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Distribution of stone cell in Asian, Chinese, and European pear fruit and its morphological changes

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Summary

This study was conducted to microscopically verify the distribution and morphological changes occurring in stone cells during fruit growth in order to determine physiological changes occurring in stone cells in pear fruits. European pear (*P. communis* L. cv. 'Bartlett'), Chinese pear (*P. bretschneideri* Rehd. cv. 'Yali'), and Asian pear (*P. pyrifolia* Nakai cv. 'Niitaka') were collected from three trees of each cultivar for microscopic observation at 60 DAFB. Also, 'Niitaka' pear fruits were harvested at development stages of 30, 60, 90 and 150 DAFB. Stone cells were observed via light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The stone cells were found to be clustered less profoundly in the 'Niitaka' and 'Yali' pears than in 'Bartlett' pears, and the sizes of the clusters were smaller. Also, the stone cells were clustered closer to the epidermis in the 'Niitaka' and 'Yali' pears than in the Bartlett pears. Stone cells appeared in cluster structures beginning at 60 DAFB. The relative decrease in the quantity of stone cell clusters in the flesh was attributed to the fact that stone cells were no longer being generated, and the flesh cells increased dramatically in size. Developing and completed stone cells existed together within the same stone cell cluster.

Introduction

The deposition of large numbers and sizes of stone cell aggregates in pears has long been known to lower the quality of pear fruit, as the existence of such cells tends to impart a gritty texture. Also, Existence of stone cells in pear fruits induced hardness increase and sucrose content decrease. Ultimately, stone cells showed negative effect for fruit quality (CHOI et al., 2007).

Stone cells are composed of lignocellulosic materials, including lignin and carbohydrates. The principal monomeric units of lignin include vanillin and syringaldehyde, and the carbohydrate hydrolysate harbours glucose and xylose residues (RANADIVE and HAARD, 1973). MARTIN-CABREJAS et al. (1994) attributed the presence of stone cells in unripened Spanish pears to the unusually high levels of glucose and xylose in the fruits.

The formation of stone cells has been described as an event occurring in the process of plant tissue lignification (CRIST and BATJER, 1931). NII (1980) reported that stone cells differentiated and enlarged between 30 days and 50 days after anthesis in *Pyrus serotina*. The first clear appearance of stone cells in the flesh of 'Niitaka' pears could not be observed until 14 days after full bloom (CHOI et al., 2003). In the *Pyrus communis* variants, STERLING (1954) verified that stone cells initially differentiated among the parenchymal cells approximately two weeks after flowering. However, DIBUZ (1998) insisted that stone cells began to appear during the flowering stage.

For the formation of stone cell in flesh, LEE et al. (2006) reported that the formation of stone cells in the pear could be resulted from water stress conditions during cell division. The decrease of water potential by water stress appeared to suppress the absorption of calcium. Increase of peroxidase activity, with the decreased in calcium absorption, was a cue of stone cell formation (LEE et al., 2006). Stone cell content and stone cell size significantly decreased in fruits treated calcium chloride by reducing the degree of lignification due to low peroxidase activity in flesh (LEE et al., 2007).

In microscopic examinations, CRIST and BATJER (1931) previously reported that the number of stone cells in a pear tended to decrease during fruit ripening, as observed in 'Bartlett' pears. Such an observation would indicate that the delignification and reduction of cell wall thickness occurred in the stone cells of the maturing fruit. However, STERLING (1954) asserted that their microscopic account did not provide a detailed picture of stone cell development, and that the subject required further investigation. In cocoa seed, about 10% of cocoa cotyledon parenchyma was formed by polyphenol cells, which occur in clusters and lines of up to ten cells (ELWERS et al., 2010).

Accordingly, knowledge of the formation and development of stone cells within the flesh is clearly necessary for efforts to improve the quality of the fruit. Micro-observations of the stone cells in pear varieties, which are known to harbour many stone cells, would be a key to our understanding of the causes of stone cell generation.

Materials and methods

Fruit materials. European pears (*P. communis* L. cv. 'Bartlett'), Chinese pears (*P. bretschneideri* Rehd. cv. 'Yali'), and Japanese pears (*P. pyrifolia* (Burm. f.) Nakai cv. 'Niitaka') were employed in this study. The fruits were collected from three trees of each cultivar for microscopic observation at 60 DAFB, at which time the stone cell distributions could be readily determined. Also, 'Niitaka' pear fruits were harvested at development stages of 30, 60, 90 and 150 DAFB in 15 year-old trees. All fruits were obtained from the orchard at the Naju National Pear Experimental Station, which is located in Chonnam, Korea. After selection for uniformity of size and freedom from defects, the harvested fruits were transported to the laboratory in plastic boxes packed with ice and stored at 0 ºC until the following morning. We collected 20 fruits at each sampling time for observations of the stone cells.

Preparation ribbon for microscopy. Small cubes of tissue were cut from the flesh of the pears and fixed in 2.5% (v/v) gluteraldehyde for 90 minutes at 4 °C. The samples were washed 5 times in 0.1 M phosphate buffer (pH 7.2) and post-fixed in 1% tetraoxide for 90 minutes at 4 °C. The samples were washed an additional 5 times with 0.1 M phosphate buffer (pH 7.2). After dehydration in a graded series of ethanol (40%, 60%, 90%, 95%, 100%), the tissues were transferred to an ethanol and propylene solution (1:1, v/v), then infiltrated and embedded in epon. For light microscopy, the samples were cut to a thickness of 1.5 µm using a microtome (Ultracut R, Leica Co., Austria) and stained with Schiff's reagent and 1% sodium bisulphite. The sections were examined and photographed under light microscopy (Axioskop 2, Karl Zeiss Co., Germany). For transmission electron microscopy (TEM), ribbons of approximately

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0.8 μm in thickness were collected on copper grids and sequentially stained with 1% uranyl acetate and lead citrate, then examined via electron microscopy (MEO 906E, Karl Zeiss Co., Germany). For scanning electron microscopy (SEM), ribbons approximately 0.8 μm in thickness were collected and stained in amylacetate, then examined via electron microscopy (2460N, Hitachi Co., Japan). The fruits were cut in half and dyed with 3% phloroglucinol-HCl added sulfuric acid, in order to confirm the existence of the stone cell clusters in the flesh.

Statistical analysis

Differences in stone cells content between cultivars were evaluated one-way ANOVA. Duncan's multiple range tests were used for the determination of significant differences among the results by using SPSS 15.0 software. A P value of ≤ 0.05 was considered to be significant.

Results

Morphological characteristics of stone cells in 'Niitaka', 'Yali' and 'Bartlett' pears

Microscopic observations, which were conducted at 60 DAFB, provided distinct information regarding morphology and stone cell distribution in the flesh of the different pear cultivars. At 60 DAFB, the LM observations showed that the majority of stone cells had gathered into clusters. The stone cell clusters were randomly distributed throughout the flesh tissues, and evidenced no detectable regular patterns. Cultivar differences were observed in the relative sizes and arrangements of the stone cells. In observations of the 'Niitaka' and 'Yali' pears, the stone cells were determined to be bigger in the 'Yali' variants than in the 'Niitaka' variants, and appeared to be concentrated to a greater degree close to the epidermis, whereas in the 'Bartlett' pears, the stone cells were the smallest among the three cultivars, and become increasingly scarce with increased proximity to the epidermis. The stone cell clusters consisted of several stone cells without concentration in the 'Niitaka' and 'Yali' pears. However, the clusters detected in the 'Bartlett' pear variants were organized into groups of many stone cells, and were located in close proximity to one another (Fig. 1).

With regard to stone cell content, which was evaluated at 160 DAFB, we determined there to be no detectable differences between the 'Yari' and 'Niitaka' pear cultivars. However, stone cell contents were significantly lower in the 'Bartlett' pears than in the 'Niitaka' and 'Yari' pears (Fig. 2).

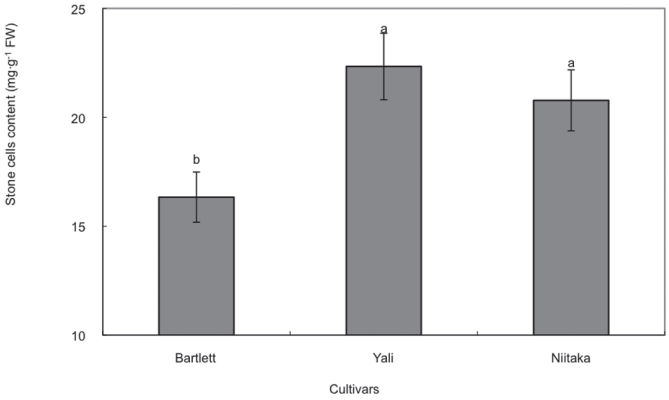
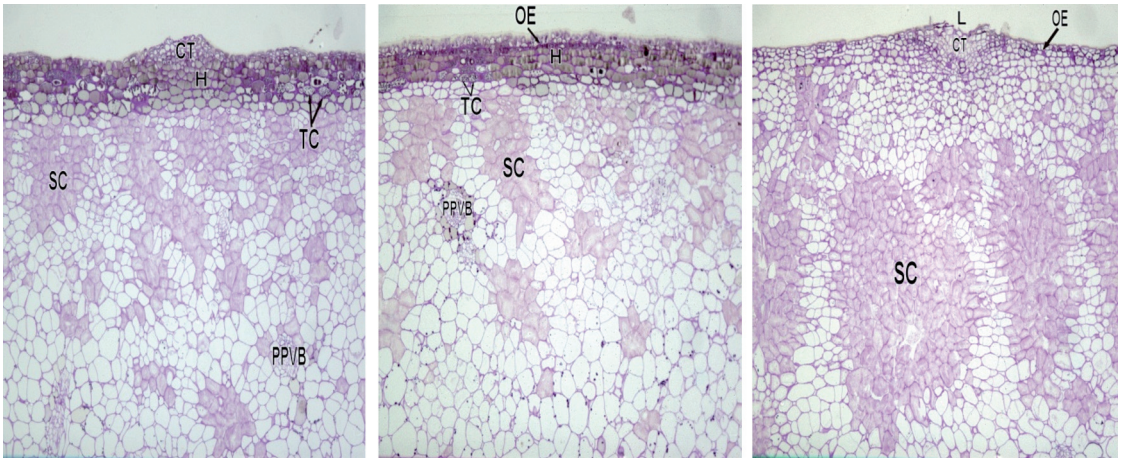


Fig. 2: Comparisons of stone cell contents in 'Bartlett', 'Yali', and 'Niitaka' pears at 160 DAFB.
[z] Vertical bars represent±SD. Results are from twenty fruits, respectively. Mean separation within columns by Duncan's multiple range test at 5 % level.

Development and distribution of stone cell clusters

The development of stone cells differed significantly according to the stages of fruit growth. At 30 DAFB, the majority of the stone cells were gathered in small clusters with a few independent cells. The stone cell clusters were spread randomly throughout the flesh tissue, and occurred with no detectable regular pattern (Fig. 3A). At 60 DAFB, stone cells tended to develop as clusters scattered irregularly throughout the fruit flesh, but appeared to be more concentrated in some areas than in others. The density of the stone cells in the flesh was the highest at 60 DAFB. The stone cells had become larger and more distinct than their adjacent cells (Fig. 3B). At 90 DAFB, the numbers of stone cells within a cluster did not increase, nor did the size of the clusters, but the density of clusters had decreased remarkably from 60 DAFB. The cells adjacent to the stone cells evidenced a spherical morphology at the initial stage (30 and 60 DAFB), but had transformed to a radial shape at



P. pyrifolia cv. Niitaka *P. bretschneideri* cv. Yali *P. communis* cv. Bartlett

Fig. 1: Comparisons of the distribution and size of stone cells in pear cultivars via light microscopy (100×) at 60 DAFB. CT: complementary cell, H: hypodermis, SC: stone cell, PPVB: peripheral vascular bundle, OE: outer epidermis, TC: tannin cell, H: hypodermis, SC: stone cell.

90 DAFB (Fig. 3C). At 150 DAFB, the fruit had attained complete maturity, and the swelling of the hypodermal cells could be clearly observed. The clusters of stone cells had dispersed throughout the entirety of the flesh, and their density had decreased due to the enlargement of the fruit (Fig. 3D). The results mentioned above can be attributed to a relative decrease in the quantity of stone cell clusters in the flesh, due to the fact that stone cells were no longer being generated, and the flesh cells increased dramatically in size.

Stone cells existed in precisely combined clusters, with no intercellular spaces. Tannin was observed embedded within the developing stone cells, and was presumably related to the thickening of the secondary cell wall (arrow). Developing and completed stone cells existed together within the same stone cell cluster (Fig. 4A; 4B). By dyeing using 3% pholoroglicinol, distribution of stone cells in flesh was easily observed (Fig. 5).

At 60 DAFB, the cell micro-organelles, including vacuoles, plastids,

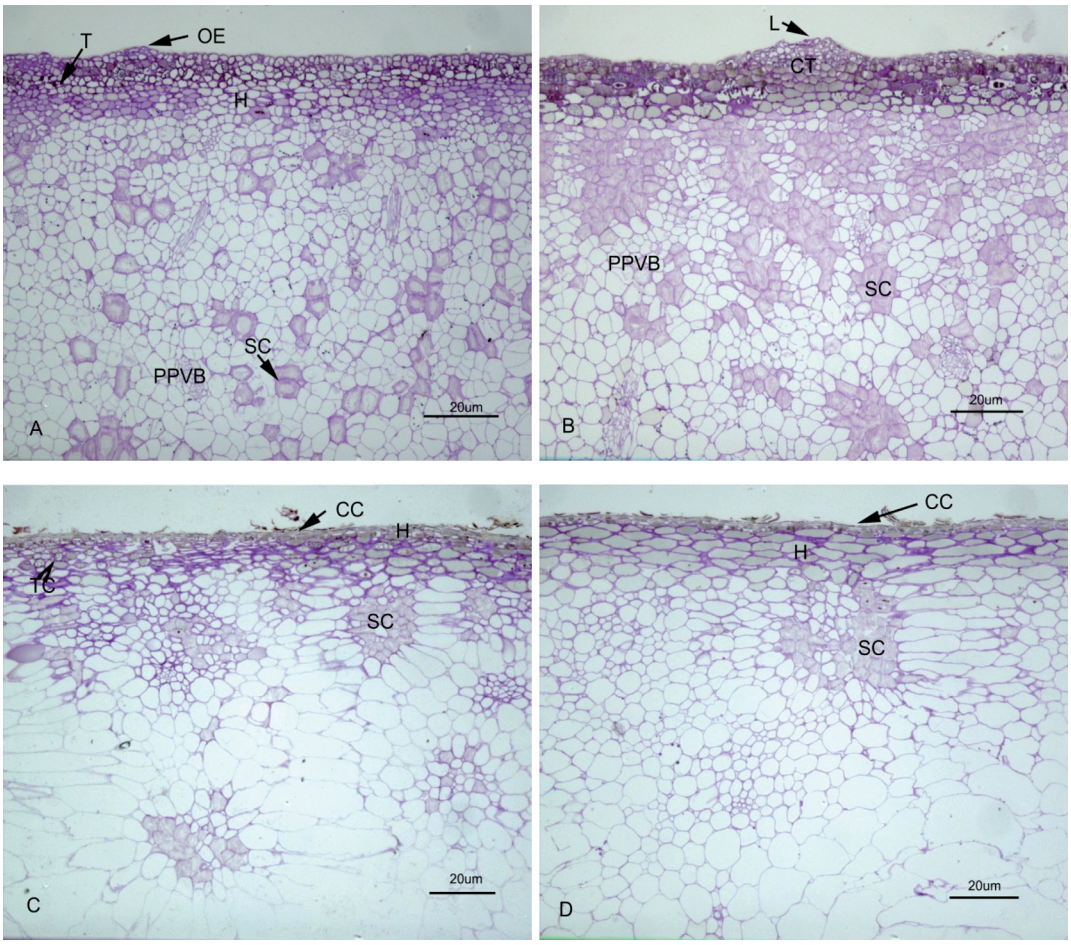


Fig. 3: Changes of flesh and epidermal tissue observed by the light microscope (100×) on 30 (A), 60 (B), 90 (C) and 150 DAFB (D) in 'Niitaka' pear. H: hypodermis, CC: cork cell, NTC: non-tannin cell, SC: stone cell, and TC: tannin cell.

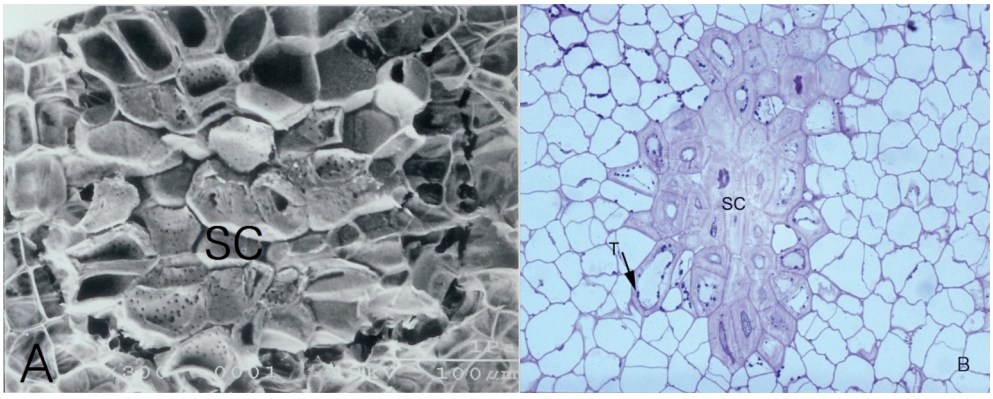


Fig. 4: Morphology of stone cells within a cluster in the flesh of 'Niitaka' pear under SEM (A: 300×) and optical microscope (B: 200×) on 60 DAFB. T: tannin, and SC: stone cell

and nuclei, were observed inside of the thickening cell wall under TEM (Choi et al., 2003). As cell wall thickening progressed, the plastids and vacuoles shrank and identification became more difficult. In the final stages of development, the cell wall thickened inwardly via deposition until the protoplast had disappeared entirely (Fig. 6A; 6B).

Discussion

Light microscopic observations revealed cultivar-based differences in the relative sizes and distribution of stone cells, as well as in the arrangement of stone cell clusters, in pears. These cultivar-predicated differences may be attributable to differences in fruit quality (Choi et al., 2007; Lee and Kim, 2001). Hiwasa et al. (2004) reported that European, Chinese, and Japanese pear fruits evidence differential softening characteristics as the result of endo-PG activity and PG gene expression occurring during ripening.

The morphological characteristics of stone cells in pear flesh were readily observable at 60 DAFB. Our observations with regard to the appearance of stone cells in the flesh were consistent with the results of Martin-Cabrejas et al. (1994), who examined the epidermal regions of ripe and 10-day-ripe Spanish pears. This means that the formation of stone cells tends to cease at 60 DAFB. Therefore, microscopic observations at 60 DAFB may constitute an improved technique for relative evaluations of stone cell contents.

Stone cells were discovered in small clusters at 30 DAFB, and occurred in larger clusters at 60 DAFB. In addition, stone cell clusters observed at 90 DAFB and 150 DAFB evidenced no form difference in either cluster size or in the number of stone cells, as compared with measurements taken at 60 DAFB. This finding indicated that the formation of stone cells and the clustering of these cells were actively realized from 30 DAFB to 60 DAFB. In addition, rows of radial parenchymal cells began to appear surrounding the stone cells by the later date. As stone cells do not divide, further cluster growth must occur at the expense of the radically oriented parenchyma cells, and this development is reflected in the outer cell arrangement of the stone cell clusters. Therefore, the occurrence of stone cell clusters in fruit flesh may constitute a depressing factor in fruit growth, as stone cell clusters repress the normal growth of adjacent parenchyma cells. Consequently, the radial construction of

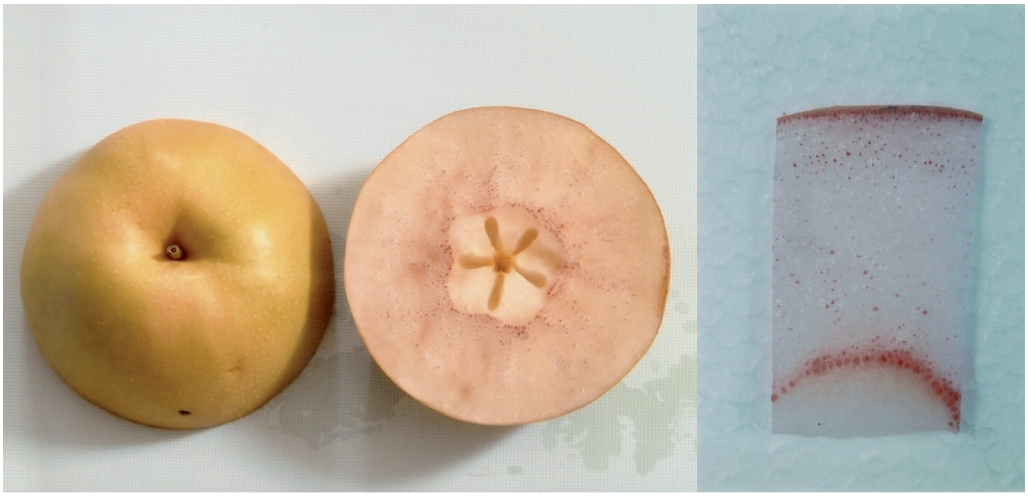


Fig. 5: Confirming of stone cells by 3% phloroglucinol-HCl dye at 150 DAFB in fresh of 'Niitaka' pear.

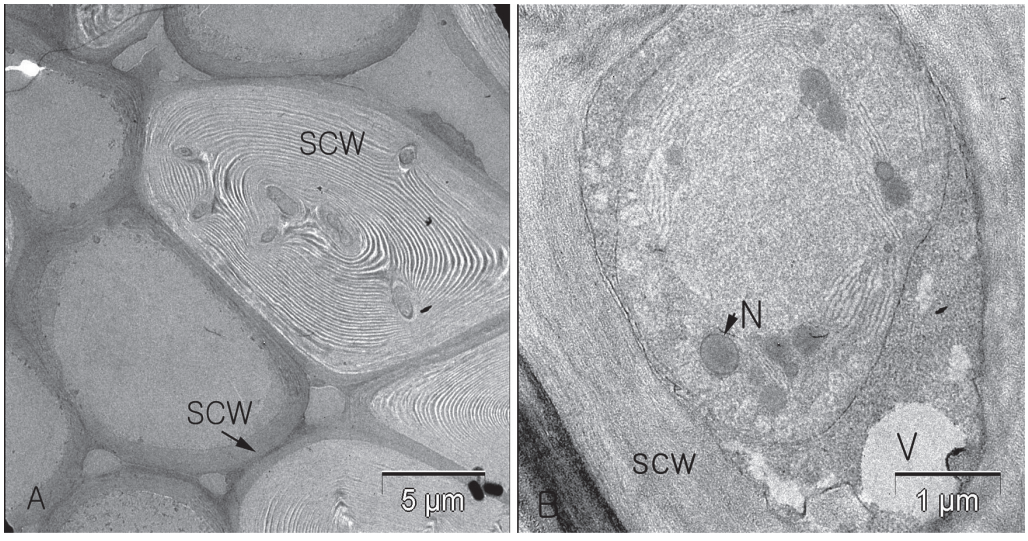


Fig. 6: Morphological characteristics of a stone cell observed in flesh of 'Niitaka' pear using TEM on 150 DAFB (A; B). N: nucleolus, SCW: secondary cell wall, and V: vacuole.

adjacent parenchymal cells at 90 and 150 DAFB can be considered proof that the stone cell clusters were not expanding. SMITH (1935) established that the quantity of stone cells in a pear depends primarily on the extent to which stone cell formation occurs during the early stages of fruit development. Also, NII (1980) insisted that the density of stone cells in fruit flesh tends to be highest after cell division has ceased. Therefore, it appears that the expansion of stone cell clusters is not evident during the later stages of fruit development.

The coexistence of developing and completed stone cells within a cluster may be the most important key to our understanding of cluster formation. Furthermore, it has been noted that the accumulation of tannin also occurs within the developing stone cells. This may be attributed to the rapid synthesis of lignin in the flesh during the early stages of fruit development, during which time the tannin and polyphenolic contents in the cell wall are relatively high (RYOGO, 1969). Therefore, the accumulation of tannin in the cell wall may be a factor in stone cell formation.

Stone cells have been shown to form via the progressive thickening of the secondary cell wall, and are linked to each other by pits between the cell walls (CHOI et al., 2003). These results were consistent with those of STERLING (1954) in a study of 'Bartlett' pears, in which the presence of a number of simple pits was observed throughout the surface of the cell wall. Connected pits may be responsible for the transformation of adjacent cells into new stone cells (CHOI et al., 2003). In addition, microorganelles have been observed within the stone cells during the early stages of growth, but had disappeared or were evident only as vestigial remains at 150 DAFB.

Consequently, stone cells evidenced an inward thickening of the cell wall after clustering, but did not exhibit any increase in cell volume. Thus, it appears likely that the formation of stone cells may be influenced by environmental conditions during the cell division period. Accordingly, the question of stone cell formation during the cell division period must be addressed.

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