Observational and Experimental Studies of the Meristem of Leguminous Plants

III. Released nucleoplasm in the central cylinder of pea root tips

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In the previous papers of this investigation, the author conformed the presence of two kinds of pericyclic cells in both bean and pea root tips (Toriyama 1978 a, b). This is ascribed to the technique-combination of Lillie's buffered neutral formalin fixation and Heidenhain's hematoxylin staining which is excellent for detecting differentiation of the α -cells and β -cells in the pericyclic layer.

By the application of the same cytological and histological technique, new findings relating to the characteristic 'released nucleoplasm' in the limited area of pea root tips are reported in the present investigation.

Material and methods

Pisum sativum, variety "Alaska", seeds were placed in large petri dishes, watered and placed in a moist chamber at 24°C; young seedlings were grown in continuous darkness, and those of 3 to 4 day old plants were used for the experiments. The distal 4 mm root tips excised from the primary root of about 40 mm length were fixed with Lillie's buffered neutral formalin (commercial formalin, 100 ml; distilled water, 900 ml; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 4 g; Na_2HPO_4 anhydrous, 6.5 g. Lillie 1948) at room temperature for 10 hours. This procedure was performed under a safety lamp of green light. The fixation was performed between 9 to 12 AM, 2 to 4 PM and 1 to 3 AM on May 20 and 21, 1977. After fixation the root tips were thoroughly washed in running water for several hours, and dehydration was performed through a series of tertiary butyl alcohols (Johansen 1937-1938), followed by embedding in paraffin in the usual way. Paraffin blocks were cut 12 μm in thickness. The sections were stained with Heidenhain's hematoxylin for 60 minutes, safranin for 30 minutes and/or 0.5 per cent methyl blue aqueous solution for 90 minutes. The tissues examined were obtained from 15 root tips.

Results

1. 'Released nucleoplasm' in the central cylinder

Experiments and observations were performed using the materials which were fixed in the diurnal periods, during 2 to 4 PM on May 20, 1977. The technique-combination of Lillie's formalin fixation and Heidenhain's hematoxylin and safra-

nin staining is excellent for detecting differentiation of the 'released nucleoplasm' as reported below.

In the longitudinal section of the central cylinder, the cytoplasm of meristematic procambial cells had no vacuolation and in addition contained a spherical-nucleus which had a black-brown nucleolus (Fig. 2a). In some meristematic cells which are localized in that area from 600 μm to 700 μm from the apical initials (D_1 in Fig. 1), a transparent layer appeared at the periphery of the nucleus (Fig. 2b). In that area from 700 μm to 900 μm from the apical initials (D_2 in Fig. 1), the nuclei became large, and their transparent layer expanded within cytoplasm (Fig. 2c). Then, following this phenomenon, the cell wall of these cells gradually disappeared, and finally, the nuclei were released in the diffused cytoplasm as indicated by the arrow (1) in Fig. 2g. In addition, in some cylindrical thin meristematic cells which were localized in D_1 -zone (Fig. 1), the transparent thin layer appeared on the periphery of the nucleus (Fig. 2d). In the D_2 -zone (Fig. 1), the nuclei became long, and the cell wall gradually disappeared (Fig. 2e). Furthermore, in the

Fig. 1. Scheme of the median longitudinal section of a primary root tip of *Pisum sativum*, variety "Alaska". TRN, the area which has thin released nucleoplasm; RN, the area which has typical released nucleoplasm, that is to say, two kinds of bodies of released nucleoplasm; D_1 , D_2 , the areas which show the differentiation of released nucleoplasm; PC, procambium; α , α -zone; β , β -zone (mentioned in Toriyama 1978b).

area about 900 μm to 2000 μm from the apical initials (RN in Fig. 1), several bodies of released nucleoplasm appeared clearly in the diffused cytoplasm (Figs. 2f, g). Therefore, there are two kinds of bodies of released nucleoplasm; one kind is thick and short (width 6–8 μm , length 15–20 μm) as indicated with arrow (1) in Fig. 2g, and the other kind is thin and long (width 4–5 μm , length 20–25 μm) as indicated

Figs. 2a–j. Part of central cylinder in the pea root tip. $\times 800$. a–h, longitudinal section; a, arrows indicate nucleus of procambial cell. b, arrow indicates transparent periphery layer of nucleus; c, arrows indicate expansion of transparent periphery layer of nucleus. d, arrows indicate transparent thin layer of nucleus. e, arrow indicates nucleus which has grown longer. f, arrows indicate typical released nucleoplasm. g, arrow (1) indicates thick and short released nucleoplasm, arrow (2) indicates thin and long released nucleoplasm. h, arrows indicate thin nucleoplasm. i, transverse

section of central cylinder, arrow (1) indicates thick released nucleoplasm, arrow (2) indicates thin released nucleoplasm. a-i, all the micrographs shown were made from tissue sections fixed with Lillie's buffered neutral formalin and stained with Heidenhain's hematoxylin and safranin; j, the same part of Fig. 2i, arrow (1) indicates thick released nucleoplasm, arrow (2) indicates thin released nucleoplasm. The tissue was fixed with FAA and stained with Heidenhain's hematoxylin.

with arrow (2) in Fig. 2g. In that area about 2000 μm to 3000 μm from the apical initials (TRN in Fig. 1), the released bodies of nucleoplasm became thin and long (Fig. 2h). And then, generally these released bodies of nucleoplasm have one to four nucleoli which were stained a black-brown color with the Heidenhain's hematoxylin. In that area more than 3000 μm from the apical initials, the released bodies of nucleoplasm almost degenerated, so that, in the transverse section, the released nucleoplasm did not appear in the pith of the central cylinder.

Figs. 3a-e. Transverse section of central cylinder of pea root tip. a, b, diurnal period. c, d, e, nocturnal period; arrows indicate released nucleoplasm. All the micrographs shown were made from tissue sections fixed with Lillie's buffered neutral formalin and stained with 0.5% methyl blue. $\times 800$.

Lastly, at the transverse section of around 1200 μm from the apical initials, several released bodies of nucleoplasm which have a nucleolus clearly appeared due to the same cytological and histological technique; this is to say, about 6-8 μm width of released nucleoplasm was observable as indicated by the arrow (1); and thin nucleoplasm of width 4-5 μm , was also observable as indicated by the arrow (2) (Fig. 2i).

The author attempted to use FAA¹ as fixing reagents and Heidenhain's hemato-

¹ FAA: formalin 5, acetic acid 5, 100% ethanol 60, and water 30, in volume.

xylın and safranin for staining. Under this experimental condition, it is possible to recognize the released nucleoplasm as indicated by the arrows in Fig. 2j. But, it seems to the author that Lillie's formalin fixation was better than FAA for fixing the released nucleoplasm in the central cylinder.

2. *Distribution of 'released nucleoplasm' in the central cylinder*

In the diurnal condition, from 9 to 12 AM, the released bodies of nucleoplasm which was deeply stained blue color with the methyl blue appeared clearly in transverse section at 1200 μm from the apical initials (Figs. 3a, b). In the nocturnal period, from 1 to 3 AM, the released nucleoplasm in the same part of the central cylinder stained lightly with the same dye solution (Figs. 3c, d, e). And also, tiny granules appeared in some released nucleoplasm (Figs. 3d, e). This released nucleoplasm which was fixed during the nocturnal condition has a tendency to stain lightly with methyl blue solution. According to these experimental data, observations were performed using the materials which were fixed in the diurnal period, from 9 to 12 AM, for the purpose of detecting the distribution of the released nucleoplasm.

By applying a combination of Lillie's formalin fixation and methyl blue staining to the transverse section at about 2500 μm from the apical initials, a central cylinder, the protoplasm which was almost totally vacuolated, was observed (Fig. 4a). But a few bodies of released nucleoplasm which were stained blue color with the dye lay scattered in the tissue (Fig. 4b). In the transverse section of about 1200 μm to 2000 μm from the apical initials, around ten to twenty bodies of released nucleoplasm which were stained blue color appeared clearly with the use of the author's technique (Fig. 4c). It is worth mentioning that the blue colored nucleoplasm mounted in the diffused cytoplasm was not covered with a cell wall (Fig. 4d). Consequently, the nucleoplasm and the diffused cytoplasm become situated in the intercellular spaces. In a non-stained preparation, which was prepared and covered with balsam after being immediately deparaffined with xylol, a body of nucleoplasm and the cytoplasm which was not covered with the cell wall were observable clearly with phase contrast microscopy (Fig. 4e).

In addition, in the transverse section at about 350 μm to 500 μm from the apical initials, non-vacuolated protoplasts which were stained blue lay scattered in the procambial central cylinder (Fig. 4f). It is probable that these non-vacuolated small meristematic cells may be initials cells which should produce the released nucleoplasm. It must now be mentioned that these non-vacuolated meristematic cells had no cell wall as indicated by the arrows (Fig. 4g). The cytological and electron microscopical details of the early stage of differentiation of these released bodies of nucleoplasm are worthwhile subjects for future investigation. The typical released bodies of nucleoplasm were localized in the area about 900 μm to 2000 μm from the apical initials (Figs. 2f, g and 4c).

Discussion

In root tips, the whole of the central cylinder is usually regarded as procam-

Figs. 4a-g. Transverse section of central cylinder of primary root tip of *Pisum sativum*, variety "Alaska". a, 2500 μ m from apical initials. $\times 300$. b, a part of the tissue of Fig. 4a, arrows indicate released nucleoplasm. $\times 800$. c, 1200 μ m from apical initials. $\times 300$. d, a part of the tissue of Fig. 4c, arrows indicate released nucleoplasm. $\times 800$. f, 350 μ m from apical initials. $\times 300$. g,

bium, whether or not a central pith eventually becomes differentiated. In the transverse section around 2500 μm from the apical initials of pea root tips, as shown in Fig. 4a, the distinct pith tissue was observable. Besides, in the transverse section of about 350 μm to 500 μm from the apical initials, procambial central cylinder meristem still appeared dense (Fig. 4f). Being in the area between these two layers, is of importance in the differentiation and in the morphogenesis of the central cylinder.

It is a noteworthy phenomenon that the differentiation of released nucleoplasm appeared in the limited area about 600 μm to 900 μm from the apical initials. These noticeable facts were detected with the technique-combination of Lillie's formalin fixation and Heidenhain's hematoxylin and safranin staining and/or methyl blue staining. The author has reported that the same cytological and histological techniques are found to detect two kinds of pericyclic cells in the pea root tips (Toriyama 1978b). Generally speaking, the author's techniques are most suitable to fix the protoplast or the nucleoplasm in the meristem of pea root tips.

Of special interest is that the cell was not formed partially in the cambial central cylinder as indicated Figs. 2f, g and 4g. As a result of this phenomenon, the cytoplasm and the nuclei lie in the intercellular spaces. The author would like to designate as 'released nucleoplasm' these nuclei which are surrounded by the diffused cytoplasm in the intercellular spaces. So far as is known, the work reported here is the first specifically designed to elucidate the released nucleoplasm in the central cylinder of the root tips. In future, the differentiation of the released nucleoplasm in the central cylinder in many species of higher plants must be estimated from the view-point of comparative histology. And also, further experiments on the cytochemical studies of the released nucleoplasm are being carried out.

Summary

In the present investigation, the released nucleoplasm in the central cylinder of primary root tips of *Pisum sativum*, variety "Alaska", was studied from the cytological and histological view point.

In the area about 900 μm to 2000 μm from the apical initials, there are two kinds of bodies of released nucleoplasm; one is thick and short (width 6–8 μm , length 15–20 μm), and the other is thin and long (width 4–5 μm , length 20–25 μm). The process of differentiation of this nucleoplasm from the procambial meristem of the central cylinder are observable in the area about 600 μm to 900 μm from the apical initials.

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a part of the tissue of Fig. 4f. arrows indicate non-vacuolated protoplasts which have a boundary without cell wall. $\times 800$. All the micrographs (except 4e) shown were made from tissue section fixed with Lillie's buffered neutral formalin and stained with 0.5% methyl blue. e, arrow indicates released nucleoplasm surrounding cytoplasm which was not surrounded by a cell wall, with phase contrast microscopy. $\times 960$.

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