Histological Observaion of Callus Formation in Mungbean (*Vigna radiata* (L.) Wilczek) Cotyledon Culture

Josefina O. NARCISO, Kazumi HATTORI and Tomikichi WADA
(Laboratory of Plant Genetics and Breeding, School of Agricultural Sciences,
Nagoya University, Nagoya 464-01, Japan)

Received January 10, 1996

Abstract : Histological observation was conducted to evaluate the origin, initiation site and development of callus in mungbean cotyledon culture. Results showed that callus formation in mungbean occurred in three sites : 1) in the peripheral area of the provascular strands where the initiation of endogenous callus was noted ; 2) at the cut portion of the cotyledon where external primary callus developed ; and 3) in the ruptured epidermal layer where the primary callus emerged. Callus formation differed in each site. Endogenous callus formed due to the rapid cell division of the cells in the peripheral zone of the provascular tissues. The primary callus from the cut portion of the cotyledon and reptured portion of the epidermal layer were mostly formed out of cell enlargement of the parenchymatous cells. Although the primary callus cells soon became highly vacuolated, this does not seem to contribute to the increase in callus cell mass. Owing to low dividing activity of the primary callus cells, the cytoplasm-rich and meristematic callus cells are apparently derived from the small callus cells which were located on the periphery of the original provascular tissues. And so, some cells in the provascular cells could be seen as the prominent origin of callus in the excised cotyledon system of mungbean.

Key words : Callus, Cotyledon, Histology, Mungbean.

リヨクトウ (*Vigna radiata* (L.) Wilczek) 子葉培養におけるカルス形成の組織学的観察 : Josefina O. NARCISO・服部一三・和田富吉 (名古屋大学農学部)

要 旨 : リヨクトウ子葉培養におけるカルスの起源、形成部位および発育を明らかにするため組織学的観察を行った。その結果、リヨクトウにおいてはカルス形成は以下のような3つの部位から生ずることが明らかとなった。すなわち、1) 内部に形成されるカルスの起源と考えられる維管束前形成層の周縁部、2) 外部に向かって形成される1次カルスが発達する子葉の切断端、および3) 1次カルスが出現する表皮の裂け目の部位からカルスが生じた。カルスの形成過程は各部位で異なっていた。内部に形成されるカルスは前形成層の周縁の細胞の急速な分裂によるものであった。子葉の切断端および表皮の裂け目から形成される1次カルスは、主に葉肉柔細胞の肥大により生じた。これらの1次カルス細胞はすぐに液胞化が著しくなったが、これはカルス細胞塊の増加にはそれほど寄与していないものと思われた。1次カルスの細胞は分裂活性が低いことから見て、細胞質に富みかつ分裂活性の高いカルスの細胞は、明らかに、もともと前形成層に由来する前形成層周辺部の小さなカルス細胞から発生したものと思われる。それ故、子葉の前形成層内の細胞がリヨクトウの子葉培養系でのカルスの主要な起源と考えられた。

キーワード : カルス、子葉、組織学、リヨクトウ。

The increasing demand for food by the world's population necessitates the rapid development of new crop varieties, particularly in mungbean. To address this challenge, the introduction of genes into cells is acknowledged by researchers as one of the most promising ways of developing new genotypes. When genes are introduced, the availability of callus with high meristematic activity or high regeneration potential may be an important prerequisite for efficient plant regeneration through embryogenesis or organogenesis.

Callus has been successfully induced using various plant explants in mungbean and other *Vigna* species^{2~4,8,10,12,15}). These studies reported plantlet regeneration through somatic em-

bryogenesis, or from organogenesis. However, in case of mungbean, a highly efficient protocol for plantlet regeneration is yet to be established. To obtain callus lines with high meristematic or embryogenic cells as well as those competent for regeneration, it is essential to advance the study of those callus lines. Histological observation can provide information on the embryogenic potential of a callus type and its growth and development, which are possibly important in the establishment of an efficient protocol for regeneration. Moreover, histological study can provide us the precise site of active callus formation, which can see as a guide in determining a morphogenetically potential site in which gene introduction can

be pursued. The work of Mendoza *et al.*¹³⁾ is the only known report which deals with the histological observation in mungbean. This examined plant regeneration in mungbean cotyledon histologically but did not look at the process of callus induction. In this regard, the present study evaluated the origin, initiation site and development of callus in mungbean through histological observation in order to recognize the embryogenic potential of a callus type.

Materials and Methods

Mungbean variety IPB22-117 was used as test material. Mature seeds of this variety were surface sterilized following the method of Mendoza and Futsuhara¹²⁾. The surface sterilized seeds were immersed sterile water for 16 hrs to allow easy removal of seed coat. The seed coat was removed and the 2 cotyledons were excised under aseptic conditions. The portion where the embryonic axis was located was removed. The cotyledon was inoculated by placing their abaxial side on the medium. The callus induction medium as described by Eapen and George⁴⁾ consisted of : MS salts (Murashige and Skoog¹⁴⁾) + B5 vitamins (Gamborg *et al.*⁷⁾) + 3% sucrose + 5 mgL⁻¹ 2,4-D + 2.5 gL⁻¹ gelrite. Ammirato¹⁾ reported that the early stages of somatic embryogenesis in legumes and non-legumes is induced by exposure to 2,4-D with or without other accompanying growth regulators. This medium was used by Eapen and George⁴⁾ for callus induction of some *Vigna* species. It was also observed in our previous work that callus induced from this medium has high embryogenic potential. The pH of the medium was adjusted to 5.8 prior to the addition of the gelling agent and autoclaved for 20 min at 120°C. The cultures were incubated at 25 ± 2°C under minimum light conditions of about 8.5 $\mu\text{Em}^{-2}\text{sec}^{-1}$. Sampling of the cultured cotyledon was done at 0, 1, 3, 5, 7, 10 and 14 days after culture and fixed immediately with FAA for 24 hr. The fixed samples were dehydrated in graded acetone series and embedded in resin, according to Mendoza *et al.*¹³⁾ The resin-embedded samples were sectioned at 6 μm using a rotary microtome. The sections were stained with toluidine blue for two minutes. For the observation of starch grains and protein bodies, samples taken at 0, 5, 10 and 14

days after culture were used. To detect starch and protein bodies, samples were double-stained with Periodic Acid-Schiff (PAS) and amido black, respectively. The prepared sections were viewed under light microscope.

Results

Cross section of mungbean cotyledon at day 0 shown in Fig. 1a revealed the presence of parenchymatous cells which were more or less isodiametric, with an average maximum diameter of 110 μm and minimum diameter of 89 μm . Embedded in the parenchymatous cells are the provascular tissues which were darkly stained and consisted of small, cytoplasm-rich cells. Covering the parenchymatous cells and provascular tissues was the epidermis composed of one layer of cells arranged parallel to the axis of the cotyledon surface. Beneath the epidermal cells was a single layer of subepidermal cells, which were relatively bigger than the epidermal cells.

There was no apparent morphological change that occurred in the cotyledon at day 1. However, histological observation showed that cotyledon at day 1 had slightly larger parenchymatous cells which may indicate the start of cell vacuolation (Fig. 1b). At day 3, very small nodular protuberances began to appear at the surface of the cotyledon (Fig. 1f). Fig. 1c showed that there was a marked cell enlargement in the epidermal and subepidermal cells at day 3. At day 5, the first visible sign of callus formation was observed. Friable callus started to emerge on the portion where the embryonic axis was cut (data not shown). During this time, further cell enlargement was observed in the parenchymatous cells as well as the subepidermal and epidermal layers (Fig. 1d). There was a 34% and 17% increase over that at day 0 in the maximum and minimum diameter of parenchymatous cells, respectively. Concurrent with rapid cell enlargement was the induction of meristematic activity in the provascular tissues. Meristematic activity shown as an increase in cell numbers was abnormally localized in the peripheral area of the provascular tissues. With the rapid cell enlargement and meristematic activity, endogenous callus formation occurred at day 3 in the periphery of the provascular tissues (Fig. 1e). The endogenous callus cells might have come as a

Fig. 1

Fig. 2

Fig. 3

Fig. 4

result of the rapid cell division of small cells in the periphery of the dedifferentiating provascular tissues. As callus induction progressed, meristematic cell mass increased in size and the provascular cells were seen to be dedifferentiated (Fig. 2a-c). Growth of endogenous callus started at the provascular tissues and expanded rapidly towards the epidermal layer. The pressure generated by the rapid growth of the endogenous callus and enlargement of parenchymatous cells led to the rupture of the epidermal layer to allow callus formation on the surface at day 5 (Fig. 3a). It can be noted that there was rapid cell division in the endogenous callus cells, including the dedifferentiating provascular cells in the area near the rupture of the epidermal layer (Fig. 3b). However, in the area that was not ruptured, both division and elongation of cells in the periphery of provascular tissues was observed (Fig. 3c). This area might not be directly involved in the rupture of the epidermal layers. The initiation of primary callus at dy 5 can be attributed to the enlargement of parenchymatous cells near the cut portion of the cotyledon.

At day 0, the parenchymatous cells were filled with starch grains and protein bodies (Fig. 4a). With the start of external callus

formation at day 5, starch grains and protein bodies have become few and were found only in cells adjacent to the epidermal layer (Fig. 4b). At day 10, starch grains were very few and it was not evident in areas where callus was formed (Fig. 4c). At day 14, starch grains were almost depleted while protein bodies were completely depleted (Fig. 4d).

Discussion

Callus formation in mungbean cotyledon occurred in three sites: 1) in the peripheral area of the provascular tissues where the endogenous callus was initiated, 2) at the cut portion of the cotyledon and where external primary callus developed and 3) at the ruptured epidermal layer, where the external primary callus emerged. At the first site, callus formation was noted to be preceded by the induction of meristematic activity at the periphery of provascular tissues. At the second and third sites, cell enlargement of the parenchymatous cells occurred prior to callus formation. Endogenous callus was formed due to the rapid cell division of the cells in the periphery of the provascular tissues. The external primary callus from the cut portion of the cotyledon and ruptured portion of the epidermal layer were formed from cell enlargement of the

Abbreviations

EC=endogenous callus, EL=epidermal layer, PC=parenchymatous cells, PT=provascular tissues, SL=subepidermal layer.

Explanations of Figures

Fig. 1a-e. Light microscopic observations of mungbean cotyledon at different days of culture. Cross sections, (a) at the day of culture showing the parenchymatous cells, provascular tissues, epidermal layer and subepidermal layer; (b) at day 1 after culture; (c) at day 3 after culture; (d) at day 5 after culture. Note the enlarged parenchymatous cells, subepidermal layer and epidermal layer. (e) Endogenous callus produced at day 3 after culture. Scale bars=100 μ m.

Fig. 1f. Mungbean cotyledon at day 3 after culture. Note the tiny protuberances (open arrows) and the ruptured central portion (closed arrow). Scale bar=1 mm.

Fig. 2a-c. Light microscopic observations of the mungbean cotyledon at: (a) day 5 after culture, (b) day 7 after culture and (c) day 14 after culture showing the dark-stained provascular tissues. Scale bars=100 μ m.

Fig. 3a-c. Light microscopic observations of mungbean cotyledon at day 5 after culture showing: (a) the ruptured epidermal layer. Scale bar=200 μ m; (b) cell division in the provascular tissues near the callus initiation site. Scale bar=100 μ m; (c) cell elongation in provascular tissues away from the initiation site. Scale bar=100 μ m.

Fig. 4a-d. Starch (open arrows) and protein bodies (closed arrows) as stained by PAS and amido black in cotyledon at: (a) the day of culture, day 5 after culture, (c) day 10 after culture and (d) day 14 after (b) culture. Scale bars=100 μ m.

parenchymatous cells. El Maâtaoui *et al.*⁵⁾ using stem fragments of cork oak also observed the formation of endogenous callus prior to callus proliferation on the surface of the explant. Their report indicated that prior to the induction of endogenous callus, dedifferentiation of the cortical parenchyma occurred and the calluses were formed as a result of repeated cell division. It was observed in this study that there were two areas where meristematic activity of provascular tissues occurred. The first was the area near the rupture of the epidermal layer, and the second distant from the ruptured site. In the area near the ruptured site, rapid cell division was observed while both cell division and elongation were observed in the remaining area.

Histological studies in other crops have pointed to the exact origin of callus using various explants. In sugarcane, Liu *et al.*¹¹⁾ traced the origin of callus from rolled young leaf to the parenchyma adjacent to the primary phloem, while callus from stem tip explants arose from the primary phloem region. Gupta⁹⁾ also noted the pericycle origin of the hypocotyl callus of *Trigonella foenumgraecum* L. White¹⁶⁾ reported that the callus of mature spruce explant was formed starting from the cambium, through the lining of the xylem resin ducts. Fasseas and Bowes⁶⁾ observed that the initiation of callus in the mature cotyledon of *Phaseolus vulgaris* L. was confined to storage parenchymatous cells. In the present study, the possible origin of mature cotyledon callus was traced to the parenchymatous cells and the cells at the periphery of the provascular tissues.

Callus formation, just like any *in vitro* process, is an energyrequiring process. This was revealed by the observation that the amount of starch grains and protein bodies in the parenchymatous cells decreased as callus induction progressed. Protein was more rapidly depleted than starch. At 14 days after culture, there were still traces of starch grains observed in some large parenchymatous cells, while protein bodies were completely depleted. Mendoza *et al.*¹³⁾ also observed rapid depletion of starch and protein in the parenchymatous cells of mungbean cotyledon during *in vitro* shoot initiation.

The discovery of the endogenous callus, which has not been reported yet in mungbean

cotyledon cultures, can be considered as the most important finding of this study. This would have not been possible if histological observation had not been done. Histological observation showed that endogenous callus was formed as a result of rapid cell division of immature parenchymatous cells in the peripheral area with high meristematic activity suggesting the high embryogenic potential of this callus. This callus type, if cultured in an appropriate culture medium and condition, could possess higher regeneration efficiency and potential for in future transformation studies.

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