The Developmental Change of Cell Structures Stained by Iron-Hematoxylin in the Cotyledon Areole of Leguminosae

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Change of cell structures stained by iron-hematoxylin in cotyledon areole (= CA) cells was observed in comparison with those structures in CA surrounding cells during seed germination and seedling development in six species of Leguminosae, i.e., Carmichaelia exsul, Glycine max, Goodia lotifolia, Melilotus altissima, Pisum sativum, and Tadehagi triquetrum ssp. pseudotriquetrum. Three patterns were recognized in the process of the change: (1) the stained structures of CA cells are different from those in CA surrounding cells throughout seed germination (Goodia lotifolia and Pisum sativum); (2) the difference is indistinct at the beginning of the germination and becomes distinct when rooting (Carmichaelia exsul and Glycine max); (3) the difference is indistinct throughout the germination (Melilotus altissima and Tadehagi triquetrum ssp. pseudotriquetrum). After unfolding of cotyledons in seedlings, most of the structures stained by iron-hematoxylin were lost, and the differences between CA cells and their surrounding cells became obscure in any cases.

Key words: cell structures, cotyledon areole, development, Leguminosae, seed germination

Cotyledon areole (= CA) is a spot existing on the abaxial side of cotyledon in some species of Papilionoideae of Leguminosae (Endo and Ohashi 1997), and has been reported from 23 tribes of the subfamily (Endo and Ohashi 1998a). CA is composed of granularly projected epidermal cells, and the hypodermis and several cell layers abutting inwards against the epidermis (Endo and Ohashi 1998a). Sizes of CA cells differ from the surrounding cells in dormant seed cotyledons (Endo and Ohashi 1998b). Stainability for iron-hematoxylin is sometimes well different between the CA cells and their surrounding cells but not different sometimes (Endo and Ohashi 1998b).

Anatomical and physiological changes of cotyledons during germination have been reported on several legume species (Bagley et al. 1963, Bain and Mercer 1966a, 1966b, Opik 1966, Smith and Flinn 1967, Smith 1974). Bain and Mercer (1966a) observed changes of subcellular organization of cotyledons during germination and seedling development in Pisum sativum. They recognized three morphological phases in development of the seedling. And they described the changes of subcellular organization at each phase; subcellular organization is reorganized during the first phase; then, the organization begins to break down and reserves begin to move from the cotyledons at the second phase; and, finally, subcellular disorganization continues and stor-
age is lost greatly (Bain and Mercer 1966a). The CAs are visible till abscission of cotyledons in *Pisum sativum*, a hypogoeously germinated species, and till unfolding of the first juvenile leaf in *Melilotus altissima*, an epigoeously germinated species (Endo and Ohashi 1998a). However, the anatomical studies of CAs have been done only with dormant seed cotyledons. How do the CAs anatomically change during the seed germination and the seedling development? In the present study, we observed changes of CA cells during the seed germination and the seedling development.

**Materials and Methods**

We selected six species: *Carmichaelia exsul* F.Muell., *Glycine max* (L.) Merr., *Goodia lotifolia* Salisb., *Melilotus altissima* Thuill., *Pisum sativum* L., and *Tadehagi triquetrum* (L.) H.Ohashi ssp. *pseudotriquetrum* (DC.) H.Ohashi. These species showed different stainability of CA cells in comparison to their surrounding cells for iron-hematoxylin in mature dormant cotyledons; i.e., stainability is similar in *Carmichaelia exsul*, *Glycine max*, and *Tadehagi triquetrum* ssp. *pseudotriquetrum* but different in *Melilotus altissima*, *Pisum sativum*, and *Goodia lotifolia* (Endo and Ohashi 1998b).

To examine changes of the stained structures of CA cells and their surrounding cells during germination and seedling development, we observed cotyledons at following three morphologically distinct stages: Stage 1, just after imbibition of water; Stage 2, at sending out of roots; and Stage 3, at unfolding of cotyledons in epigoeously germinated species, i.e., *Carmichaelia exsul*, *Glycine max*, *Goodia lotifolia*, *Melilotus altissima*, and *Tadehagi triquetrum* ssp. *pseudotriquetrum*, and at unfolding of the first scaly leaf in hypogoeously germinated species, i.e., *Pisum sativum*.

For imbibition of water, we soaked seeds in water. The seed coats, which could not imbibe water within one day, were harmed so that all seeds imbibed water. After the imbibition, the seeds were planted in moist vermiculite.

For observations of the serial sections with LM (= light microscope), the materials were fixed in FAA and dehydrated in a t-butyl alcohol series and embedded in “Hist-Paraffin” (m.p. 52–54°C, Wako Pure Chemical Industries, Ltd., Japan). After the embedding, the materials were cut into serial sections with 5–7 μm thickness. The sections were stained by a combination of iron-hematoxylin–safranin and fast green FCF.

<p>| Table 1. The list of seed sources, voucher specimens, and cultivating periods of the species examined |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Tribe</th>
<th>Seed source; voucher specimen</th>
<th>St 1</th>
<th>St 2</th>
<th>St 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Carmichaelia exsul</em> F.Muell.</td>
<td>Carmichaelieae</td>
<td>Seeds obtained from Royal Bot. Gard. Sydney, Australia; Y. Endo 3133 (CBM)</td>
<td>1 d</td>
<td>4 d</td>
<td>14 d</td>
</tr>
<tr>
<td><em>Glycine max</em> (L.) Merr.</td>
<td>Phaseoleae</td>
<td>Seed source unknown; Y. Endo 3132 (CBM)</td>
<td>1 d</td>
<td>2 d</td>
<td>13 d</td>
</tr>
<tr>
<td><em>Goodia lotifolia</em> Salisb.</td>
<td>Bossiaeae</td>
<td>Seeds obtained from Royal Bot. Gard. Melbourne, Australia; n. v. s.*</td>
<td>1 d</td>
<td>6 d</td>
<td>19 d</td>
</tr>
<tr>
<td><em>Melilotus altissima</em> Thuill.</td>
<td>Trifolieae</td>
<td>Seeds obtained from Hort. Bot. Univ. Osloensis, Oslo, Norvegia; Y. Endo 3135 (CBM)</td>
<td>7 h</td>
<td>2 d</td>
<td>5 d</td>
</tr>
<tr>
<td><em>Pisum sativum</em> L.</td>
<td>Vicieae</td>
<td>Seed source unknown; Y. Endo 3134 (CBM)</td>
<td>1 d</td>
<td>3 d</td>
<td>6 d</td>
</tr>
<tr>
<td><em>Tadehagi triquetrum</em> (L.) H.Ohashi ssp. <em>pseudotriquetrum</em> (DC.) H.Ohashi</td>
<td>Desmodieae</td>
<td>Seeds collected in Taiwan, Pingtung, Kenting; H. Ohashi et al. 14717 (TUS)</td>
<td>7 h</td>
<td>2 d</td>
<td>9 d</td>
</tr>
</tbody>
</table>

*aSt 1 = the period between the initial imbibition of water by the seeds and Stage 1; bSt 2 = the period between the imbibition and Stage 2; cSt 3 = the period between the imbibition and Stage 3; d = day(s); h = hours; *n. v. s. = no voucher specimen.*
The sources of the seeds, their voucher specimens, and cultivating periods between the initial imbibition and the fixation are presented in Table 1.

**Results**

*Melilotus altissima* and *Tadehagi triquetrum* ssp. *pseudotriquetrum*: structures stained by iron-hematoxylin of CA cells were similar to those in their surrounding cells at Stage 1 and 2 (Fig. 1), and most of the structures of the cotyledon cells were unstained at Stage 3 (Fig. 2).

*Carmichaelia exsul* and *Glycine max*: structures stained by iron-hematoxylin of CA cells were similar to those of their surrounding cells at Stage 1 (Fig. 3), at Stage 2 CA cells had larger unstained spaces than the surrounding cells and the CA parts seemed more whitish (Figs. 4, 5, 7). Most of the structures of the cotyledon cells were unstained at Stage 3 (Figs. 6, 8).

*Pisum sativum*: at Stage 1 and 2 (Fig. 9) the CA cells were smaller than their surrounding cells and had unstained granules fewer than the surrounding cells. At Stage 3 the boundary of the two parts became more distinct than the stage 1 and 2 because the structures of CA cells were more deeply stained by iron-hematoxylin than those of the surrounding cells (Fig. 10).

*Goodia lotifolia*: some granules were stained deeply in the CA cells without epidermis but whole cell structures were stained deeply in their surrounding cells at Stage 1 (Fig. 11), at Stage 2 only one granule was stained deeply in the CA cells (Figs. 12, 13) and several granules, unoccupied or semi-occupied by stained structures, were gathered in the surrounding cells (Figs. 12, 14), and at Stage 3 most of the structures of the cotyledon cells were unstained except in epidermal cells of CA part and in the surrounding cells of the midvein, which had one stained granule (Fig. 15). At all three stages, the cell wall of CA cells (Fig. 13) was thinner than that of their surrounding cells (Fig. 14).

**Discussion**

We compared the three phases of Bain and Mercer (1966a) and three stages of the present study in the case of *Pisum sativum* in Fig. 16. We presumed that Stage 1 in our study may be the beginning of the Phase 1, at which the subcellular organizations of cotyledons start to reorganize; Stage 2 may be near and before the boundary between the Phase 1 and 2, at which the subcellular organizations begin to breakdown and reserves begin to move from the cotyledons; and Stage 3 may be near the boundary between the Phase 2 and 3, at which the subcellular disorganization continues and storage is lost greatly.

At Stage 3 in not only *Pisum sativum* but also all other species examined, the most structures became unstained by iron-hematoxylin in CA cells and other cotyledon cells. From the comparison between the Phases of Bain and Mercer (1966a) and the Stages of the present study, we consider that the change of stainability of structures in CA cells and other cotyledon cells may be caused by subcellular disorganization and great loss of storage in these cells. And we consider that the most stained structures observed before Stage 3 may be lost. As a result of the loss, the differences between CA cells and the other cotyledon cells became obscure. And we think that specific function of CA cells, if present, may be lost by the stage.

In *Carmichaelia exsul*, all cotyledon cells are stained by iron-hematoxylin in same degree at Stage 1 (Fig. 3), and at Stage 2 unstained spaces increase in CA cells and the cotyledon cells at a distance from the epidermis (Figs. 4, 5). This fact suggests that subcellular organization breaks down at these regions earlier.

From the histological study of the cotyle-
Figs. 1–8. LM photos of sections of cotyledons of *Melilotus altissima* Thuill. (1, 2), *Carmichaelia exsul* F. Muell. (3–6), and *Glycine max* (L.) Merr. (7, 8), showing CA and its surrounding cells. 1–4, 5. Stage 2. 2, 6, 8. Stage 3. 3. Stage 1. 5. Enlargement of the part enclosed with the square in Fig. 4. Upper is the abaxial side of the cotyledons. Scale bars = 0.2 mm in 1–4, 6–8, = 0.1 mm in 5. Abbreviations: CA = Cotyledon areole; N = nucleus; MV = midvein; MVp = primordia of midvein.
Figs. 9–15. LM photos of sections of cotyledons of *Pisum sativum* L. (9, 10) and *Goodia lotifolia* Salisb. (11–15), showing CA and its surrounding cells. 9, 12. Stage 2. 10, 15. Stage 3. 11. Stage 1. 13, 14. Enlargement of the parts enclosed with the square "a" (to 13) and square "b" (to 14) in Fig. 12. Upper in 9–14 and left in 15 are the abaxial side of cotyledons. Scale bars = 0.2 mm in 9, 10, = 0.1 mm in 11, 12, = 0.05 mm in 13–15.
dons of *Phaseolus vulgaris* L. of Papilionoideae during germination, it was reported that nuclei and cytoplasm are still present in the cells near vascular bundles at abscission (Smith 1974). In *Goodia lotifolia* at Stage 3, only one granule was stained at epidermis of CA part and the cells near midvein (Fig. 15). We presume that the granules may be a nucleus. The granule was also reported from CA cells of dormant cotyledons in the species (Endo and Ohashi 1998b). Therefore, the nucleus in CA cells is distinct during the dormancy and germination. This fact may mean that most of the structures stained by iron-hematoxylin, which hide a nucleus, are absent in the CA cells.

In *Goodia lotifolia* at Stage 1, small granules were stained well in CA cells except for the epidermis, and whole cell structures were stained well in the epidermis (Fig. 11). Functions of these two parts in CA may be different from each other at the stage. In the species at Stage 2, the epidermal cells of CA show some features as other CA cells, i.e., nucleus is distinct in these cells (Figs. 12, 13). All CA cells may have a common function at Stage 2 in this species.

On the processes of the changes of the structures stained by iron-hematoxylin of CA cells and their surrounding cells during seed germination and the seedling development, we recognized the following three patterns: (1) the stained structures of CA cells are different from those of their surrounding cells during the germination in *Goodia lotifolia* and *Pisum sativum*; (2) the difference becomes more distinct at rooting than at the beginning of the germination in *Carmichaelia exsul* and *Glycine max*; (3) the difference is indistinct during the germination in *Melilotus altissima* and *Tadehagi triquetrum* ssp. *pseudotriquetrum*. In the species with the second pattern, to recognize the boundary of CA and the surrounding part it may be better to examine CAs when rooting than beginning of seed germination.

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**References**


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