled border-like cells at lower temperatures (Fig. 4A). The projected areas of peeled border-like cells at 21 °C and 18 °C were, respectively 33.3 % and 69.7 % fewer than those at 25 °C (Fig. 4B). This reduction in peeled border-like cells was associated with the inhibition of root elongation, which was 42 % less at 21 °C and 65 % less at 18 °C (Fig. 4C).

Next the effect of root-tip removal on the formation of border-like cells and their attachment to the root was examined. After removal of the sheath, the root apex was excised 0-5, 1-0 or 1-5 mm from the tip. The root growth period was set to 48 h to evaluate the attachment position of the peeled border-like cells to the root surface. At 48 h after the removal of the sheath, roots without the 0.5-mm tip region accumulated similar amounts of border-like cells, peeling at the terminal position of the root compared with those in roots with tips (Fig. 5A). In contrast, roots without the 1-0-mm and 1-5-mm tip regions had few border-like cells on their surfaces at 48 h, though a small quantity of peeled border-like cells was present at 24 h. At both 24 h and 48 h, the border-like-cell area was the same in control roots with the tips and the roots without the 0-5-mm tip region (Fig. 5B). Conversely, the areas of peeled border-like cells in the roots that lacked the 1-0-mm and 1-5-mm portions of the tip were, respectively, 10 % and 55 % fewer than that of the control at 24 h and both were \(<20\%\) of control by 48 h (Fig. 5B). Root elongation was unaffected by the removal of 0.5 mm of the tip but was nearly arrested when the tips were excised at the 1-0-mm or 1-5-mm position over the 24- to 48-h period (Fig. 5C). These results further support the hypothesis that root elongation drives the peeling of border-like cells until the distal portion of the split position reaches the 0.5- to 1.0-mm tip region.

**Border-like cell detachment under friction**

In contrast to hydroponically grown roots, no sheath of peeled border-like cells surrounded the tip region of *A. mangium* roots in vermiculite soil (Fig. 6A). A root-box system was used to investigate the detachment pattern of border-like cells in filter paper and in vermiculite soil. In filter-paper conditions, the root system was held between two sheets of moist filter paper with a damper of polyurethane-form blocks in a plastic box. After growth in filter papers for 24 h or in vermiculite soil for 72 h, the roots were stained with fluorescein diacetate. Observations by both fluorescence and light microscopy confirmed that at least one sheet of peeled border-like cells was attached to the surface of all of the *A. mangium* roots after growth in both vermiculite soil and in filter paper (Fig. 6B, C). However, the distribution and numbers of peeled border-like cells on each root were variable (data not shown), possibly due to multiple factors (e.g. contact, moisture and root elongation rate) in the microenvironment. Meanwhile, no border-like cells were peeled from any of the soybean roots (n = 4; Fig. 6D). These results indicate that basipetal peeling by friction during root elongation may sever the connection between the root surface and border-like cells in the tip region, which could result in a lack of sheath development.

To understand the effect of different patterns of border-like cell peeling on the growth and development of *A. mangium* roots, root elongation rates and root cap parameters were compared with those of soybean under various growth conditions. In all sections of root apex of *A. mangium*, the numbers of cell layers of lateral root cap in solution were 1.3–1.7 times larger than those in moist filter paper (Table 1). In the filter-paper group, the number of cell layers in the 1- to 3-mm tip region of *A. mangium* (range 3.7 ± 0.6 to 1.7 ± 0.6) were comparable to those of soybean (3.5 ± 0.5 to 1.3 ± 0.5). In *A. mangium* seedlings, the root cap length from the columella initials to the tip was slightly shorter in the filter-paper group (724 ± 48 μm) than in the hydroponic group (923 ± 54 μm; Table 1). Under both growth conditions, the root cap lengths in the roots of *A. mangium* were longer than those of soybean. The root elongation rates were faster in moist filter papers than in solution in both species, although the increase in the proportion of the root elongation rates was greater in seedlings of *A. mangium* (206.7 %) than soybean (142.9 %; Table 2). Together, these results support the idea that detached border-like cells on the surface of *A. mangium* root apex may contribute to structural support for the control of root growth and columella root-cap development.

**Comparison to border cells**

To understand the factors that affect the formation and development of border-like cells in *A. mangium* roots, the aggregation patterns of cells detached from different zones of the root apex were profiled. The untouched tips of roots germinated in a moist Petri dish were used to collect border cells effectively (Hawes and Puppe, 1986). The exposure of a radicle root of *A. mangium* to solution instantly induced the release of border-like cells, which were partially attached around the 1-mm tip region (Fig. 7A). Meanwhile, border cells were dispersed from the 0- to 3-mm tip region in soybean, though no border-like cells were apparently detached (Fig. 7B).

To dispatch the border-like cells clogging the root surface, root apices were excised at the 1-mm and 3-mm tip positions before being directly immersed in the solution. Under a light microscope, the cells detached into the solution were classified according to cell type and spatial root zone. In the root sections
Fig. 4. Low temperature reduces the peeling of border-like cells in *A. mangium* roots. (A) Bright-field images of root apexes with peeled border-like cells at 25, 21 and 18°C at 0 h (top) and 24 h (bottom) after the removal of existing sheaths. Scale bar = 500 μm. (B) Projected areas of peeled border-like cells outside the roots are reduced at low temperature. The projected areas in bright-field images before and after temperature treatment were measured using WinRHIZO, and the incremental areas during the treatment are shown. (C) Effect of temperature on root elongation. Incremental root lengths at different temperatures for 24 h are shown. Bars represent the mean ± s.d. (n = 3–7).

Fig. 5. Peeling and accumulation of border-like cells in tipless roots of *A. mangium*. (A) Accumulation of peeled border-like cells in roots in which tip portions were cut 0, 0.5, 1.0 or 1.5 mm from the tip. Bright-field images of the roots 24 h (top) and 48 h (bottom) after the sheath removal and tip excision are shown. Scale bar = 1 mm. (B) Quantification of the projected areas of peeled border-like cells outside the roots. The increase in the areas in bright-field images during the 0–24 h and 0–48 h periods of tip excision was determined with WinRHIZO. (C) Root elongation after excision of the tip. Incremental root lengths during the 0–24 h and 24–48 h periods after excision treatment are shown. Bars represent the mean ± s.d. (n = 3–7).
of *A. mangium*, a similar number of both single border cells and border-like cells were released from the 0- to 1-mm tip section, whereas only border-like cells were released from the 1- to 3-mm section (Table 3 and Fig. 8A, D, E). In border-like cells from both root sections, the intercellular connection along the longitudinal lineage was minimally disturbed (Fig. 8A, D). Meanwhile, in soybean, border cells were released either singly or in aggregates from both the 0- to 1-mm and 1- to 3-mm tip sections (Fig. 8B, C, F, G). The proportions of singular border cells and aggregated cells were similar between the 0- to 1-mm (25/75%, single/aggregated) and 1- to 3-mm (38/62%) regions in soybean root apex (Table 3). The numbers of border-like cells detached from the roots of *A. mangium* were similar to those of soybean in the corresponding sections. These results suggest that the dissociation of intercellular connections is specifically repressed in the lateral root cap of *A. mangium*.

Finally, mucilage production was evaluated in the detached cells in a solution containing Indian ink. The blockage of dye intrusion into cells demonstrated that mucilage around the border-like cells of *A. mangium* was slightly obscured, as compared with those in single border cells of both species (Fig. 8H, J, K, L, N). Mucilage also surrounded soybean border cells in the aggregated form, in which each cell was attached to another in random positions (Fig. 8I, M). These results suggest that mucilage plays a minor role in the intercellular connections in the lateral root cap of *A. mangium*.

**DISCUSSION**

In the present study, it was found that the root apex of the woody plant *A. mangium* is covered with border-like cells with a distinct type of structure, which is quite different from those previously described (Kawata *et al.*, 1979; Gunawardena and Hawes, 2002; Vicré *et al.*, 2005; Hamamoto *et al.*, 2006). In the *A. mangium* root apex, a constitutive peeling of the border-like cells occurs irrespective of iron status or microbial association (Fig. 1B and Supplementary Data Fig. S1). Labelling of roots with a fluorescent dye revealed that the border-like cells bilaterally peel from the entire root apex, mainly in a region 1–3 mm behind the tip (Fig. 3A, C). Time course and tip-excision experiments demonstrated that the peeled border-like cells remain attached in the 0.5- to 1.0-mm tip region, forming a sheath around the root apex (Fig. 5A, B). Root structural analysis and the tip-excision experiments further confirmed that the lateral root cap is responsible for the peeling of border-like cells (Figs 2B and 5A, B). These results indicate that the border-like cells maintain strong intercellular connections even after detachment. Studies of root growth under different conditions suggested that shear stress caused by soil friction

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**Table 1. Quantification of columella root cap length and the number of lateral root cap layers in roots grown under hydroponic and filter paper culture conditions**

<table>
<thead>
<tr>
<th>Growth condition/species</th>
<th>Columella cap length (μm)</th>
<th>No. of cell layers of the lateral root cap at each distance (mm) from the quiescent centre</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
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<tr>
<td><strong>Hydroponic</strong></td>
<td></td>
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<tr>
<td><em>A. mangium</em></td>
<td>923 ± 54</td>
<td>9.0 ± 1.7</td>
</tr>
<tr>
<td>Soybean</td>
<td>472 ± 40</td>
<td>6.7 ± 1.2</td>
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<tr>
<td><strong>Filter paper</strong></td>
<td></td>
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<tr>
<td><em>A. mangium</em></td>
<td>724 ± 48</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Soybean</td>
<td>585 ± 22</td>
<td>5.7 ± 1.4</td>
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Using longitudinal histological sections as in Fig. 2, the distance from the tip to the columella root cap initials after 48 h of growth under the respective conditions was determined. The number of layers of the lateral root cap 1, 2, 3 or 4 mm away from the quiescent centre, just after the removal of the sheath, was counted.

Data are the mean ± s.d. (*n* = 3).
or inner cell expansion triggers bilateral peeling of border-like cells (Figs 3, 4 and 6). Spatial differences in the aggregation pattern of border cells between A. mangium and soybean seedlings implied that inhibition of cell dissociation is specific to the lateral root cap in the former species (Fig. 8A, D). Thus, this finding of border-like cells with strong intercellular connectivity could provide a new key phenotype to help uncover the underlying mechanisms in border cell formation for root growth and development.

Profiling detached cells from radicle roots revealed that the root tip region (0–1 mm) of A. mangium, which has open-type RAM, releases both single border cells and border-like cells (Table 3 and Fig. 8D, E). The number of single border cells from the root apex of A. mangium was comparable to that of soybean, which also has open-type RAM but releases no border-like cells. The production of both single border cells and border-like cells only in the tip region suggests that single border cell production is spatially repressed in the lateral root cap of A. mangium, especially far from the columella root cap. Previously, Hamamoto et al. (2006) found that the number of border cells released from the roots of 26 plant species is much higher in open-type RAM plant species than in closed-type RAM ones. To date, border-like cells have been identified in the root tip of A. thaliana and Nicotiana tabacum, both of which have closed-type RAM and release few single border cells (Vicre´ et al., 2005; Hamamoto et al., 2006). Tiers of border-like cells in A. thaliana are attached to the outermost columella daughter cells (Wenzel and Rost, 2001; Vicre´ et al., 2005). In the present study, the peeled border-like cells of A. mangium remained attached to the lateral side (0.5–1.0 mm) of the columella root cap region, which surrounded the entire root apex (Figs 1A, 2B and 5A, B). These differences suggest that a factor independent of RAM status may control border-like cell formation in A. mangium. In maize roots, dehydrated mucilage quickly expands to cover the whole root tip during hydration (McClully and Boyer, 1997). In the present study, however, it was found that less mucilage is deposited in the border-like cells, and that border-like cell detachment is constitutive irrespective of hydration status (Figs 6A–C and 8H, K). These findings support the idea that mucilage is unlikely to be the major cause of border-like cell development in the roots of A. mangium.

The lack of single border-cell detachment in the 1- to 3-mm region and the peeling pattern at the root tip suggest that the cell walls of the columella root cap may be hydrolysed into single border cells in A. mangium. Indeed, border cell production is linked to the spatial control of cell wall modification (Hawes and Lin, 1990; Durand et al., 2009). Expression of UDP-glucosyl transferase in roots is specific to the root cap (Woo et al., 1999). Conversely, border-like cell formation in the A. mangium root apex may be due to genetic factors that repress cell wall hydrolysis at the outer lateral root cap during root development. Furthermore, the presence of UV-reactive substances in the cell walls of detached border-like cells (Fig. 1F) supports the previous finding that secondary wall components, such as suberins and lignins, may be involved in reinforcing intercellular attachment (Clark and Harris, 1981). In arabidopsis, BRN1 and BRN2, which encode proteins similar to the class IIB NAC-type transcription factor that is associated with secondary-wall synthesis, are involved in increased adhesion of border-like cells at the root tip (Bennett et al., 2010).

### Table 2. Increase in elongation rates of A. mangium roots grown in filter paper

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth condition</th>
<th>Root elongation (mm 24 h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. mangium</td>
<td>Filter paper</td>
<td>19.3 ± 1.6</td>
</tr>
<tr>
<td>A. mangium</td>
<td>Hydroponic</td>
<td>9.3 ± 1.2</td>
</tr>
<tr>
<td>Soybean</td>
<td>Filter paper</td>
<td>65.2 ± 3.0</td>
</tr>
<tr>
<td>Soybean</td>
<td>Hydroponic</td>
<td>45.6 ± 4.2</td>
</tr>
</tbody>
</table>

Root elongation rates during 24 h of treatment under hydroponic or filter-paper conditions. Numbers in each column represent the mean ± s.d. 

### Table 3. Number of cells detached from the root apex of A. mangium and soybean

<table>
<thead>
<tr>
<th>Root tip section (mm)</th>
<th>Species</th>
<th>Number of single cells n (± s.d.)</th>
<th>Number of aggregated cells n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>A. mangium</td>
<td>3390 ± 2080 (53)</td>
<td>3030 ± 2710 (47)</td>
</tr>
<tr>
<td>Soybean</td>
<td>940 ± 610 (25)</td>
<td>2810 ± 1480 (75)</td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>A. mangium</td>
<td>0 (0)</td>
<td>2380 ± 670 (100)</td>
</tr>
<tr>
<td>Soybean</td>
<td>1100 ± 680 (38)</td>
<td>2640 ± 2120 (62)</td>
<td></td>
</tr>
</tbody>
</table>

Detached cells were divided into two groups (single cells and aggregated cells) according to cell profiling under light microscopy. Aggregates with less than four associated cells were counted as single cells. Numbers for n represent the mean ± s.d. (n = 3–6), and the ratio of the number to the total detached cells of the respective root sections is given as a percentage.
The outer periphery of the root cap continually receives various inputs from the soil during root elongation (Iijima et al., 2003). The peeling of border-like cells presumably contributes to the protection of the root elongation zone from pathogens or toxic metals via the replacement of old root surfaces with new, smooth ones. This function is expected to be more effective against adverse soil factors that are less invasive to the inside of the roots, because the root epidermis and outer cortex are effective in blocking the permeation of Al into the inner root (Jones et al., 2006). In soil, the basipetal peeling of border-like cells occurred in a ‘landslide’ manner associated with contact friction (Fig. 6A–C). This may have a ‘tunnel’ effect, reducing friction between the root and soil by passage of the root apex inside a tunnel of peeled border-like cells.

Roots of *A. mangium* have a long, well-developed columella root cap structure in which ties of peripheral cells overlap with those of lateral root cap cells (Table 1 and Fig. 2A, B). The cells of the lateral root cap and columella root cap in the adjacent tiers are synchronically propagated in arabidopsis roots (Dolan et al., 1993). In the *A. mangium* root apex, lateral root cap cells in an un-dissociated state may help support the structure of the columella root cap. A long columella root cap could also be effective in reducing the impact of soil compaction or pathogen attack. Conversely, strong intercellular connections in the border-like cells may negatively regulate root elongation. Indeed, a similar finding was reported in maize seedlings, in which the removal of the root cap temporally accelerated root elongation (Pilet, 1986).

The lateral root cap functions in root gravitropism through sedimentation of statoliths (Blancaflor et al., 1998) and the basipetal flow of auxin (Dolan et al., 1993; Friml et al., 2002; Swarup et al., 2005). In the present study, it was also confirmed by Lugol staining that statoliths accumulate in border-like cells up to the 1.0-mm tip region (data not shown). The mass peeling of the border-like cells by friction suggests that they may affect root bending via thigmotropic signalling and/or basipetal auxin flow. The removal of sheath of border-like cells makes the root more vulnerable to Al-induced bending (Endo et al., 2009). Auxin negatively regulates the peeling of arabidopsis border-like cells by repressing gene expression of endo-beta-1,4-D-glucanase (del Campillo et al., 2004).

Border cells are produced in most plant species, from gymnosperms to the most recently diverged clade of angiosperms (Hawes and Pueppke, 1986; Hamamoto et al., 2006). However, among 133 woody plants, including both gymnosperms and angiosperms, only the Japanese cedar, *Cryptomeria japonica*, and several species of the genus *Acacia* have similar border-like cells to those of *A. mangium* (H. Osawa et al., unpubl. res.). In addition to spatial regulation in root tissues, these phenotypic differences among plant species may be useful for determining genetic factors that regulate the formation of border-like cells.

In conclusion, a distinct type of border-like cells that have strong intercellular connections at the root apex was identified...
in A. mangium. Cell wall hydrolysis by the lateral root cap may regulate the development of the border-like cells through a repressive factor. Additionally, the covering and uncovering of border-like cells may play a role in the protection of root elongation from adverse growth conditions.

SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: Stereomicroscope images of the root apices, showing that peeling of border-like cells is unrelated to fungal colonization. Figure S2: Longitudinal and transverse sections of the root apex showing intracellular connections of border-like cells abundant with phenolic substances.

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