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Structure and Development of Stomata on the Primary Root of *Ceratonia siliqua* L.

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Stomata of various sizes are produced on the primary root of *Ceratonia siliqua* L. Most are generated during embryogenesis, prior to seed desiccation. They can be detected on the dry embryo in a wide zone just above the root tip. Initially, large stomata are formed. These have the ability to induce divisions of their neighbouring cells, creating particular cell patterns around them. Later, small perigenous stomata are generated. As the root grows following seed germination, the stomatal zone overlaps with that of the root hairs. Although root stomata of *C. siliqua* undergo a structural differentiation that seems almost identical to that of the elliptical stomata formed on leaves, they are unable to move and remain permanently open. Polarizing microscopy of fully differentiated stomata and young stomata at the stage of stomatal pore formation revealed deposition of radial cellulose microfibril systems on their periclinal walls. However, these systems were less developed than those on leaf stomata, a feature that might be responsible for their inactivity. Besides, plastids of the root guard cells (GCs) do not differentiate into chloroplasts but function solely as amyloplasts. Root stomata have a short life span. During rapid and intense root growth, GCs cannot keep pace with the elongation of their neighbouring rhizodermal cells. They therefore split in their mid-region, transversely to the stoma axis. The two parts of the transversely torn stoma are dragged apart and a large opening is formed on the root surface, just above the substomatal cavity. The root stomata, together with these openings, may facilitate increased gaseous exchange during respiration and/or an increased transfer of some nutrients and water in the rapidly growing primary root.

Key words: Root, stomata, structure, development, *Ceratonia siliqua.*

INTRODUCTION

As regulators of gaseous exchange in plants, stomata have developed a specific structure and unique physiology, both functionally coordinated to result in movement of guard cells (GCs) (Meidner and Mansfield, 1968; Galatis and Mitrakos, 1980; Palevitz, 1982; Sack, 1987). Stomata are ubiquitous in the epidermis covering all above-ground plant parts of primary structure, but, as a rule, they are absent from the rhizodermis (Esau, 1965; Fahn, 1982). However, stomata have been reported on the rhizodermis of the primary root of a few plant species (Tietz and Urbasch, 1977; Lefebvre, 1985; Christodoulakis and Psaras, 1987; Tarkowska and Wakowska, 1988).

Ceratonia siliqua (carob tree; Leguminosae) is a Mediterranean evergreen sclerophyllous species. The morphology and anatomy of the leaf bud, leaf, stem and root of this species and of other xeromorphic Mediterranean shrubs were studied in detail by Christodoulakis (1992). Stomata were noted to be scattered on the primary root of germinating seedlings of *C. siliqua*, above the root hair zone. The orientation of these stomata did not necessarily follow that of the rhizodermal cells. In all cases described to date, the stomata have been open and most of them have been suspended over a discrete substomatal cavity (Christodoulakis and Psaras, 1987). Although the occurrence of stomata on the root is an interesting and peculiar phenomenon, there is as yet no information on the structure, development and fate of these stomata, or their functional significance.

In this paper the structure and development of root stomata of *C. siliqua* are investigated. The probable function of these stomata during their short existence on the root is also discussed.

MATERIALS AND METHODS

Seeds of *C. siliqua* were treated with 65 % sulfuric acid for 10 min at room temperature to hydrolyse their impermeable waterproofing perisperm and to permit imbibition. Seedlings were allowed to grow for 2–7 d after germination and roots reached over 3 cm in length. Roots were then cut below the hypocotyl and the root tip was also excised at the distal boundary of the root hair zone.

Fresh whole mounts of roots were observed under a high magnification OLYMPUS stereomicroscope after staining briefly with 0·5 % toluidine blue 'O' in 1 % borax solution. Root paradermal sections were treated with 0·05 ppm abscisic acid (ABA) solution (Jang *et al*., 1996) and stomatal response to the hormone was observed under a ZEISS light microscope.

Roots, intact developing embryos and dry embryos were prepared for scanning electron microscopy (SEM). They were fixed in phosphate-buffered 3 % glutaraldehyde (pH

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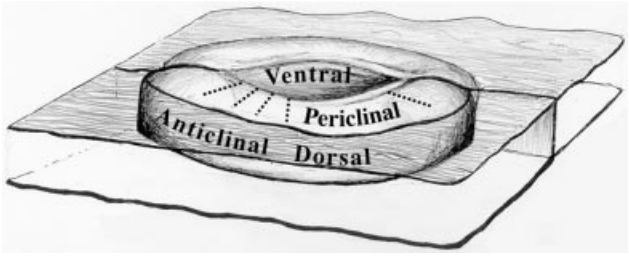

FIG. 1. Illustration of a root stoma. Guard-cell walls parallel to the root surface are considered periclinal. Walls perpendicular to the root surface are considered anticlinal. The inner anticlinal wall of the guard cell is the ventral wall. The outer anticlinal wall is the dorsal wall. Cellulose microfibrils within the guard cell wall are considered radial if they are oriented in the same way as the dotted lines (converging at the centre of the pore).

6·8) for 2 h, dehydrated in a graded series of acetone solutions, critical point dried, coated with gold–palladium and viewed in a Cambridge S150 Stereoscan SEM.

Root pieces (with root tip removed) were fixed in phosphate-buffered 3 % glutaraldehyde (pH 6·8) at 4 °C, for 2 h. They were post-fixed overnight with 1 % OsO_4, dehydrated in a graded ethanol series and embedded in Durcupan ACM (Fluka, Steinheim, Switzerland). Semithin sections were stained with 0·5 % toluidine blue 'O'. Ultrathin sections, double stained with uranyl acetate–lead citrate, were examined in a Phillips 300 transmission electron microscope (TEM).

To investigate the alignment of cellulose microfibrils in the periclinal walls of the GCs, free-hand paradermal sections and epidermal tissue peels (root pieces treated in 10 % nitric acid for 3 min at 100 °C) from 2- to 3-d-old roots were examined with a Zeiss Axioplan microscope equipped with a polarizing optical system. Epidermal tissue peels from mature leaves (leaf pieces treated with 30 % nitric acid for 5 min at 100 °C) were examined in the same way.

Light micrographs were taken in digital form and as conventional black and white negative and colour transparencies. Selected frames were printed on high quality, glossy photographic paper. Figure 1 illustrates some of the terms used to describe the orientation of stomatal walls and microtubules.

RESULTS AND DISCUSSION

SEM observations of dry embryos revealed that stomatal formation takes place during embryogenesis, before seed desiccation. Stomata are evenly distributed, in a normal pattern, across a wide zone over the tip of the radicle (Fig. 5A). Stomata also occur on the adaxial surface of the cotyledons of the dry embryo which, in *C. siliqua*, become the first pair of photosynthetically active leaves of the young plant. Root hairs were not observed on dry embryos. Examination of primary roots and free-hand paradermal root sections with the stereomicroscope revealed that stomata are distributed on the root surface just above the root hair zone (Fig. 2B). Some stomata appear elevated (Fig. 2F), while others are on the same level as rhizodermal cells. Stomata have not been found below the root hair zone, close to the root tip.

Careful examination of the rhizodermal cell lineages in free-hand and semithin sections from young primary roots of 2- to 5-d-old seedlings of *C. siliqua* revealed that stomata of various shapes and sizes are formed during different stages of embryo development (Fig. 3A). Initially, large stomata are formed. Their ventral wall is usually parallel to the root axis. In maturity, they are usually surrounded by rhizodermal cells that form a rosette pattern: two—rarely three—very elongated cells at each pole and up to five cells on each lateral side (Fig. 3B, C). Large stomata appear to have the ability to induce orientated divisions in their immediate rhizodermis. Rarely, one or both of the GCs of these stomata divide again, yielding stomata consisting of three or four GCs, respectively (Fig. 3D). Root stomata consisting of four guard cells have also been observed in *Helianthus annuus* (Tarkowska and Wacowska, 1988). To the best of our knowledge, such stomata have never been observed on leaves of *C. siliqua* or other plants. Large stomata degenerate quickly, leaving large rhizodermal gaps in their positions (Fig. 3E).

Small stomata are formed later in the same root zone. They are initiated by an asymmetrical cell division, producing a smaller cell—the GC mother cell—and a larger rhizodermal cell (Fig. 3F). These GC mother cells divide symmetrically in various orientations. In a few of them the ventral wall is oriented transversely to the root axis. Their stomatal pore opens before GCs assume a kidney-like shape (Fig. 3G). In roots of 2- to 3-d-old seedlings we observed stomata at different stages of differentiation as well as a few GC mother cells (Fig. 3H).

In young GCs, distinct thickenings are deposited at the junction of the mid-region of the periclinal walls with the ventral wall. The mid-region of the latter wall is also thickened along its whole depth (Fig. 5C). Formation of the stomatal pore starts from the internal- and/or the external-thickened portions of the ventral wall, usually the internal ones, and proceeds inwards (Fig. 4B, H). Ledges projecting over the stomatal pore are also formed (Fig. 5B, C). The dorsal walls remain thin. The young GCs possess a ribosomal cytoplasm and the expected complement of organelles, i.e. plastids, many mitochondria and endoplasmic reticulum membranes, active dictyosomes, microbodies and relatively small vacuoles. In angiosperm leaf stomata, the plastids undergo a particular differentiation into chloroplasts containing some grana and functioning as amyloplasts (Galatis and Mitrakos, 1980). In the root stomata of *C. siliqua*, plastids become loaded with starch but display very few internal membranes (Figs 4G and 5B, D, E). They never differentiate into chloroplasts even when roots develop under continuous illumination. Examination of free-hand sections from such roots using a fluorescence microscope revealed that the GC plastids are devoid of detectable quantities of chlorophyll.

The fact that GCs of the root stomata assume a typical kidney-like shape and form typical wall thickenings suggests that the morphogenetic mechanism functions normally. It is well known that GC shaping and stomatal pore formation are causally related to the deposition of

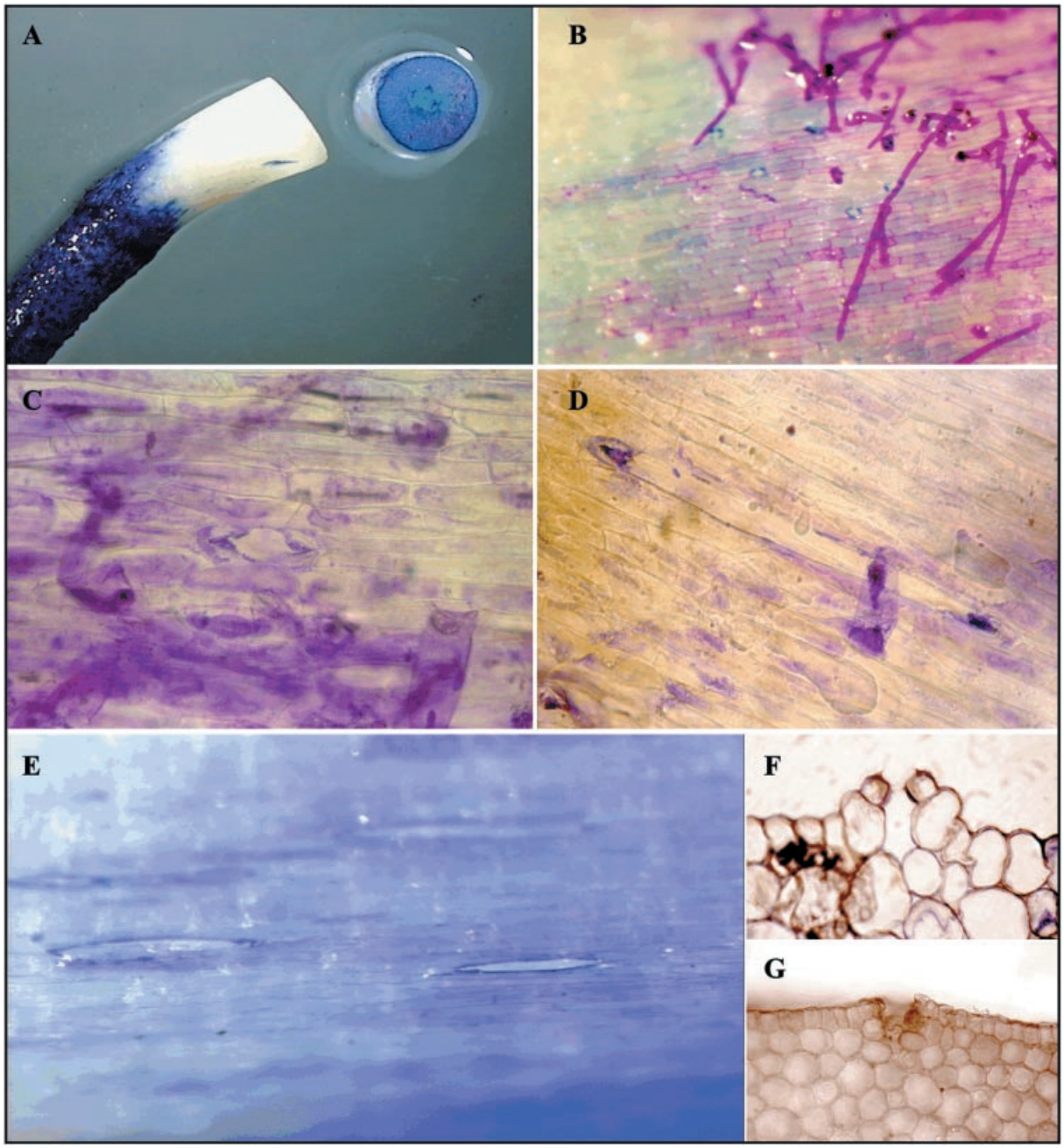

FIG. 2. A, Root tip following brief staining with 0·5 % toluidine blue 'O'. The protodermal cells are intensely stained. The radial arrangement of the conductive bundle (arrow) confirms that stomata are arranged on the root rather than on the shoot of the seedlings. B, Surface view of the rhizodermis stained by toluidine blue 'O' as seen under a stereomicroscope. Root hairs (red) and guard cells (blue; arrows) are differentially stained. C, Root stoma at the first stage of breaking apart (arrows indicate the two broken parts). D, Two halves (arrows) of the broken stoma have moved apart. E, Long fissures are produced by the broken stomata. The cavities formed are easily visible on the root surface (arrows). F, A typical elevated root stoma as observed on a paradermal section of a fresh root. G, A fissure and the cavity formed as observed in a cross-section of the root.

radial cellulose microfibril systems in the periclinal walls (Galatis and Mitrakos, 1980; Galatis *et al.*, 1983; Sack, 1987; Galatis and Apostolakos, 1991). These cellulose microfibrils converge on the junctions of the mid-region of the periclinal walls with the ventral one, where local wall thickenings emerge and, later, on the rims of the stomatal pore. Examining young root stomata at the stage of stomatal pore formation with a polarizing microscope revealed that the periclinal walls display a weak birefringence mirroring the deposition of radial cellulose microfibrils in them (Fig. 4A). This birefringence was also found in all differentiated stomata examined in acid-cleared rhizodermal sections (Fig. 4C, D)—an observation suggesting that after the stomatal pore opens the periclinal walls are reinforced with

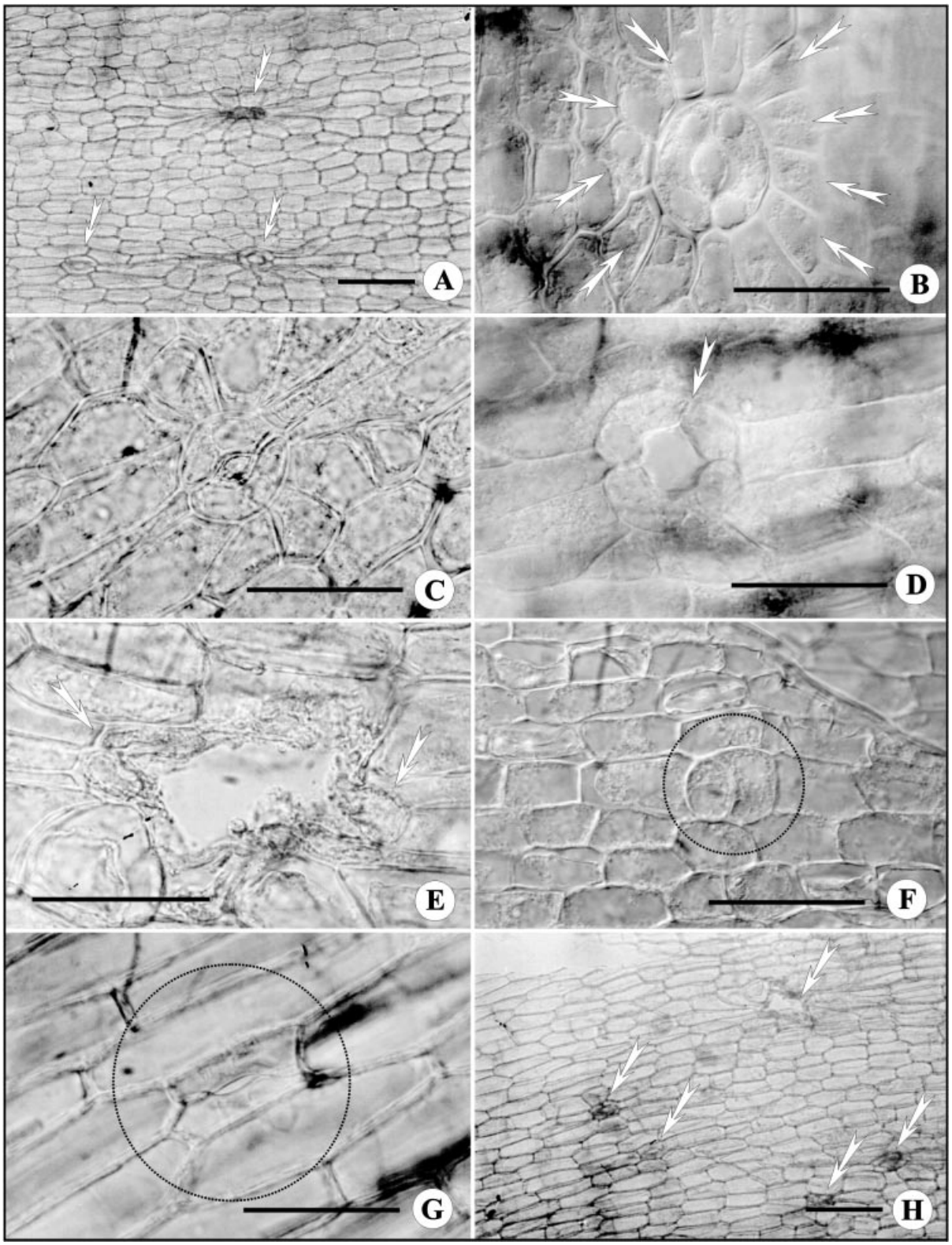

FIG. 3. A, Light micrograph of the root epidermis showing the various types of stomata. B, The rosette-like arrangement of the subsidiary cells (arrows) is clearly demonstrated using Nomarski optics. C, One of the large stomata of the root epidermis. The result of the oriented divisions induced by the GCs can be observed. D, A root stoma with three guard cells. The arrow indicates the abnormal transverse wall. E, A large, degenerated stoma. The rhizodermal gap left can be observed between the remnants of the guard cells (white arrows). F, A GC mother cell (circled). G, The pore between the GCs is open before the guard cells acquire their kidney-like shape. H, A part of the rhizodermis peeled off a 3-d-old root. Stomata at various stages of differentiation can be seen (arrows).

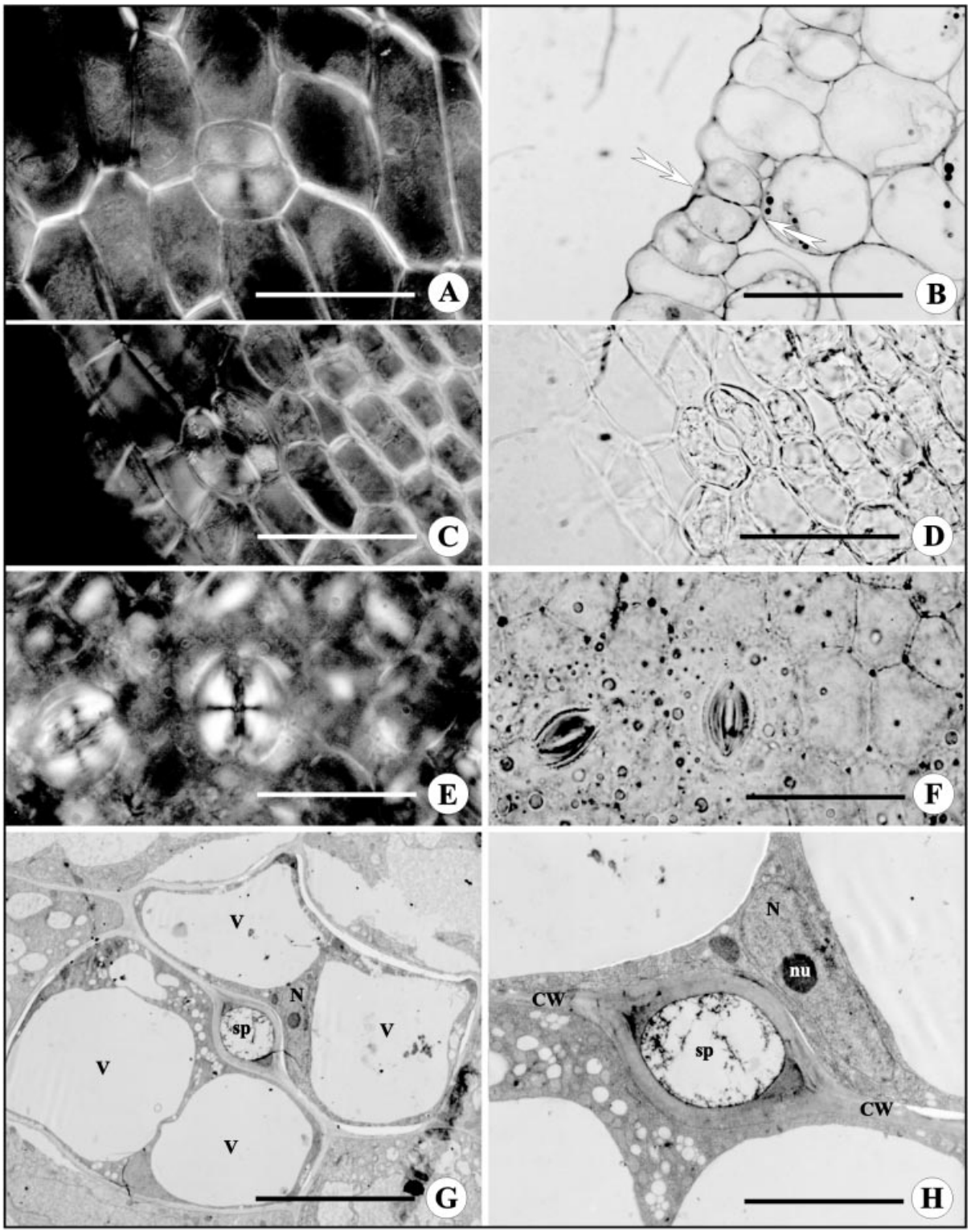

FIG. 4. A, A young root stoma at the stage of the stomatal pore formation. Polarizing microscopy reveals a weak birefringence of the periclinal walls mirroring the deposition of radial cellulose microfibrils. B, Cross-section of a root stoma indicating that stomatal pore formation starts from the internal- and the external-thickened portions of the ventral wall and proceeds inwards. C, Differentiated root stomata viewed with a polarizing microscope reveal a weak birefringence of their periclinal walls. It seems that following stomatal pore opening the periclinal walls have been reinforced with additional radial cellulose microfibrils. D, The same portion of the rhizodermis as in C, viewed with normal optics. E, Leaf stomata viewed with a polarizing microscope. The periclinal walls display a strong birefringence. F, The same portion of the leaf epidermis as in E, viewed with normal optics. G, Transmission electron micrograph of a stoma in a tangential section of the root. H, Detail of G. The stomatal pore and the cell wall with its peculiar thickening can be observed. V, Vacuole; sp, stomatal pore; N, nucleus; CW, cell wall; nu, nucleolus.

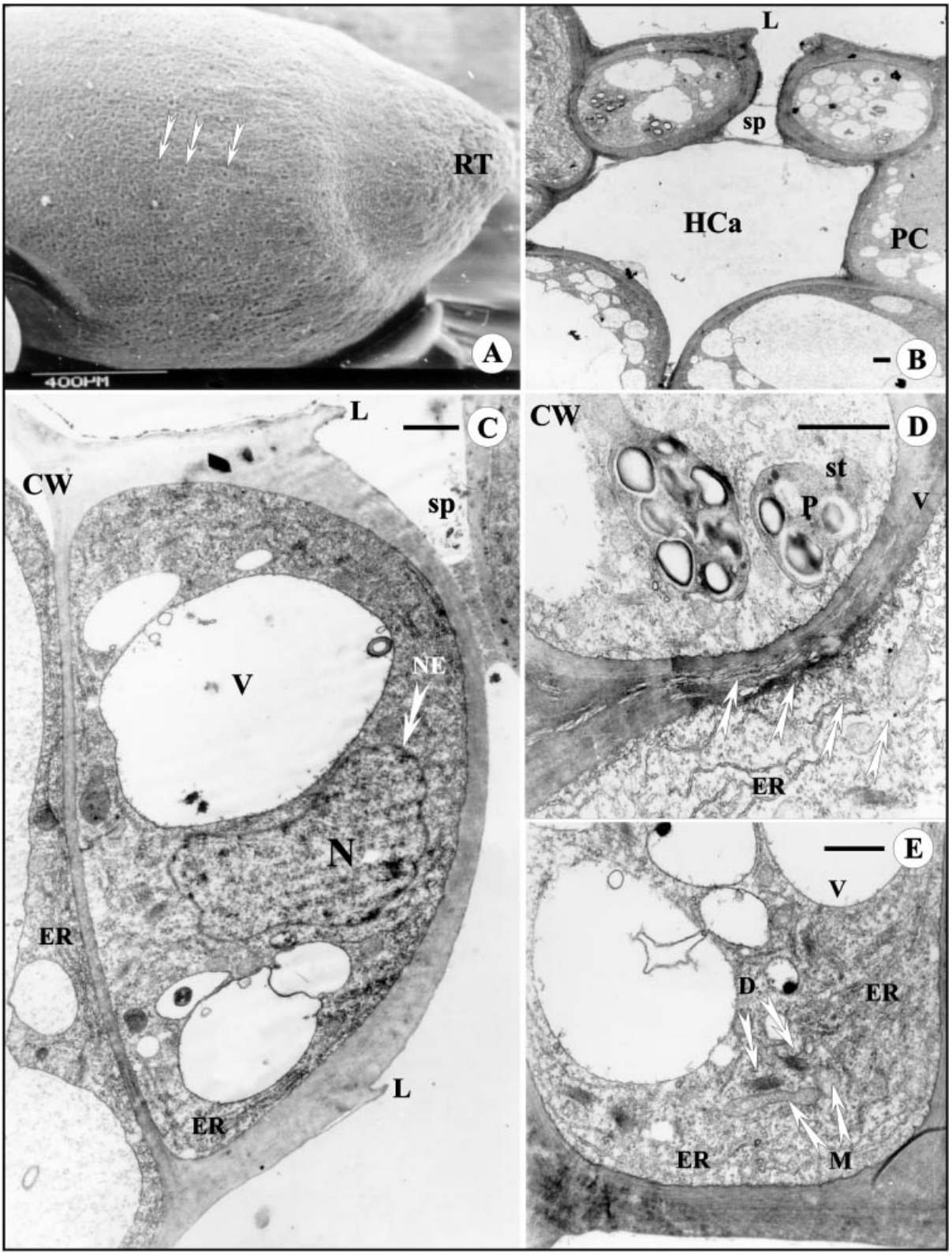

FIG. 5. A, The radicle of the embryo from a seed of *Ceratonia siliqua* observed in an SEM. Arrows indicate stomata. B, Section through the mid-region of a root stoma as viewed with a TEM. Cell walls, ledges, plastids, vacuoles, stomatal pore, hypostomatal cavity and subsidiary cells are clearly visible. C, Median cross-section of a root guard cell. D, Detail of the guard cell in B. Arrows indicate the fractured middle lamella. E, Detail of a guard cell. CW, Cell wall; D, dictyosome; ER, endoplasmic reticulum. Hca, hypostomatal cavity; L, ledge; M, mitochondrion; N, nucleus; NE, nuclear envelope; P, plastid; PC, subsidiary cells; st, starch granule; sp, stomatal pore; V, vacuole.

additional radial cellulose microfibrils. However, compared with leaf stomata (Fig. 4E, F), those of the root exhibit a weaker birefringence, suggesting that their radial cellulose microfibril systems are less well developed. As expected, the dorsal and ventral walls possess anticlinal cellulose microfibrils.

The dorsal walls of GCs seem to be forced to separate from the partner walls of the adjacent rhizodermal cells at the layer of the middle lamella (Fig. 5D). This 'violent' phenomenon can be explained only if the development of the root and the fate of the stomatal complexes are followed carefully. Root stomata have a short life span. During root elongation the rhizodermal cells elongate rapidly. However, the intervening GCs fail to follow this intense elongation and break in the middle. 'Half' of the stoma—composed of two halves of GCs, which are no longer alive, seems to move apart from the other 'half' (Fig. 1C). In this way, a long fissure is produced between the rhizodermal cells parallel to the root axis (Fig. 2D). Below that fissure a hollow area is created, obviously from the forcibly extended hypostomatal cavity (Fig. 2E, G).

Graminaceous GCs, before becoming dumb-bell shaped, transiently acquire a typical kidney-like shape. Highly coordinated elongation forces exerted on the young elliptical stomata, accompanied by intense wall deposition on the expanding wall areas, transform the kidney-like GCs into a dumb-bell-like shape. The radial cellulose microfibril systems in the periclinal walls, in association with the anticlinal ones in the dorsal and ventral walls, allow the GCs to elongate at their mid-region only (Galatis, 1980; Palevitz, 1982). In the root stomata of *C. siliqua*, GC elongation is unable to keep pace with the rapid and intense elongation of the adjacent rhizodermal cells and therefore the stomata break in the mid-region.

Formation of extensive epidermal openings by transverse disruption of stomata has not been described in roots or in other plant organs examined to date. Via these large openings, the internal tissues of the primary root are in direct contact with the external environment. Thus, increased gaseous exchange between the root and its environment, as well as increased transfer of some nutrients and water into the primary root, may be facilitated.

Root stomata of *C. siliqua* do not seem to respond to ABA treatment (which usually induces stomatal closure) and, like the hydathode stomata, they remain open permanently. The same is true for root stomata of *Pisum arvense* and *Ornithopus annuus* (Tarkowska and Wacowska, 1988). Lefebvre (1985) reported both open and closed stomata in roots of *Pisum sativum*. However, it is not clear whether the *Pisum* root stomata are inactive or whether some of them do not form a stomatal pore. It is well established that changes in turgor pressure induce alterations in GC shape, which bring about opening and closing of the stomatal pore (Galatis and Mitrakos, 1980; Jarvis and Mansfield, 1981).

The inability of the *C. siliqua* root stomata to move may be caused by: (*a*) malfunction of the biochemical mechanism controlling the turgor changes underlying stomatal movement; (*b*) inability of the GC wall structure to transform the turgor changes into the appropriate changes in GC shape; or (*c*) because the rhizodermal cells adjacent to the GCs are devoid of the appropriate shape and/or wall structure to support stomatal movement. Since the periclinal walls of the differentiated root GCs display a less-developed radial cellulose microfibril system compared to that of leaf stomata (see above), the second hypothesis seems most plausible. The radial cellulose microfibril systems are of fundamental importance not only for GC morphogenesis–stomatal pore formation (Galatis and Mitrakos, 1980; Galatis, 1980; Galatis *et al*., 1983; Galatis and Apostolakos, 1991; Apostolakos and Galatis, 1998, 1999), but also for stomatal movement (Meidner and Mansfield, 1968; Aylor *et al*., 1973).

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