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BRIEF COMMUNICATION

DEVELOPMENTAL PROGRAMMED CELL DEATH IN PRIMARY ROOTS OF SONORAN DESERT CACTACEAE[1]

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Primary roots of two species of Sonoran Desert Cactaceae, *Stenocereus gummosus* and *Pachycereus pringlei*, have a determinate pattern of growth: meristematic cells divide only for a limited time and then differentiate. Detecting DNA fragmentation by *t*erminal deoxynucleotide transferase-mediated d*U*TP *n*ick-*e*nd *l*abeling (TUNEL), we have shown that programmed cell death (PCD) was not involved in meristem exhaustion. However, we found TUNEL-positive nuclei in the root hair and root cap cells of both species. Programmed cell death in root hair cells has not been previously reported, and the pattern of PCD events in the root cap differed from that described earlier. These data suggest that in the studied Cactaceae, PCD is involved in developmental adaptations related to the formation of a compact root system important for rapid seedling establishment in a desert environment. Participation of PCD in developmental loss of the root cap and in root hair renovation proposed in the current study implicates an evolutionary conserved link between PCD and differentiation processes in plants.

Key words: Cactaceae; determinate root growth; *Pachycereus pringlei*; plant development; programmed cell death; *Stenocereus gummosus*.

Programmed cell death (PCD) is an essential process during plant growth and development. It occurs in response to pathogen invasion (reviewed in Greenberg and Yao, 2004) and various forms of abiotic stress, for example, high saline concentrations (Katsuhara and Kawasaki, 1996). A variety of tissues, including tissues of germinating seeds, xylem tracheary elements, reproductive organs or their primordia, senescent leaves, and petals, undergo PCD as a normal part of their development (see Pennel and Lamb, 1997; Jones, 2001; Kuriyama and Fukuda, 2002; for reviews). Recently, PCD was shown to be involved in the elimination of all but one embryo in a polyembryonic pine seed (Filonova et al., 2002) and of weaker shoots in pea seedlings (Belenghi et al., 2004).

The most extensively studied form of PCD is animal cell apoptosis, which is associated with a distinct set of biochemical and physical changes involving the cytoplasm, nucleus, and plasma membrane (see Pennel and Lamb, 1997; Lawen, 2003; for reviews). At the cellular level, apoptotic animal cells shrink and cell junctions are disintegrated. Within the nucleus, their chromatin condenses, aggregates into dense compact masses, and is fragmented by endonucleases. The nucleus itself becomes convoluted and buds off into several fragments (Lawen, 2003). Different forms of plant PCD share some of these features with animal apoptosis. Although plant PCD may be categorized by cytological features into at least three different types (Fukuda, 2000), fragmentation of nuclear DNA, while not always resulting in internucleosomal fragmentation (Gunawardena et al., 2004), is detectable by *t*erminal deoxynucleotide transferase-mediated d*U*TP *n*ick-*e*nd *l*abeling (TUNEL) for all these types. A number of nucleases have been shown to play a role in plant PCD (Muramoto et al., 1999; Ito and Fukuda, 2002).

We previously found a determinate growth pattern in primary roots of some Sonoran Desert Cactaceae (Dubrovsky, 1997; Dubrovsky and North, 2002; Dubrovsky and Gómez-Lomelí, 2003). In these species meristematic cells divide only for a limited time followed by a short period of rapid differentiation. In the species studied in this work, primary roots grow up to 3 d in *Stenocereus gummosus* (Engelm.) Gibson & Horak (Dubrovsky, 1997) and up to 8 d in *Pachycereus pringlei* (S. Watson) Britton & Rose (Dubrovsky and Gómez-Lomelí, 2003). We demonstrated that one of the mechanisms involved in the meristem exhaustion is the temporary establishment (*P. pringlei*) or complete lack of (*S. gummosus*) the quiescent center in the root apical meristem (Rodríguez-Rodríguez et al., 2003). We also hypothesized that PCD events may be involved in the primary root meristem determinacy in the studied species. Here we report that the PCD occurs in the root hairs and in the sloughing root cap of these Cactaceae and that PCD is not involved in the meristem exhaustion.

MATERIALS AND METHODS

Plant growth conditions and microscopy—Fruit of *Stenocereus gummosus* (Engelm.) Gibson & Horak and of *Pachycereus pringlei* (S. Watson) Britton & Rose were collected from plants growing in native populations near La Paz, Baja California Sur, Mexico. Seeds were sterilized in 60% commercial bleach supplemented with 0.08% Triton X-100, washed four times in sterile water, and germinated in 15-cm petri dishes on 20% Hoagland's medium (Sigma-Aldrich, St. Louis, Missouri, USA) solidified with 0.8% agar. Petri dishes with growing plantlets were maintained vertically in a growth chamber at 28° ± 0.5°C under light of 190 μmol · m^{-2} · s^{-1} and a 12-h photoperiod. The roots were fixed in ice-cold fixative (2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4), gradually dehydrated and embedded in Historesin

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(Leica Instruments, Heidelberg, Germany), and sectioned with a glass knife on a Leica RM2155 microtome (Cambridge Instruments, Nussloch, Germany) as described (Rodríguez-Rodríguez et al., 2003). Sections of 3 μm were mounted on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, Missouri, USA). For scanning electron microscopy, the roots were fixed in 1.5% glutaraldehyde and 0.3% paraformaldehyde in 25 mM PIPES buffer, postfixed in 1% osmium tetroxide, dehydrated, critical-point dried, sputter-coated, and observed with a Hitachi S-3500N scanning electron microscope (SEM; Hitachi, Ibaraki, Japan).

For detection of dead cells, live roots of *S. gummosus* plants of 2–10 d after germination were incubated for at least 5 min with 1 $\mu g \cdot mL^{-1}$ propidium iodide (Sigma), which stains nuclei of only damaged or dead cells and excludes from the living plant cells (Oparka and Read, 1994). To avoid detection of cells damaged during handling, great care was taken during image acquisition. Because root hairs could be damaged by the coverslip itself, no images of root hairs lying within 33 μm from the coverslip were taken. Roots were observed under a laser scanning confocal microscope LSM 510 Meta (Zeiss, Oberkochen, Germany) using the 543-nm line of a He/Ne laser, BP565-615 filter, and two-channel mode (red channel of propidium iodide emission, and bright field). Zeiss Image Examiner software version 3.2, was used for image analysis.

In situ detection of DNA fragmentation (TUNEL assay)—Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling was performed using an ApopTag fluorescein in situ apoptosis detection kit for indirect immunofluorescence staining (Intergen Co., New York, New York, USA) with minor modifications of the manufacturer's instructions. Briefly, root sections were treated with proteinase K for 15 min, with TdT enzyme for 1.5 h at 37°C, and with anti-digoxigenin-fluorescein isothiocyanate (FITC) conjugate for 1 h at room temperature. Either root sections of salt-treated plantlets or DNAse I treatment were used as a positive control for the TUNEL assay. For salt treatment, *S. gummosus* seedlings (24 h after germination) were transferred to the medium supplemented with 500 mM NaCl and cultivated for another 8 h. For DNAse I treatment, the sections were preincubated in DN buffer (30 mM Tris-HCl pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT) for 5 min and incubated with DNAse I (500 μg/mL in DN) for 15 min at room temperature. Omission of TdT enzyme in the reaction served as a negative control. For *S. gummosus*, six roots at late stage one, 12 roots at stage two (early and late), and 12 roots at stage three (early and late) were analyzed using four sections of 1–2 mm apical portion of each root. For *P. pringlei*, four roots at stage two were analyzed using 6–8 sections of 1–2 mm apical portion of each root. After TUNEL staining, sections were mounted in 50% glycerol, 0.15% *n*-propylgallate, containing 0.5 μg/ml 4′6-diamidino-2-phenylindole (DAPI) and observed under an epifluorescence Zeiss Axioskop microscope.

RESULTS AND DISCUSSION

Stenocereus gummosus and *P. pringlei* grown under the described conditions exhibited the typical determinate growth pattern reported earlier (Dubrovsky, 1997; Dubrovsky and Gómez-Lomelí, 2003). Three stages have been defined during root development in these species (Rodríguez-Rodríguez et al., 2003). The SEM image of a recently emerged *S. gummosus* primary root at stage one demonstrates typical growth and the beginning of root hair formation (Fig. 1A). At this stage, the meristem, the elongation zone, and the differentiation zone can be recognized. The root apical meristem is maintained at approximately the same length (Dubrovsky, 1997). At stage two, roots continue growth, but the meristematic activity begins to decrease, both the meristem and elongation zone become shorter, and root hair formation is approaching the root tip. At this stage, the roots become thinner as the number of cell layers within the meristem is decreasing due to the lack of activity in the initial cells (Rodríguez-Rodríguez et al., 2003). At stage three, root growth is completed, the root apical meristem is exhausted, the elongation zone is not present, and the root hairs cover the root up to the very tip (Fig. 1B). In *S. gummosus*, the TUNEL assay was performed on roots over all three developmental stages. In an analysis of stage two roots of *P. pringlei* DNA fragmentation was found in the same tissues as for *S. gummosus*.

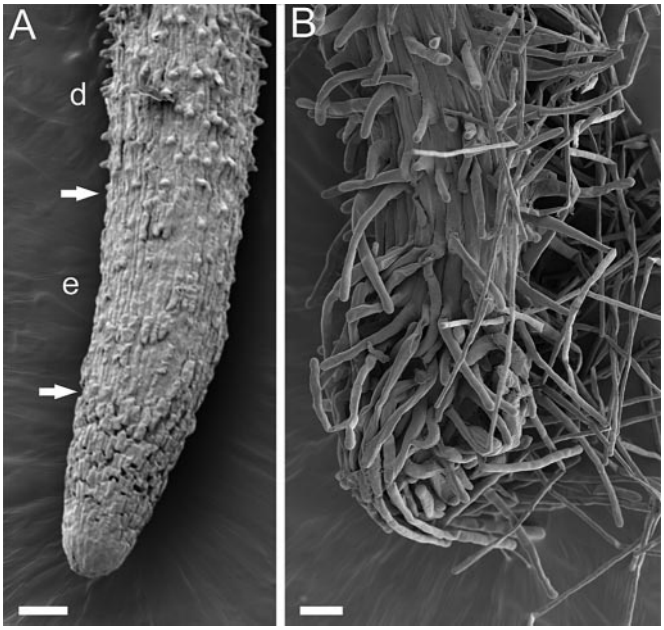

Fig. 1. Scanning electron microscopy (SEM) images of *Stenocereus gummosus* root tips. (A) A recently emerged primary root at stage one. Lower arrow marks the approximate border between the root apical meristem and the elongation zone (e), the upper arrow is between the elongation and differentiation zones (d). (B) A primary root at stage three that has terminated growth. Scale bars: (A) 100 μm. (B) 50 μm.

We first analyzed whether PCD plays a role in meristem exhaustion. We detected DNA fragmentation in meristematic cells of roots treated with 500 mM NaCl (Fig. 2A, B), but did not find any TUNEL-positive nuclei in the primary meristem of untreated roots, neither at developmental stages one and two nor in the ex-meristematic cells at stage three ($N = 30$ for *S. gummosus*, $N = 4$ for *P. pringlei*). Salt stress is known to induce PCD in roots of different plant species (Katsuhara and Kawasaki, 1996; Huh et al., 2002), and Cactaceae in general are considered to be salt-sensitive species (Nobel and Bobich, 2002); therefore, we used sections of salt-treated roots as a positive control for DNA fragmentation. Non-apoptotic PCD without DNA degradation has been described in animals (Sperandio et al., 2000; Castro-Obregón et al., 2002), but no analogous mode of PCD has been reported to date in plants. These data suggest that PCD, belonging to those types described for plants, is not involved in the meristem exhaustion of determinate roots in these Cactaceae. The meristem exhaustion and root determinacy in these species may result from the absence of a permanent quiescent center in the root apical meristem (Rodríguez-Rodríguez et al., 2003).

Using the TUNEL assay, we did find DNA fragmentation in the root hair and root cap cells. The TUNEL-positive nuclei were found in root hair cells in both species (Fig. 2C–J). The majority of these nuclei had characteristics of chromatin condensation (Fig. 2E–H) or nuclei fragmentation (Fig. 2C, D), features shared by distinct types of plant PCD and animal ap-

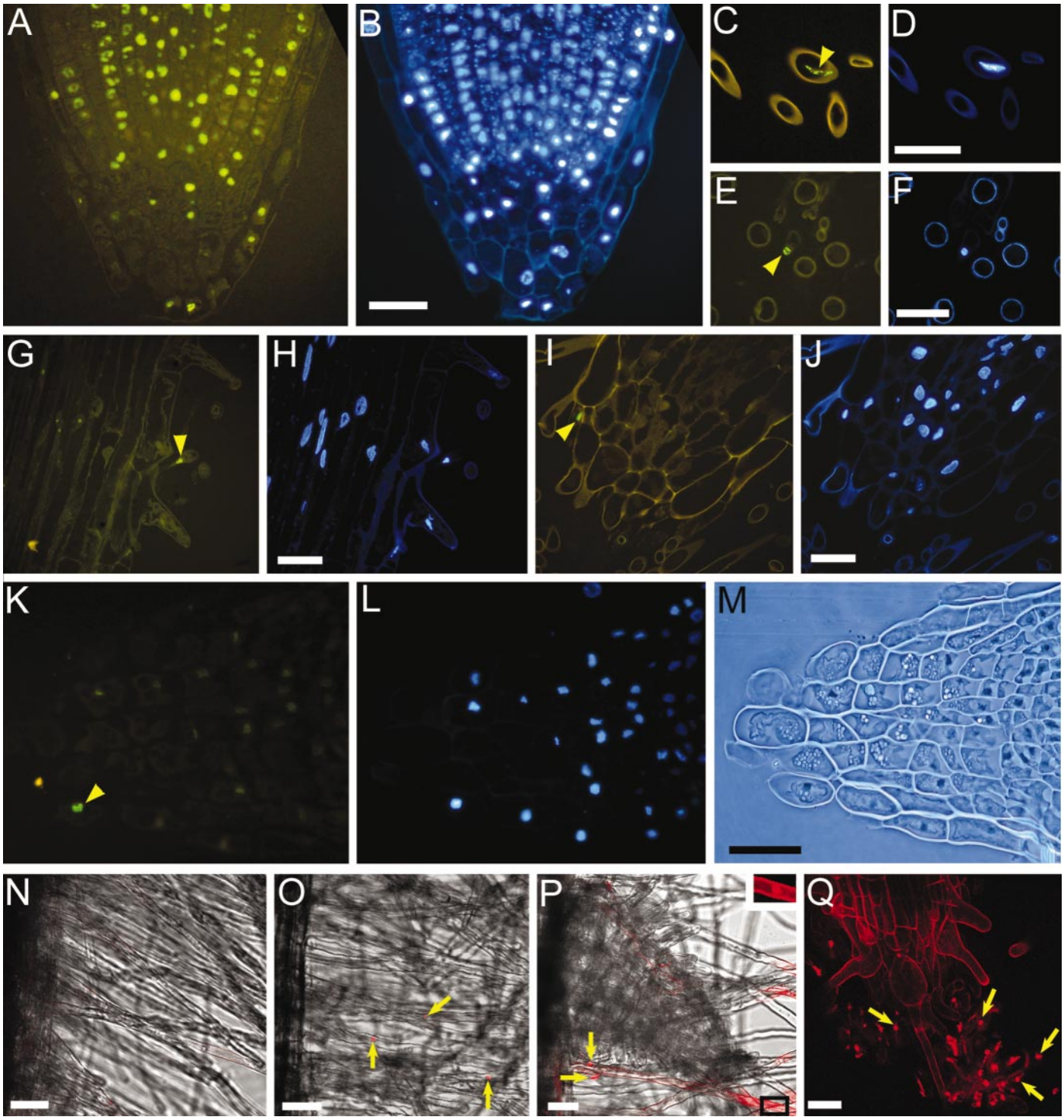

Fig. 2. Programmed cell death in determinate primary roots. (A–D, G–J) *Stenocereus gummosus*. (E, F, K–M) *P. pringlei*. (A–L) TUNEL-positive nuclei that fluoresce green were labeled with fluorescein-isothiocyanate (FITC) using TUNEL reaction; nuclei stained with 4′-6-diamidino-2-phenylindole (DAPI) fluoresce blue. Paired micrographs are of the same section but with filters set for each stain. (A) FITC and (B) DAPI, positive control for PCD: apex of a salt-treated root at stage one. (C) FITC, (D) DAPI, nuclear fragmentation in a root hair cell. (E) FITC, (F) DAPI, TUNEL-positive nucleus in a root hair cell. (G) FITC, (H) DAPI, TUNEL-positive nucleus in a root hair cell. (I) FITC, (J) DAPI, DNA fragmentation in a root hair cell near the apex after meristem exhaustion and growth cessation. (K) FITC, (L) DAPI, Single TUNEL-positive nucleus in the root cap. (M) Phase contrast image of the same section as in K and L. Open meristem organization and the root cap are shown. (N–Q) Laser scanning confocal microscope images of *S. gummosus* roots stained with propidium iodide. Nuclei of dead cells are red. (N–P) Merged images of bright field and red channel. (Q) Red channel image only. (N) Optical section of a young 2-d-old root, the lack of red nuclei means that no cells are dead. (O) Root hair cells in 8-d-old plants (projection of four serial, optical sections) with dead cells. (P) Optical section of root hairs close to a recently emerged lateral root in 10-d-old plant. Inset is a red channel close-up of outlined area with the nucleus of the root cap cell stained with propidium iodide. (Q) Root tip of an 8-d-old plant (projection of 12 serial, optical sections) with dead cells of the sloughed root cap. Arrowheads indicate TUNEL-positive nuclei; arrows indicate the propidium-iodide-stained nuclei. Scale bars: (A–M) 50 μm. (N–Q) 200 μm.

optosis (Pennel and Lamb, 1997; Lam, 2004). Cell walls of the root hair cells containing TUNEL-positive nuclei typically had less autofluorescence than walls of root hair cells with intact nuclei (Fig. 2E–J). These three additional features further suggested that the cells with TUNEL-positive nuclei did undergo PCD. To date, developmental PCD in root hair cells has not been reported in plants.

In the studied species, the determinate nature of primary-root growth is important for the induction of lateral roots. In turn, most lateral roots terminate their growth shortly after emergence and form laterals of the next order. This iterative process leads to the formation of a compact root system, which is required for successful seedling establishment in the desert environment (Dubrovsky, 1997; Dubrovsky and Gómez-Lomelí, 2003). Recently emerged lateral roots are densely covered with new root hairs and thus contribute to ecological fitness. In Cactaceae, each epidermal cell in each cell file is capable of forming root hairs (Fig. 1 and Pemberton et al., 2001). To maximize water and nutrient use efficiency, the maintenance of existing root hairs on lateral roots of previous orders appears to be less important than the formation of new ones on emerging lateral roots. This may explain why root hair cells were subjected to PCD. The root hairs are important for mineral uptake, especially for phosphorous, which is a limited resource in desert soils (Bates and Lynch, 2000). Optimal periods for seedling establishment in the Sonoran Desert are very short. Part of the general survival strategy of these succulent plants may be the ability to adapt to environmental changes through the rapid formation of clusters of sympodially branched short lateral roots, as well as the loss of older root hairs via PCD concurrent with the formation of new root hairs developed on recently emerged laterals, referred to here as ''root hair renovation.'' Also, when seedlings are established, PCD in the root hair cells may occur during a long drought, a question that requires further study.

The percentage of the TUNEL-positive nuclei in the root hair cells during stages two and three was 2.2 $\pm$ 0.3 and 3.6 $\pm$ 1.1, respectively (percentages estimated in the roots where TUNEL-positive nuclei were found; means $\pm$ SE). We observed a tendency towards an increased percentage of such nuclei and an increased incidence of roots containing such nuclei (Fig. 3) with more advanced stages. When data for all roots were analyzed, including those with no TUNEL-positive nuclei (0%), an increase in the percentage from stage two to stage three was significant at $P < 0.1$ (Student's independent *t* test). Because the initial objective of this work was to investigate whether PCD was involved in root meristem exhaustion, a detailed TUNEL analysis was performed for sections of 1–2 mm apical portion of roots of *S. gummosus* at all three developmental stages. In addition, we also analyzed 1–2 mm basal portion of some roots of *S. gummosus* at developmental stage three. Basal portions of three of four analyzed roots at late stage three had TUNEL-positive nuclei (5–13% of total nuclei). These values are comparable with the percentage of root meristematic cells (5–10%) undergoing mitosis (mitotic index), although all meristematic cells in growing roots are proliferatively active (Baskin, 2000).

Because apoptosis in animals is recognized as a rapid process once it is invoked (Wang et al., 1996), DNA fragmentation in plant cells undergoing PCD probably takes place in a very short time (D. Gilchrist, University of California, Davis, California, USA, personal communication). Therefore, a relatively low percentage of TUNEL-positive nuclei in root hair and root cap cells could be explained by the fact that PCD in these cells is not synchronized.

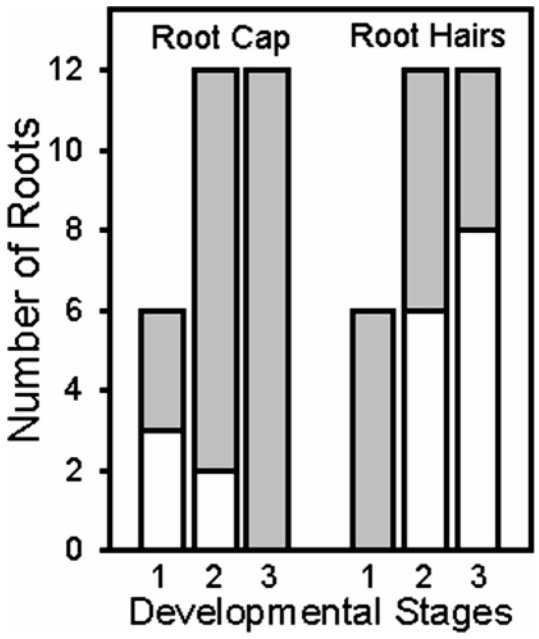

Fig. 3. Incidence of roots of *Stenocereus gummosus* with TUNEL-positive nuclei in the root cap or root hair cells. Shaded bars, total number of analyzed roots per stage; open bars, number of roots with TUNEL-positive nuclei. Four sections of each root were analyzed. Root caps were absent in some roots at late stage two and in all roots during stage three.

We also have found TUNEL-positive nuclei in root cap cells in both species (Fig. 2K–M). In the studied Cactaceae, the root cap was present only during the first two stages of primary root development. During stage one, the columella comprised 4–5 and 5–7 tiers of cells in *S. gummosus* and *P. pringlei*, respectively. During stage two, the number of cell tiers progressively diminished as a result of the loss of peripheral cells in the columella and lateral root cap usually resulting in a complete loss of the root cap sometime during the late second or early third stages. In the Cactaceae studied here, the root cap is a temporary structure that consists of a relatively small number of cells compared to other species, and it becomes smaller before it is lost. In onion roots, for which PCD in the root cap was first reported (Wang et al., 1996), the root cap is rather large, and almost all nuclei in several outer tiers of the root cap cells were TUNEL-positive, indicating that fragmentation of nuclei is synchronized in the onion root cap cells. Similarly, in the root cap of *Arabidopsis thaliana*, TUNEL-positive nuclei were found in various cells of the two outer layers (Zhu and Rost, 2000). In this study, TUNEL-positive nuclei were found in the outermost layer of the root cap, but in contrast to the previous studies on onion and *Arabidopsis*, nuclei of only a few root cap cells were TUNEL-positive, and therefore, PCD events did not appear to be synchronized (Fig. 2K–M). The percentage of TUNEL-positive nuclei in the root cap cells was not estimated because of the very low number of nuclei in the root cap cells per section.

The root cap is maintained as a result of the activity of the root cap initial cells. These cells are in direct contact with the quiescent center, which maintains their nondifferentiated state and proliferative activity (Van den Berg et al., 1997; Aida et al., 2004). In the species studied here, the quiescent center, if

established, exists for only a short time (Rodríguez-Rodríguez et al., 2003). The loss of the root cap apparently is related to the loss of meristem integrity that results from absence of a permanent quiescent center. Therefore, mechanisms of PCD regulation in the root cap appear to differ in species with determinate and indeterminate root growth.

Propidium iodide, a dye that only stains the DNA of damaged or dead cells (Oparka and Read, 1994), was used as an additional means to visualize dead cells in the live roots. In young roots of *S. gummosus*, 2–4 d after germination, stained nuclei were not generally found (Fig. 2N). In root hairs of 8–10-d-old plants, 1–5 stained nuclei could be found within the field of view (Fig. 2O). In recently emerged lateral roots, the nuclei of neither the root hairs nor the root cap were stained (Fig. 2P). Close to the lateral root, in the area where root hairs are protected from possible damage caused by handling, root hairs with stained nuclei were found (Fig. 2P). Stained nuclei of already sloughed root cap cells were frequently observed similar to those depicted in Fig. 2Q. This data confirms that cell death occurs in the same cell types for which we detected TUNEL-positive nuclei.

In summary, we showed that root hair and root cap cells of *S. gummosus* and *P. pringlei* appear to undergo PCD. The pattern of PCD events in the root cap of these cactus species differs from that described in other species. Establishment of a temporary quiescent center or its absence leads to meristem exhaustion and growth cessation in determinate roots of Sonoran Desert Cactaceae (Rodríguez-Rodríguez et al., 2003). This study indicates that PCD is not involved in this process. Participation of PCD in the developmental loss of the root cap and in root hair renovation proposed in the current study implicates an evolutionarily conserved link between PCD and differentiation processes.

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