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SUBCELLULAR CHANGES DURING PROTOPLAST ISOLATION OF *ORYZA SATIVA L.*[1],[2]

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Abstract

Protoplasts were enzymatically isolated from the developing young leaves of field grown rice plants in their final stage of vegetative growth. Among seven cultivars used, two indica type plants, Taichung Native No. 1 and Hsinchu Ai-chao-chien, produced a higher percentage of intact protoplast. Double nuclei in protoplasts were observed that may be originated from either fusing of two protoplasts during isolation or from the dividing cell that fail to separate during enzymatic incubation. The number of lipid droplets were increased in the cytoplasm of isolated protoplasts while pro-lamellar bodies were formed in plastids. The results were discussed.

Introduction

As a potential tool for distant hybridization as well as genetics, physiology and cell biology research, the isolated plant protoplast has been a center of attention recently. The isolation, culture and in some cases, fusion of protoplast has been reported in wide variety of plants including carrot, soybean, asparagus, and other crop plants (see review by Bhojwani *et al.*, 1977). Using cellulase and pectinase to digest cell wall, Tseng and Shiao (1976) of this institute has obtained a high yield of protoplast from leaf blades of eight weeks seedling of various rice species. These isolated protoplasts remained alive for two weeks or more under 4°C incubation.

It is of interest to know how the cell organells react during protoplast isolation. In this experiment, we used five rice cultivars, studied their protoplast of the tender leaf tissue near apical region before and after enzymatic digestion of the cell wall. The aim of this study were to establish a proper method of electron microscopic observation of isolated rice protoplast; to provide information for improving protoplast isolation as well as to obtain clues of future success in protoplast culture, fusion and plantlet induction

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Materials and Methods

Field grown rice plants prior to their flower initiation were pulled out from the ground. Around 2.5 cm of the tender leaves near the shoot tip were excised and soaked in 4°C distilled water. The cultivars used including three japonica types : Taichung No.65, Tainan No.5, Kaohsiung No.139 and two Indica type : Taichung Native No.1 and Hsinchu Ai-chao-chien.

For pre-enzymatic-treatment studies, the tissue were further cut into 1 mm size in 2.5% glutaldehyde in 0.1M phophate buffer. After fixing for two hr, the tissues were washed with buffer and postfixed with 1% OsO_4 for additional two hr. The tissues were then washed with distilled water and dehydrated with acidified dimethoxy propane (DMP) (Muller and Jacks, 1975) and transfered into dry acetone. They were embeded in Spurr's plastics and cured overnight in 60°C oven.

Protoplast isolation was conducted according to that of Tseng and Shiao (1976). Briefly, the tissue were further dissected and incubated in a 37°C medium consisting 2% pectinase (NBC), 4% cellulase (Onozuka, R–10), 20% sucrose and 0.2 mg/ml $CaCl_2$. After 80 min of incubation, the isolated protoplasts were filtered through a 325 mesh (45 micron) nylon cloth. The filtrate that consisted protpplasts were kept in 4°C refregilator for 2 to 3 hr to allow protoplast to float on the surface.

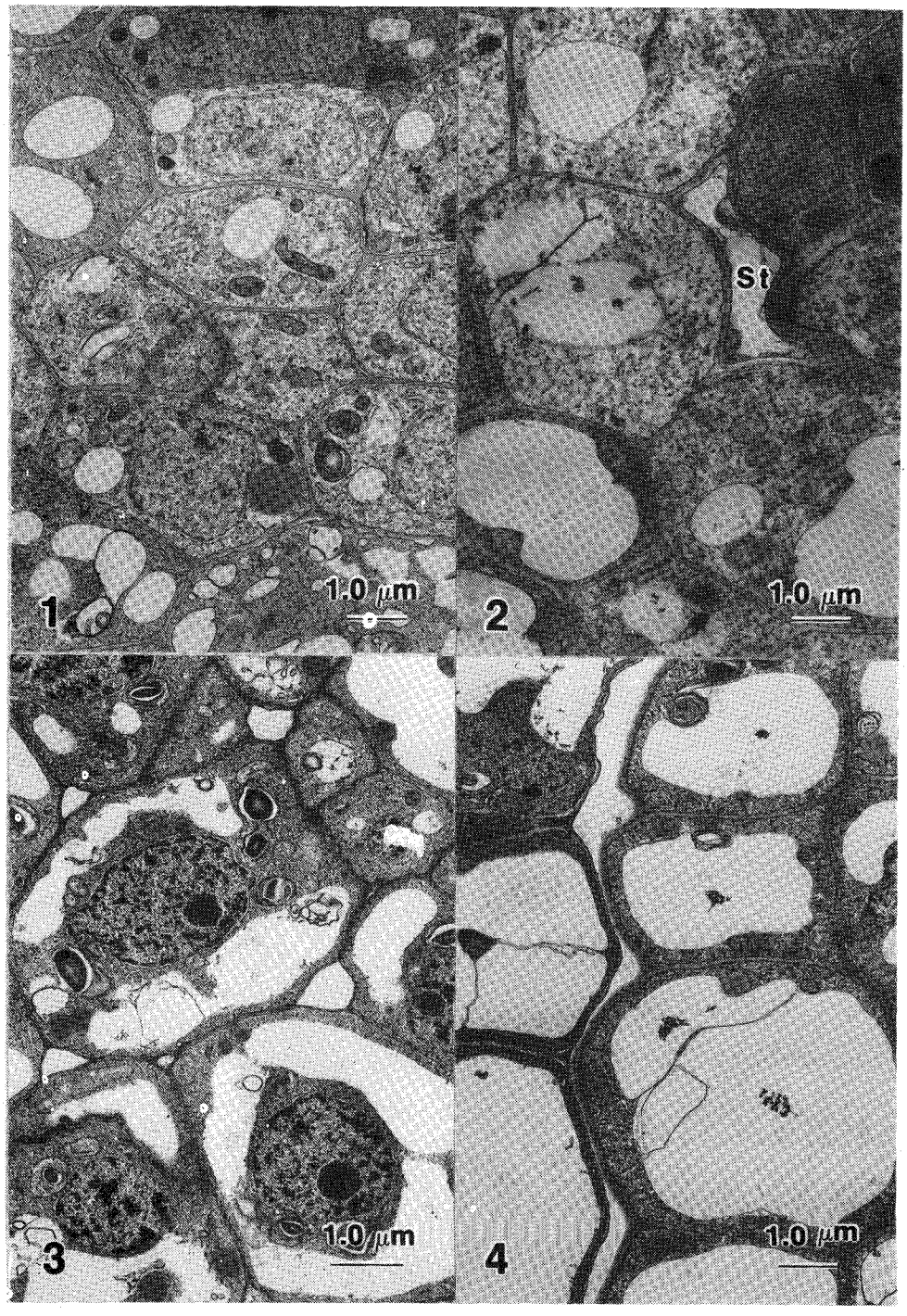
The protoplast were fixed according to the method discribed by Fowke (1975). Essentially glutaldehyde were added to the suspension to make final solution consisted of 1% glutaldehyde and 0.1M phosphate buffer. After fixed for an hr they were centrifuged (200 rpm) and transfered to a 2.5% glutaldehyde, phosphate buffered solution. the rest of the steps were the same as described for the intact tissue except in each step the protoplasts were centrifuged.

Section were cut with glass knives or a diamond knife using a Porter-Blum II microtome. They were post-stained with uranyl acetate and lead citrate and observed with a Hitachi HU–9 or a RCA 4A microscope.

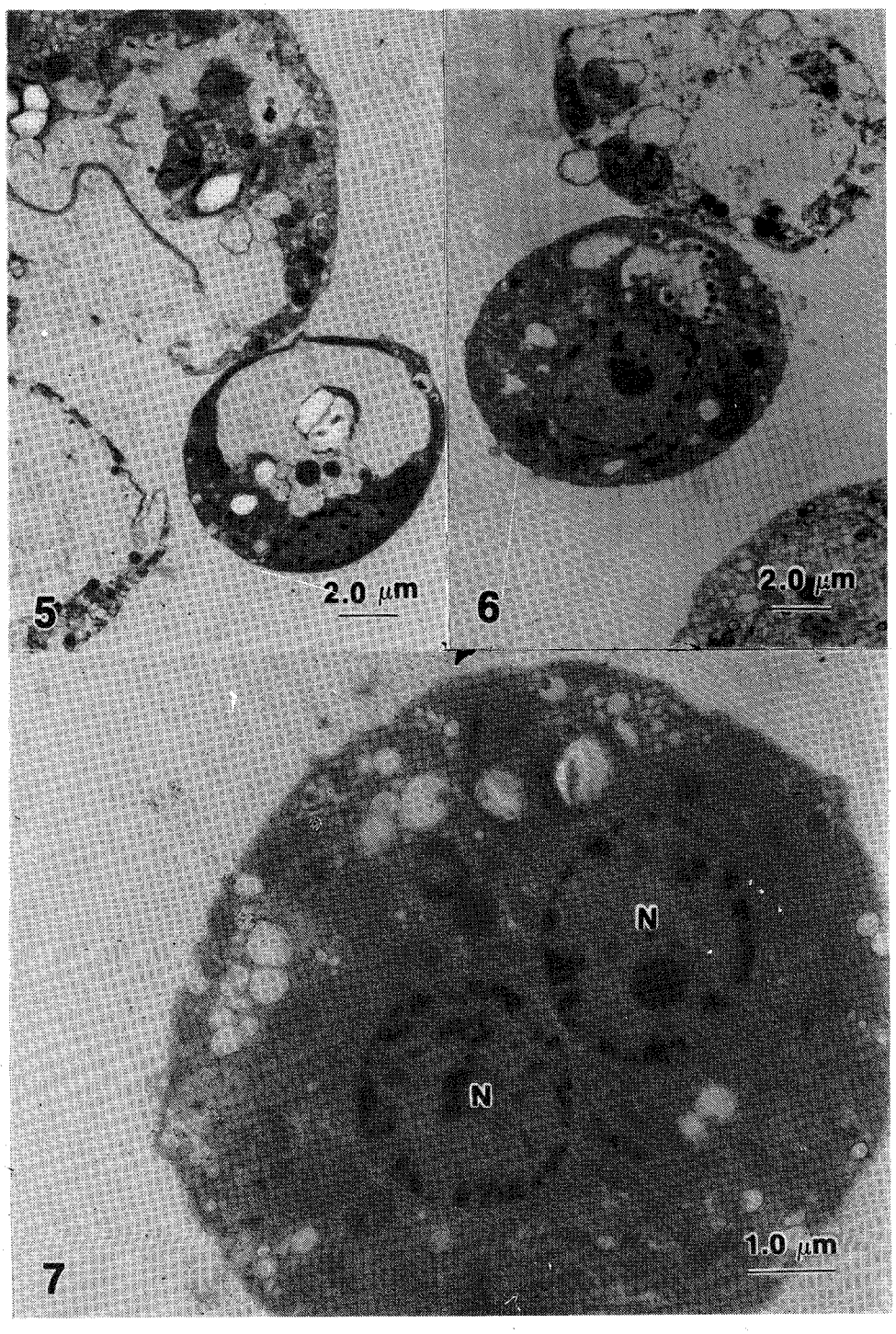
Results

Electron microscopic observation of the tissue used for the protoplast isolation revealed the diversity of cells in size and cytoplasmic content. The cell size could be from 2 to 25 microns. In general, cells in vascular tissue has dense cytoplasm with a few small vacuoles (Fig. 1). Well differentiated sieve tube and companion cell were also observed (Fig. 2). Parenchyma cells in leaf blades, on the other hand, has more vacuolated area especially in epidermal cells (Fig. 4). The plastids in leaf parenchyma mostly consisted of starch grains (Fig. 3).

As a result, the isolated protoplasts also differed in size and cell contents. Among them the meristmatic cells or young small cells with dense cytoplasm withstood well during protoplast isolation. Large, vacuolated cells tended to degenerate or break down (Figs 5 and 6). Multinucleated cells such as in Fig. 7 or in Fig. 9 could be found

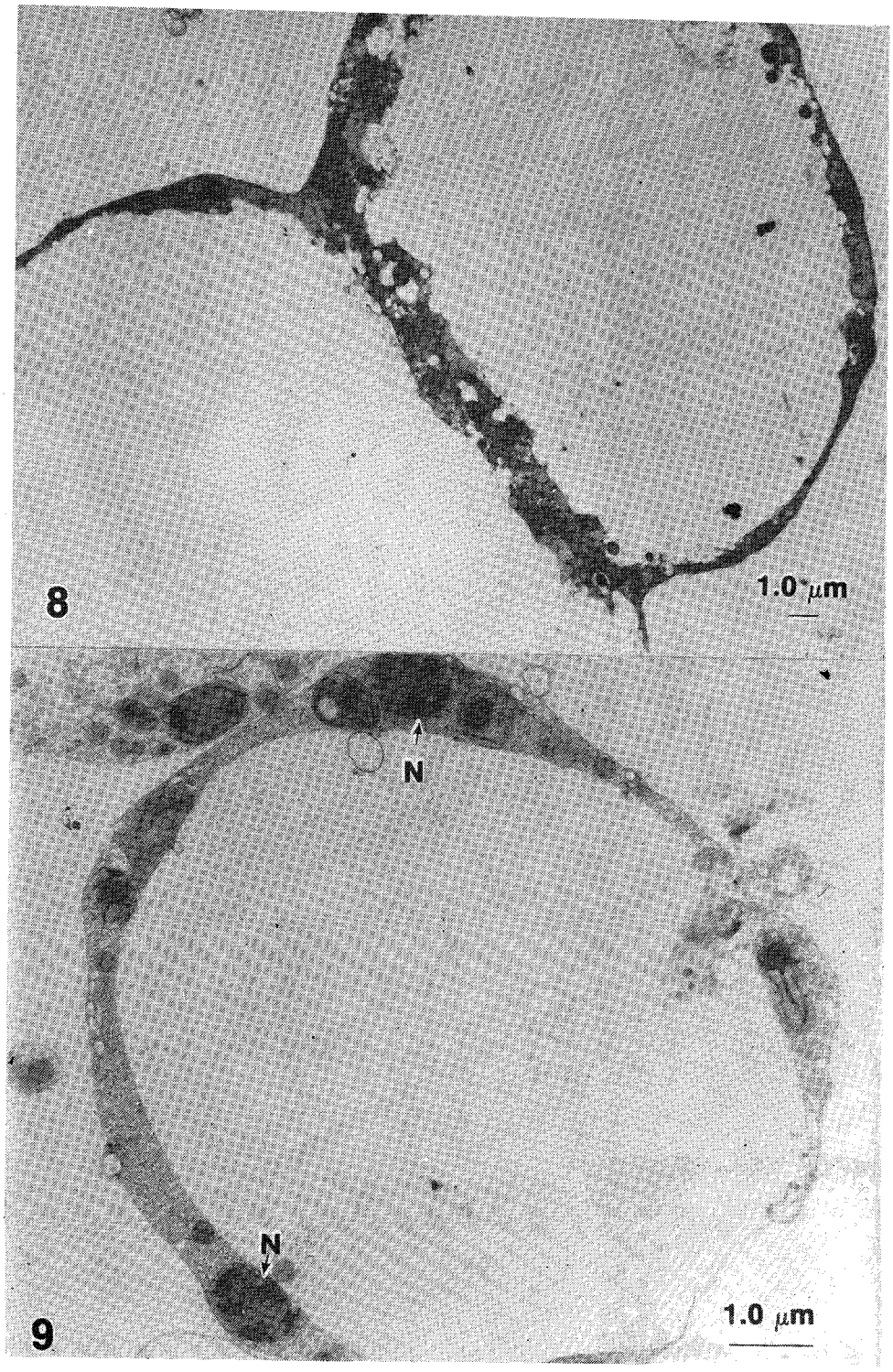


Figs. 1–4: Young leaf of rice tissues used for protoplast isolation
1. Vascular tissue from the leaf of cultivar Taichung No. 65.
2. Vascular tissue from cultivar Taichung No. 65. Sieve tube (St) is present.
3. Leaf parenchyma cells from Kaoshiung No. 139 cultivar.
4. Leaf epidermal cells from cultivar Hsinchu Ai-chao-chien.



Figs. 5–7: Isolated rice protoplasts. Note the protoplast filled with cytoplasm has a better preservation of membrane system during isolation.

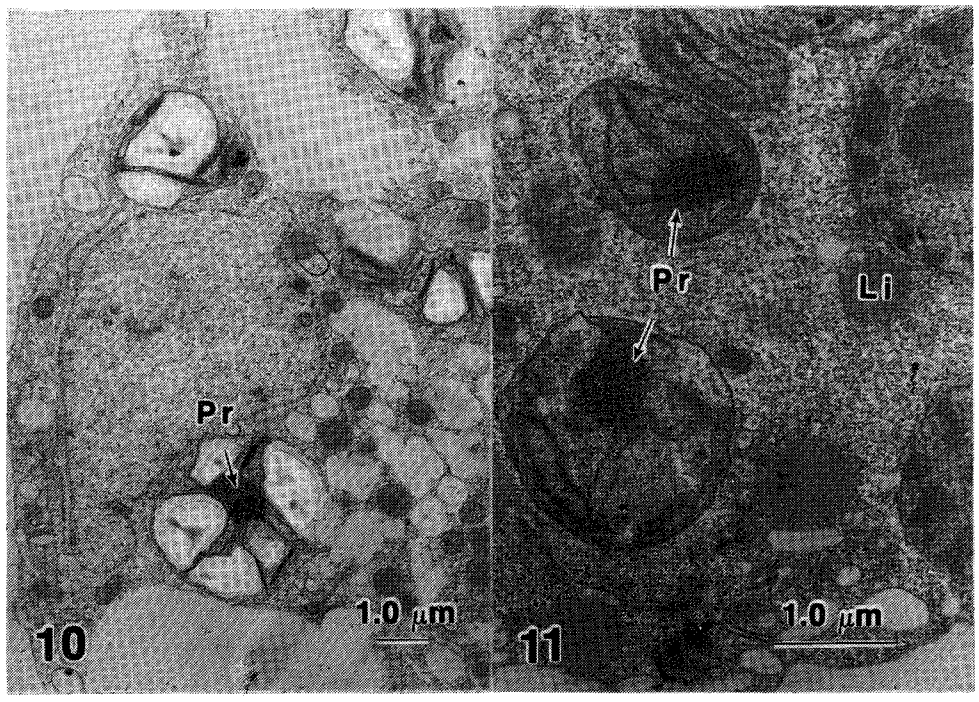
5. Protoplasts from cultivar Taichung No. 65.
6. From cultivar Taichung No. 1.
7. A protoplast from cultivar Kaoshung 139 with two nuclei (N).



Figs. 8–9: Spotaneous fusing of two rice protoplasts during isolation resulting in forming a duo nucleated protoplast. Both figs from cultivar Hsingchu Ai-chao chen.

occasionally. Two cells in the process of fusion were also observed (Fig. 8).

The formation of prolamellar bodies were observed in the plastids of isolated protoplasts, both in the plastids consisted with or without starch grains. Numerous oil-droplets were also present in the cytoplasm of isolated protoplasts (Figs 10 and 11).



Figs. 10–11.
Prolamella bodies (Pr) formed in plastids of isolated rice protoplast. Fig. 10 from cultivar Hsingchu Ai-chao-chen. Fig. 11 from cultivar Taichung No. 1.

Discussion

The results indicated that the isolation of protoplast from the unfolded young leaf of mature field grown rice plant is feasible. Of five rice cultivars used, the Indica type, Taichung Native No.1 and Hsinchu Ai-chao-chien produced a higher percentage (30%) of intact protoplasts. Tainan No. 5, a Japonica type, produced least (10%), on the other hand. Thus different cultivars exhibited different responses to the enzymatic isolation of protoplast. The viable protoplasts mostly are small in size and dense in cytoplasm with small vacuoles. These protoplasts mainly came from the meristmatic tissue or developing vascular tissue. Using young leaves from the mature plant instead of mature leaves from rice seedlings may increase the yield of these viable protoplasts to ensure the future success in fusion, culture and plantlet induction. However, the problem may lay in the separation and concentration of these protoplasts. A 'step-density gradient' method developed by Harms et al., (1978) would be useful in this regard.

About the preparatory procedures of protoplast for electron microscopic study, the method provided by Fowke (1975) is adaptable for preparing rice protoplast. To decrease the loss during dehydration, DMP was used in our experiment. The results were quite satisfactory except a rapid dehydration may cause residual culture media to precipitate and become the cause of striation in thin sections.

The multinucleated rice protoplast has been reported (Lai and Liu, 1978). In our observation, they may be originatied from two sources, one from the dividing cells that failed to separate during enzymatic digestion as in Fig. 7, the others from spontaneous fusing as clearly indicated in Fig. 8. This type of fusion may be undesirable if the isolated protoplast is intended for somatic hybridization with other species. The control of this type of fusion is desirable.

The increase in number of oil droplets in cytoplasm of isolated rice protpplast is also reported in tobacco messophyll protoplasts (Gigot *et al.*, 1975). Subject spinach leaf to a high speed centrifugation also result in increasing the number of oil droplet in cytoplasm of mesophyll cell (Beams *et al.*, 1979). Apparently it is a product of cell response to an environmental shock and may relate to the membrane metabolism. The formation of prolamellar bodies in plastids is another distinct change during protoplast isolation. Gigot *et al.*, (1975) in tobacco protoplast also reported the apparent changes in chloroplast with the appearance of pseudocrystals followed by reduction in thylacoids. Obviously the plastids is very sensitive to the environmental changes including temperature, light and nutrient supplies.

No pseudocrystalline inclusion were observed in the cytoplasm of isoloate rice protoplast as reported by Lai and Liu (1978). Apperently the using of sucrose instead of salts in our digesting medium has a more gentle effect on protoplast.

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水稻原生質體在分離過程中之微細構造變化

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本試驗使用五種在田間栽培之水稻品種，取其發育中之嫩葉以酵素作原生質體之分離後固定，切片觀察，並與未分離前之組織細胞比較，以建立使用電子顯微鏡判斷其活性之方法，期供進一步作融合及培養原生質體時之檢定依據。試驗結果顯示細胞質較充滿之原生質體較能忍受酵素分離而保持原有之結構。各品種中以台中在來一號及新竹矮脚尖產生較高百分率之完整原生質體，分離出之原生質體常見有雙核者存在，其來源可能有二：一來自兩個相同原生質體之融合，另一爲正在分裂之細胞因暴露在果膠及纖維分解酵素之下未能形成細胞壁來加以分隔者。分離之原生質體中，細胞質有許多 lipid droplets 之出現，其原質體（plastids)則有 prolamella body之形成。