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RESEARCH ARTICLE

Ultracytochemical localization of Ca^{2+} during the phloem ganglion development in *Phyllostachys edulis*

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Abstract Ultracytochemical localization of Ca^{2+} was investigated using the potassium pyroantimonate precipitation method during the development of phloem ganglion. The result showed that Ca^{2+} was mainly localized in the cell wall and intercellular spaces in the initiating phase. With the development of the phloem ganglion, the distribution of Ca^{2+} transferred to the vacuole, and the Ca^{2+} deposits in the cell wall and intercellular space decreased. At the later stage of the developmental phase, Ca^{2+} was distributed in the tonoplast and vacuole phagocytosis, and the vacuole became the main calcium storage in this phase. At the early stage of maturation of the phloem ganglion, most of the phloem ganglion cells' vacuoles cracked, and the cytoplastic Ca^{2+} content increased in large number. In the mature phloem ganglion, not only were there a few Ca^{2+} localized in the cytoplast of mature cells, but also in the differentiating cells in the vacuoles. Ca^{2+} was distributed in the tonoplast and vacuole contents; initiating cells almost had no Ca^{2+}. In general, Ca^{2+} concentration in mature phloem ganglion cells was at a low level. The results indicated that the changes in Ca^{2+} distribution evoked the phloem ganglion generation, and Ca^{2+} regulated the physiological function of the phloem ganglion.

Keywords phloem ganglion, development, Ca^{2+}, physiological function

1 Introduction

The node is crucial to the development of bamboo. The phloem ganglion is a special structure of the phloem that is only found in the nodal area of the bamboo. There are some investigations on the structure and development of the phloem ganglion (Ding and Liese, 1995; Ding et al., 2000), but there is no report on the cytochemistry during the development of the phloem ganglion. Ca^{2+} is the second message in the development of the cell, and it is important to plant growth (Bush, 1995). We expect that the study on Ca^{2+} in the phloem ganglion can reveal some mechanism of developmental biology and physiological function of phloem ganglion.

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2 Materials and methods

Healthy and vigorously growing shoots (approximately 100 cm) and 2-year old, 4-year old and 6-year old culms of *Phyllostachys edulis* (Carr.) H. de Lehaie were chosen. The eighth, seventeenth, twenty-ninth, forty-first and forty-eighth nodes were selected from the young shoot. The samples for old culms were selected from the node in the middle part of the culms. These samples, about 1 mm^3, were prepared and were fixed immediately in 3% glutaraldehyde solution, which was confected with 2% potassium pyroantimnate buffer solution at 4ºC. In succession, the samples were washed in 2% potassium pyroantimnate buffer solution, and then fixed again in 1% OsO_4 at 4ºC. The fixed samples were washed in redistilled water, then were dehydrated in a graded ethanol series and were transferred to Epon812 to be embedded. Ultrathin sections were cut with a diamond knife on a LKB-V ultramicrotome. After post-staining with 2% aqueous uranylacetate, sections were examined and photos were taken with a transmission electron microscope (model H-600). The comparative sections were treated with 100 mmol / L EGTA (pH=8.0) and examined and photos were taken with a transmission electron microscope.

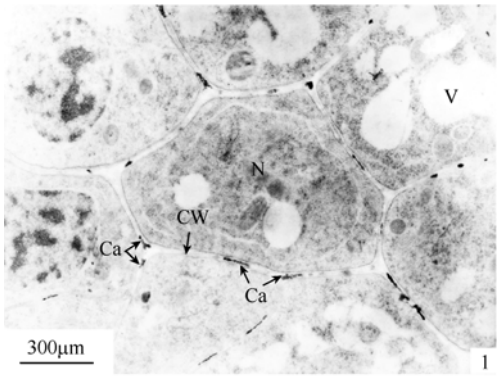
3 Results

The development process of phloem ganglion could be

divided into three phases: initiating phase, developmental phase, and maturation phase.

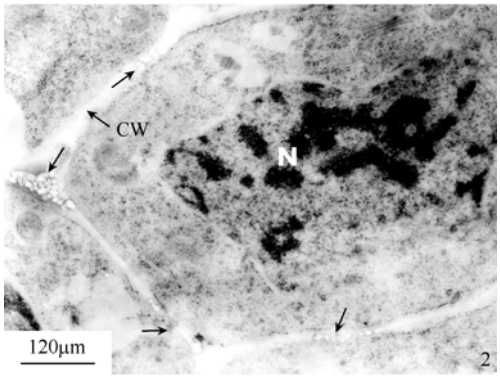
3.1 The distribution of Ca^{2+} in initiating phase

In the procambium and phloem ganglion archaeocyte, Ca^{2+} was observed in the cell wall and intercellular space, but almost no Ca^{2+} was distributed in the cytoplasm (Fig. 1). After treating with EGTA, the sites where Ca^{2+} is located became translucent, which indicated that the black deposit was Ca^{2+} deposition (Fig. 2).



CW: cell wall; N: nucleus; V: vacuole

Fig. 1 Ca^{2+} localized in the cell wall and intercellular space of phloem ganglion initials



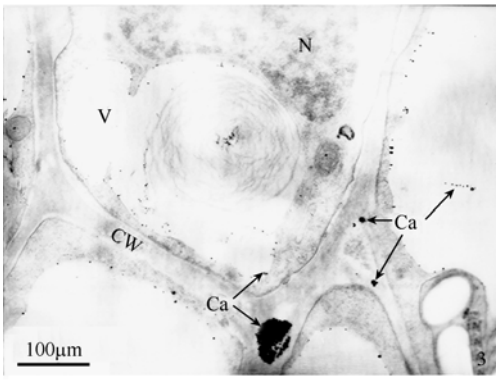
CW: cell wall; N: nucleus;

Fig. 2 Ca^{2+} positions became inanitions after chelation

3.2 The distribution of Ca^{2+} in developmental phase

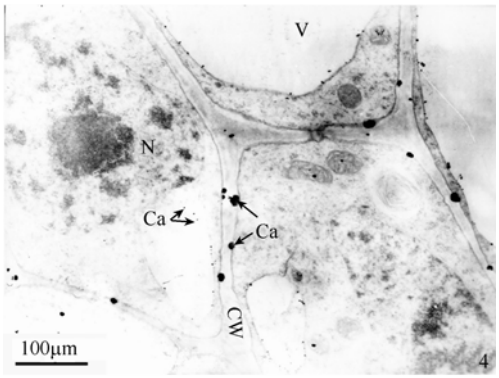
With the development of the phloem ganglion, the Ca^{2+} granule became bigger and concentrative, and transferred near the plasma membrane (Figs. 3 and 4). Then, the plasma membrane invaginated and Ca^{2+} was transferred into the cells by the Ca^{2+} transport vesicle (Fig. 5).

At the early stage of developmental phase, small vacuoles began to amalgamate into a large central vacuole, and phagocytic vesicles and membrane matter were observed in vacuoles. In this stage, Ca^{2+} were localized in the tonoplast and vacuole phagocytosis, and few Ca^{2+} deposits could be observed in the mitochondrion (Fig. 4).



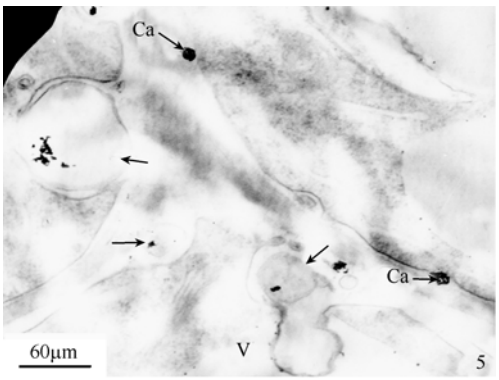
CW: cell wall; N: nucleus; V: vacuole

Fig. 3 Ca^{2+} distributed on the tonoplast and vacuole contains



CW: cell wall; N: nucleus; V: vacuole

Fig. 4 Ca^{2+} deposited as spotted and some located in vacuoles and mitochondrion in at the early stage of developmental phase

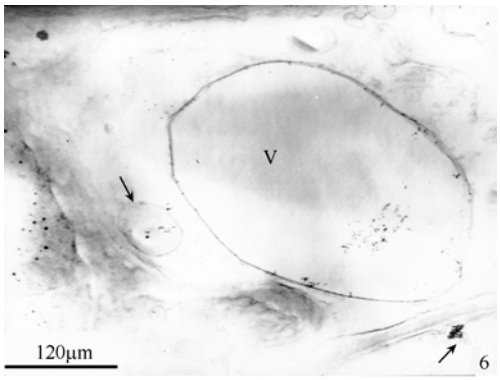


V: vacuole

Fig. 5 Transfer vesicles of Ca^{2+} (arrow) transported into the cells

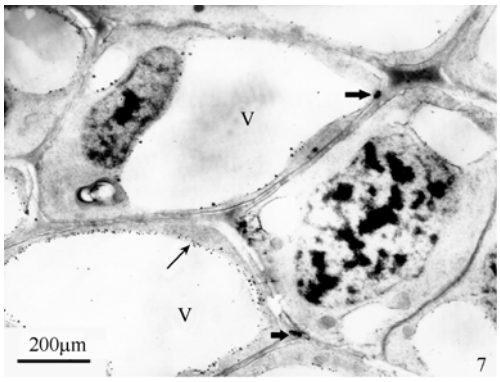
At the later stage of the developmental phase, cell walls became obviously thicker, and the large central vacuole appears. Therefore, the cytoplasm and the nucleus were pressed to the marginal area. With the differentiation of the phloem ganglion, Ca^{2+} deposits in the cell wall became less and less, whereas more Ca^{2+} were distributed in the vacuole (Figs. 6 and 7). The process of Ca^{2+} transferring from the outer to the cytoplasm of the cell was first observed with transmission electron microscopy of the vesicles containing Ca^{2+}, it was seen that the plasma membrane was invaginated. Therefore, the Ca^{2+} transport vesicles were transferred into

the cytoplasm (Fig. 5). Then the Ca^{2+} transport vesicles amalgamated with the vacuole. Lastly, the Ca^{2+} was transferred into the vacuole (Figs. 6, 7 and 8); Ca^{2+} was mainly distributed in the vacuoles at the late stage of the developmental phase. The result showed that Ca^{2+} transferred gradually from the cell wall and intercellular space to the vacuole, whereas few Ca^{2+} were localized in the cytoplasm and organelles.



V: vacuole

Fig. 6 Ca^{2+} transfer vesicles, tonoplast and its contains with Ca^{2+} localized



V: vacuole

Fig. 7 During the late stage of developmental phase, Ca^{2+} in vacuole increased and that of cell wall and intercellular space decreased

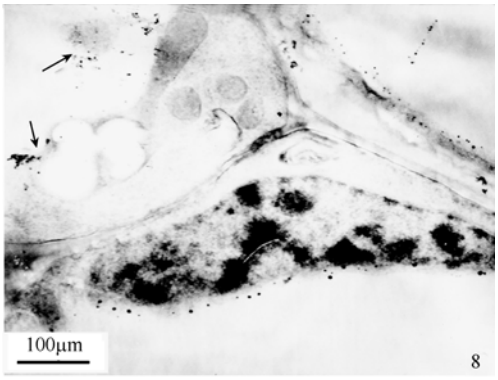
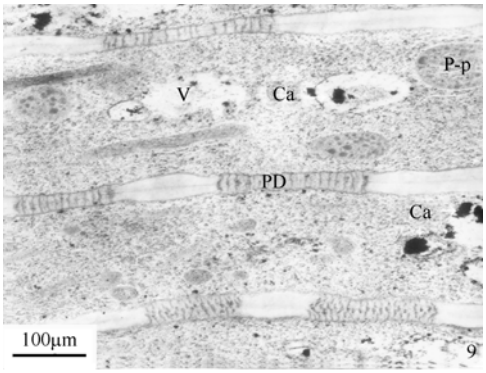


Fig. 8 Ca^{2+} transfer vesicles fused with vacuole and Ca^{2+} went into vacuole

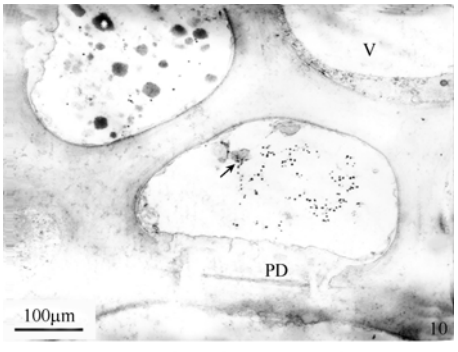
3.3 The distribution of Ca^{2+} in the maturation phase

The vacuoles were the main calcium storage of the phloem ganglion at the early stage of the maturation phase (Fig. 9). With the maturation of the phloem ganglion, the tonoplast of most cells of the phloem ganglion broke down, and Ca^{2+} concentration in the cytoplasm increased to a high level (Fig. 10), then the cytoplasm begun selective autolysis and the organelles degraded. A lot of Ca^{2+} were localized in the degradations of the cytoplasm (Figs. 11, 12 and 13). P-plastids degraded in two ways, and the dispersed proteinous crystalloid and the membrane of the P-plastids were both with Ca^{2+} deposits (Figs. 9, 10 and 11). Dispersed proteinous crystalloid degraded into filiform protein with a large number of Ca^{2+} localizing in the cytoplasm (Figs. 13 and 14). Many paramural bodies came into being in mature phloem ganglion cells, and Ca^{2+} deposits were observed in the membrane of the paramural bodies (Fig. 12). Ca^{2+} concentration in mature phloem ganglion cells was at a low level. Mature phloem ganglion was composed of not only mature cells but also the differentiating cells and initiating cells. The distribution of Ca^{2+} in these three kinds of cells was different. Few Ca^{2+} deposits localized in the cytoplasm in the mature cells of phloem ganglion (Fig. 10), but Ca^{2+} was mainly distributed in the tonoplast and phagocytosis in the differentiating cells whose vacuoles did not collapse (Figs. 15 and 16), and almost no Ca^{2+} was observed in the initiating cells (Fig. 16).



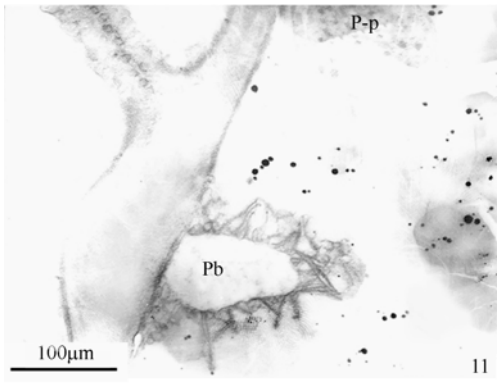
PD: plasmodesma; P-p: P-plastid; V: vacuole

Fig. 9 At the early stage of maturation, Ca^{2+} localized mainly in vacuoles



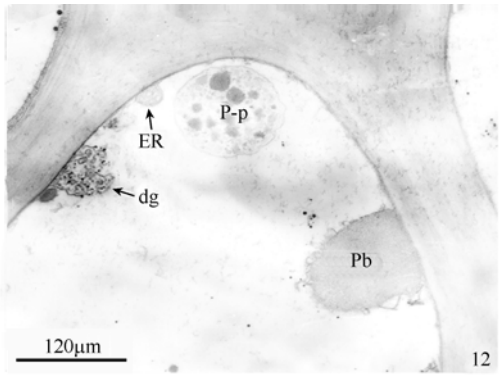
PD: plasmodesma; V: vacuole

Fig. 10 Degrading proteinous crystalloids and organelles with Ca^{2+}



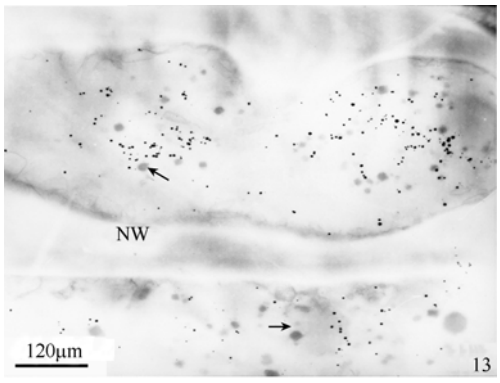
Pb: paramural body; P-p: P-plastid

Fig. 11 The degrading and paramural bodies membrane with Ca^{2+} deposited on them



dg: degradation; ER: endoplasmic reticulum; Pb: paramural body; P-p: P-plastid

Fig. 12 The degradations and paramural bodies membrane with Ca^{2+} deposited on them

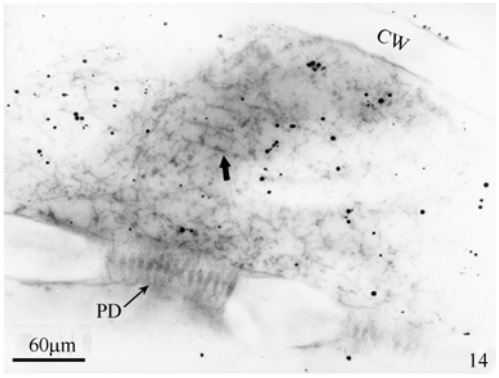


NW: nacreous wall

Fig. 13 In 6-year-old phloem ganglion, much Ca^{2+} localized in proteinous crystalloids spilling out into the cell lumen

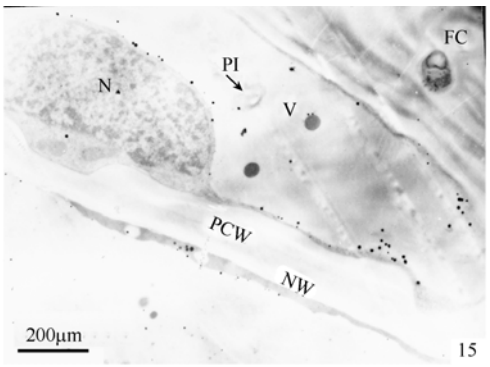
4 Discussion

Since the phloem ganglion was first found in the node of *Dioscorea* spp. by Mason, the characteristic of the phloem ganglion was deeply investigated (Brouwer, 1953; Braun, 1957; Behnke, 1965; Ding et al., 2000). The activity of phosphatase



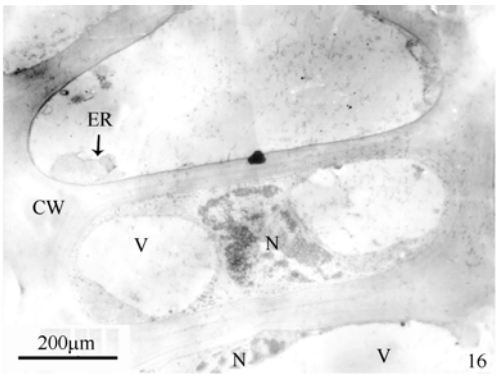
CW: cell wall; PD: plasmodesma

Fig. 14 In 6-year-old phloem ganglion, much Ca^{2+} localized in filiform protein in cell lumen



FC: fiber cell; NW: nacreous wall; PCW: primary cell wall; PI: plastid; V: vacuole

Fig. 15 Ca^{2+} distribution in phloem ganglion neighbored to the fiber, vacuole membrane and vacuole inclusions (arrow) with Ca^{2+}



CW: cell wall; ER: endoplasmic reticulum; N: nucleus; v: vacuole

Fig. 16 In 2-year-old phloem ganglion, Ca^{2+} localized in vacuoles of cells which is differentiating, no Ca^{2+} visualized in archaeocyte and Ca^{2+}

in the phloem ganglion was also studied (Braun and Sauter, 1964). Ca^{2+} is a crucial regulator of growth and development in plants. We have investigated Ca^{2+} in the phloem ganglion, and the results indicate that Ca^{2+} plays an important role during the development of the phloem ganglion.

4.1 Changes of Ca^{2+} distribution make the phloem ganglion generate

Ca^{2+} is the second message in plant growth and development, because cells must respond to an array of environmental and developmental cues by Ca^{2+}. Therefore, Ca^{2+} plays a key role in plant growth, development, physiological metabolism, and function. It is essential for cells' normal physiological activities that Ca^{2+} concentration in the cytoplasm maintains a low level.

From the results, we know that no Ca^{2+} deposits could be observed in the cytoplasm of the procambium and phloem ganglion archaeocytes;Ca^{2+} was dispersely distributed in the cell wall and intercellular space. With the development of the phloem ganglion, Ca^{2+} localizing in the cell wall and intercellular space became lumpish and partial, and began to transfer into the cytoplasm and the vacuole became the main calcium storage. So, we speculate that the change of Ca^{2+} distribution might be the initiation factor of phloem ganglion generation, which is the same as the previous studies. Investigation on the vessels indicated that the differentiation of the vessel needed the influx of Ca^{2+} (Roberts and Haigler, 1989). With the differentiation of phloem ganglion, Ca^{2+} transfers from the outer to the vacuole. Ca^{2+} plays a crucial role in determining the structural rigidity of the cell wall, and low Ca^{2+} concentrations in the cell wall may make the wall plastic and able to elongate (Hepler, 2005). At the early stage, deposited in the degradations in mature cells of maturation phase, Ca^{2+} can only been observed in the vacuole, while no Ca^{2+} is distributed in the cell wall and intercellular spaces, then the tonoplast collapses, and the free Ca^{2+} concentration in the cytoplasm obviously increases which leads the cytoplasm to selective autolysis. A lot of Ca^{2+} comes into the vacuole and makes the vacuole crack, subsequently, the hydrolase in vacuoles mixes with the cytoplasm, which leads the cytoplasm to selective autolysis, and most organelles to degrade. Many researches showed that increase of Ca^{2+} in cytoplasm generated and quickened the death of cells. For example, investigation about programmed cell death of the vessels showed that the transferring of large numbers of Ca^{2+} from the outer into the cell triggered the programmed cell death of vessels (Groover and Jones, 1999; Zhang et al., 2001). Groove and Jones (1999) suggested that the influx of Ca^{2+} led the tonoplast to collapse, and the cytoplasm to stop flowing, which triggered the death of cells, then the vacuoles cracked and the vacuole contents mixed with the cytoplasm, which led the cytoplasm to selective autolysis. The result indicates that the opening of Ca^{2+} crossroad is the key to programmed cell death of vessel elements. In phloem ganglion cells, Ca^{2+} concentration maintains a low level before the vacuole collapses, and Ca^{2+} localizes mainly in the tonoplast and the vacuole phagocytosis. But after the vacuole cracks, free Ca^{2+} concentration increases largely in the cytoplasm, whereas no Ca^{2+} deposits is observed in the nucleus at all times. At the later stage of the maturation phase, free Ca^{2+} concentration in the cytoplasm decreases to a low level. By maintaining the steady low level concentration, Ca^{2+} can play the role in plant physiological metabolism as a second message. Mature phloem ganglion is composed of cells in different phases, so their Ca^{2+} distribution is different. In the cells which have initial characters, almost no Ca^{2+} could be observed, which was different from that of the procambium and the phloem ganglion initials. This shows the function of these cells is mainly to maintain active physiological metabolism and function of mature phloem ganglion, which is very important for perennial monocots, e.g., *Phyllostachys edulis*.

4.2 Ca^{2+} regulated physiological function of phloem ganglion

Ca^{2+} is an important regulator of the plasmodesmata. Investigations show that plasmodesmata contract or close when cytoplasmic Ca^{2+} concentration increases to a high level, and intercellular communication is blocked. The reasons may be the following: (1) The elevation of cytosolic Ca^{2+} content may lead the ER contraction through the disassembling of the cytoskeleton–ER complex, and the plasmalemma around the plasmodesmata fused together, leading to the disassociation of the plasmodesmata (Jian et al., 2000). (2) High Ca^{2+} concentration may make the cell wall thicker, which squeezes the plasmodesmata and makes it contract even close (Eklund, 1991; Eklund and Eliasson, 1990). (3) Ca^{2+} may activate some kinases of the plasmodesmata structure and lead the phosphorylation of cytoskeletal proteins localizing in the cytoplasmic anulus and results in permeation pore blockage (Tucker and Boss,1996; Holdaway-clarke et al., 2000). (4) High Ca^{2+} concentration may evoke the callose deposition around the plasmodesmata neck and lead to the closure of the plasmodesmata (Rinne and Var der Schoot, 1998). During the development of phloem ganglion, there are no Ca^{2+} in the plasmodesmata, but at the late stage of phloem ganglion development, intracellular Ca^{2+} content increases, and the callose deposits around the plasmodesmata neck. Phloem ganglion belongs to the secondary one. We speculate that callose deposition and degradation might be an important reason for the regulation of plasmodesmata permeability. In mature phloem ganglion cells, Ca^{2+} concentration maintains a steady low level, and the plasmodesmata opens, which is important for phloem ganglion to normal metabolism and transportation.

Ca^{2+} is vital to the physiological function of the phloem ganglion. Knoblauch et al. (2001) studied on the sieve tubes of legumes (Fabaceae), and found changes in free Ca^{2+} regulated reversibly P-proteins between a crystalloid and dispersed filiform state. When concentration of free calcium in the cytoplasm increases, crystalloid P-protein disperses and moves to plug sieve pores blocking the flowing transport. Mature phloem ganglion cells have both crystalloid P-protein and dispersed filiform protein. So changes of free Ca^{2+} concentration may regulate the conductance through the phloem ganglion, and we speculate the role of Ca^{2+} might be related to its role of the second message. The con-

centration of free calcium in sieve tubes of *R. communis* has been found to be 20—100 times higher than that of the surrounding tissue (Brauer et al., 1998). We found free Ca^{2+} steady concentration in mature and functional phloem ganglion cells is higher than that of the surrounding tissue, so we speculate the transport direction of organic substance might relate to Ca^{2+} concentration, but the deduction needs future studies.

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