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## Involvement of inorganic elements in tissue reunion in the hypocotyl cortex of *Cucumis sativus*

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**Abstract** Cucumber (*Cucumis sativus* L.) hypocotyls were transversely cut to half their diameter, and morphological analyses of the tissue-reunion process in the cortex were conducted to elucidate the involvement of root-derived factors. Cell division in the cortex commenced 3 days after cutting, and the cortex was nearly fully united within 7 days. In shoots from which the roots were removed and which were cultured in water, cell division occurred during tissue reunion; however, thick-wall layer formed in the reunion region, and intrusive cell elongation and interdigitation of cortex cells at the cut surface did not occur, even after 7 days. Interdigitation of cells, followed by normal tissue reunion, was observed in shoots from which the roots were removed and which were cultured in squash xylem sap or Murashige and Skoog (MS) medium. The same effect was observed with the simultaneous application of B, Mn, and Zn, which are the major inorganic microelements of MS medium. Our results suggest that application of these inorganic elements, which are taken up from the soil and transferred to the xylem sap, are required for interdigitation of cells during tissue reunion in the cortex of cucumber hypocotyls, possibly because they are required for cell wall function and metabolism.

**Key words** Inorganic elements · Interdigitation of cells · Murashige and Skoog medium · Root · Tissue-reunion of cut hypocotyl · Xylem sap

### Introduction

In higher plants, growth is thought to depend on interactions among organs such as roots, shoots, and leaves. Roots

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and aboveground organs are connected by xylem and phloem in vascular bundles, and vascular tissues provide long-distance transport of water, nutrients, and various organic and inorganic materials within the plant (Jesko 1989; Satoh et al. 1998; Sakuta and Satoh 2000). When the original vascular connection is interrupted by wounding or grafting, the formation of new vascular tissue should be induced. Although the molecular mechanisms controlling vascular reunion are not yet fully understood, the involvement of phytohormones such as auxin and cytokinin, in xylem and phloem differentiation has been suggested (Roberts 1988; Mattsson et al. 1999; Sachs 2000). Studies of graft union and repair in cut tissues have focused on the differentiation of vascular elements in the tissue-reunion process, but the process of reunion in the cortex of cut tissues is not well understood.

Our previous study of tissue reunion in cucumber and tomato seedlings showed that the division and elongation of cortex cells begins at 3 days after cutting, and that the cortex is nearly completely united within 7 days (Asahina et al. 2002). Gibberellin (GA) is required for cell division during tissue reunion in the cortex of cut hypocotyls, and cotyledons are involved in GA production (Asahina et al. 2002).

In contrast, in plants from which the roots are removed and the shoot is cultured in water, cell division occurs during tissue reunion, but intrusive cell elongation and the interdigitation of cells between confronted cut cortex cells does not occur, even after 7 days. The interdigitation of cells during tissue reunion may play a role in the re-generation of the physiological and physical connections between separated tissues and cells. Such connections include intercellular attachment through the cell wall.

Plant cell walls are composed primarily of cellulose microfibrils, hemicellulose, pectic polysaccharides, and small amounts of structural proteins (Carpita et al. 1993; Ridley et al. 2001; Willats et al. 2001). Pectin is thought to be involved in intercellular attachment because it is localized mainly in the primary cell wall, middle lamella, and cell corners. Pectin consists mainly of three structurally characterized polysaccharides: homogalacturonans (HGs) and

highly branched rhamnogalacturonans-I and -II (RG-I and RG-II). Borate cross-linking of RG-II generates complex pectin networks (Iwai et al. 2003; O'Neill et al. 2004). Adequate supplies of boron to growing portions of the plant are necessary to facilitate formation of borate cross-linked RG-II for normal plant growth (Takano et al. 2005). In addition, 13 mineral elements (N, K, Ca, Mg, P, S, Cl, Fe, B, Mn, Zn, Cu, and Mo) are universally essential in seed plants to provide the constituents of organic compounds such as protein and nucleic acids, osmotica, enzyme cofactors, and electron transport proteins (Denny 2002).

Xylem sap contains inorganic elements and organic materials such as phytohormones (Nooden et al. 1990; Kuroha et al. 2002), polyamines (Friedman et al. 1986), proteins (Biles and Abeles 1991; Satoh et al. 1992; Campbell et al. 1995; Sakuta et al. 1998; Masuda et al. 1999; Oda et al. 2003), and oligo- and polysaccharides (Satoh et al. 1992; Iwai et al. 2003). It is possible that xylem sap in the roots contains some inorganic and/or organic substances essential for interdigitation of cells during tissue reunion in the cortex.

In this study, we performed morphological and histochemical analyses of the tissue-reunion process in the cortex of cut cucumber hypocotyls with removal of the roots to examine the contribution of roots in providing unidentified factor(s) that promote tissue reunion in the hypocotyl. Our results suggest that inorganic elements, including boron, zinc, and manganese, taken up from the soil and found in the xylem sap, are required for interdigitation of cells during tissue reunion in the cortex of cucumber hypocotyls.

## Materials and methods

### Plant materials and growth conditions

Cucumber (*Cucumis sativus* L., cv. Shimoshirazu-jibai) seeds were obtained from Sakata Seed Co. (Kanagawa, Japan). The seeds were germinated and grown in artificial soil (Kurehakagaku, Tokyo, Japan) under white fluorescent light ( $32 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 16 h/day at 28°C. After 7 days of growth, plants were carefully removed from the soil, the roots were eliminated using a razor blade, and the hypocotyl was transversely cut to half its diameter 3 cm from the base using a razor blade (0.1 mm thick). The shoots were then placed in 5-ml polypropylene tubes (Abbott Laboratories, North Chicago, IL, USA) and supplemented with 1–2 ml of distilled water, squash xylem sap (Satoh et al. 1998),  $10^{-5}$  M *trans*-zeatin riboside (*t*-ZR) solution (a major cytokinin in squash xylem sap; Nooden et al. 1990; Kuroha et al. 2002), or 1/20-strength modified Murashige and Skoog (MS) liquid medium or its components (Murashige and Skoog 1962). The 1/20-strength modified MS liquid medium contains macroelements ( $82.5 \text{ mg l}^{-1} \text{ NH}_4\text{NO}_3$ ,  $95 \text{ mg l}^{-1} \text{ KNO}_3$ ,  $22 \text{ mg l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $18.5 \text{ mg l}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $8.5 \text{ mg l}^{-1} \text{ KH}_2\text{PO}_4$ ), microelements ( $0.31 \text{ mg l}^{-1} \text{ H}_3\text{BO}_3$ ,  $1.115 \text{ mg l}^{-1} \text{ MnSO}_4 \cdot 4\text{H}_2\text{O}$ ,  $0.43 \text{ mg l}^{-1} \text{ ZnSO}_4 \cdot \text{H}_2\text{O}$ ,  $41.5 \mu\text{g}$

$\text{l}^{-1} \text{ KI}$ ,  $12.5 \mu\text{g l}^{-1} \text{ Na}_2\text{MoO}_4 \cdot 5\text{H}_2\text{O}$ ,  $1.25 \mu\text{g l}^{-1} \text{ CoCl}_2 \cdot 6\text{H}_2\text{O}$  and  $1.25 \mu\text{g l}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), Fe-EDTA ( $1.865 \text{ mg l}^{-1} \text{ Na}_2\text{EDTA}$ ,  $1.39 \text{ mg l}^{-1} \text{ FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) and organic elements ( $5 \text{ mg l}^{-1}$  *myo*-inositol,  $0.25 \text{ mg l}^{-1}$  nicotinic acid,  $0.1 \text{ mg l}^{-1}$  glycine,  $25 \mu\text{g l}^{-1}$  pyridoxine hydrochloride,  $25 \mu\text{g l}^{-1}$  thiamine hydrochloride,  $25 \mu\text{g l}^{-1}$  folic acid, and  $2.5 \mu\text{g l}^{-1}$  biotin). The pH of media was adjusted to 6. Sugar was not added to each media.

### Light microscopy of cut cucumber hypocotyl

Removal of root and application of water, xylem sap or MS medium were performed in 10–15 plant materials at each treatment and then embedded in resin. Sectioning and observation were made from randomly selected materials.

Preparation of sections was made as described previously in Asahina et al. (2002), and observations were made using a light microscope (DMRB, Leica, Wetzlar, Germany). Experiments were performed twice using different seed batches with similar results.

### Quantification of the thick-wall layer

To quantitatively determine the inhibition of interdigitation of cells, the length of the thick-wall layer at the cut surface was measured using longitudinal sections. The lengths of the cortex and thick-wall layer between two vascular bundles were measured and inhibition rates and standard errors were calculated for each treatment (Fig. 2e).

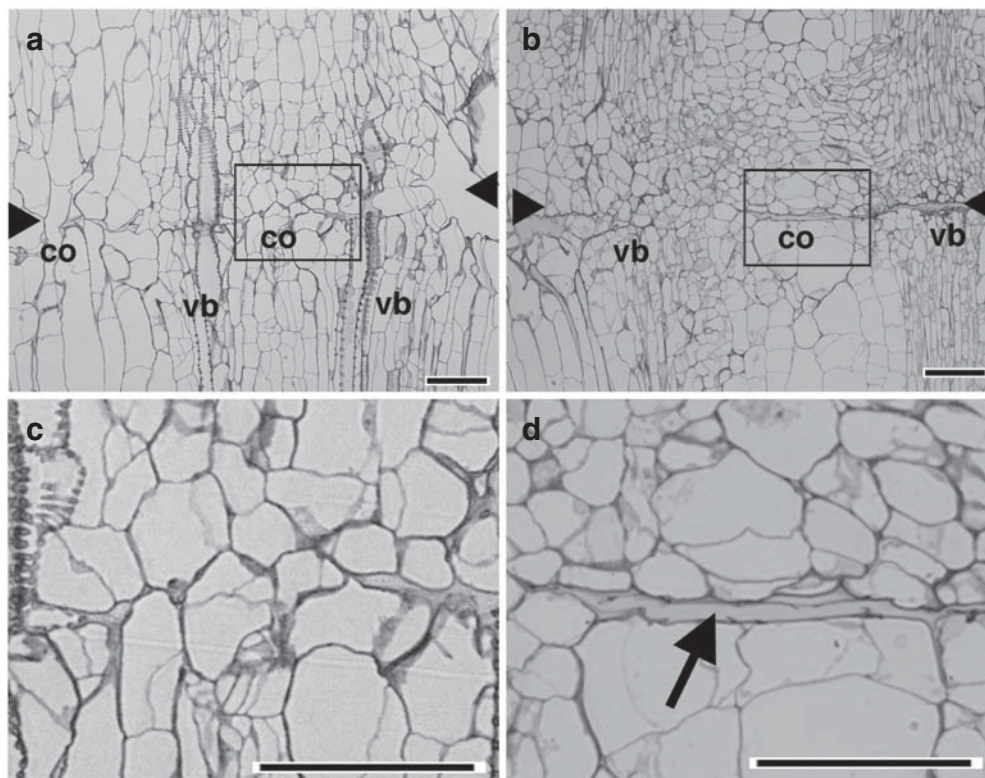
## Results

Our previous study showed that cortex cells near the cut surface initiated transverse cell division and longitudinal cell elongation towards the cut surface 3 days after cutting, followed by the formation of a cell wall layer between adjoining cortex cells. Subsequently, randomly directed cell division and intrusive cell elongation occurred. Finally, cortex cells near the cut surface were interspersed, the cell wall layer that formed at the cut surface disappeared, and interdigitation of cells was observed (Asahina et al. 2002).

When the shoots with roots removed were cultured in water, although cell division during tissue reunion in the cut hypocotyls was observed 3 days after cutting the hypocotyl, the interdigitation of cut cortex cells did not occur, even after 7 days (Fig. 1b, d, 2a). The thick-wall layer stained strongly with aniline blue, indicating that callose was abundant in this region; the layer also stained well with coomassie brilliant blue, toluidine blue, and ruthenium red, suggesting the presence of protein, polysaccharides, and pectins, respectively (data not shown).

When the shoots with roots removed were supplemented with squash xylem sap (Fig. 2b, f) or 1/20 MS medium (Fig. 2d, f), the interdigitation of cells and normal tissue reunion was restored. However, normal tissue reunion was not restored by supplementation with *t*-ZR (Fig. 2c), a

**Fig. 1.** Effects of root removal on the tissue reunion process in the cortex of cucumber hypocotyls after 7 days of growth of the cut hypocotyl. Cut hypocotyls were trimmed to 5-mm segments surrounding the cut surface and observed under a light microscope. **a** Initial plant with root, 7 days after sowing in soil; **b** shoot with root removed, after 7 days of culture in water after the hypocotyl was cut; **c-d** magnified images of the area indicated in **(a)** and **(b)**, respectively. Sections were stained with toluidine blue O. An arrow indicates the thick-wall layer; *co* cortex, *vb* vascular bundle. Scale bars indicate 100  $\mu\text{m}$



major cytokinin in squash xylem sap (Kuroha et al. 2002). Because the application of 1/20 strength MS showed enough efficiency for the interdigitation of cells in the dilution series, 1/20 MS medium or its compounds at the same concentrations were used for further experiments.

Since many adventitious roots generated on the hypocotyl when the seedling was cultivated with xylem sap or 1/20 strength-MS medium (Fig. 2b, d), adventitious roots were removed immediately after its formation in order to evaluate more clearly the effect of the root removal. Normal tissue-reunion process of the cortex was also observed even when adventitious roots were removed (data not shown).

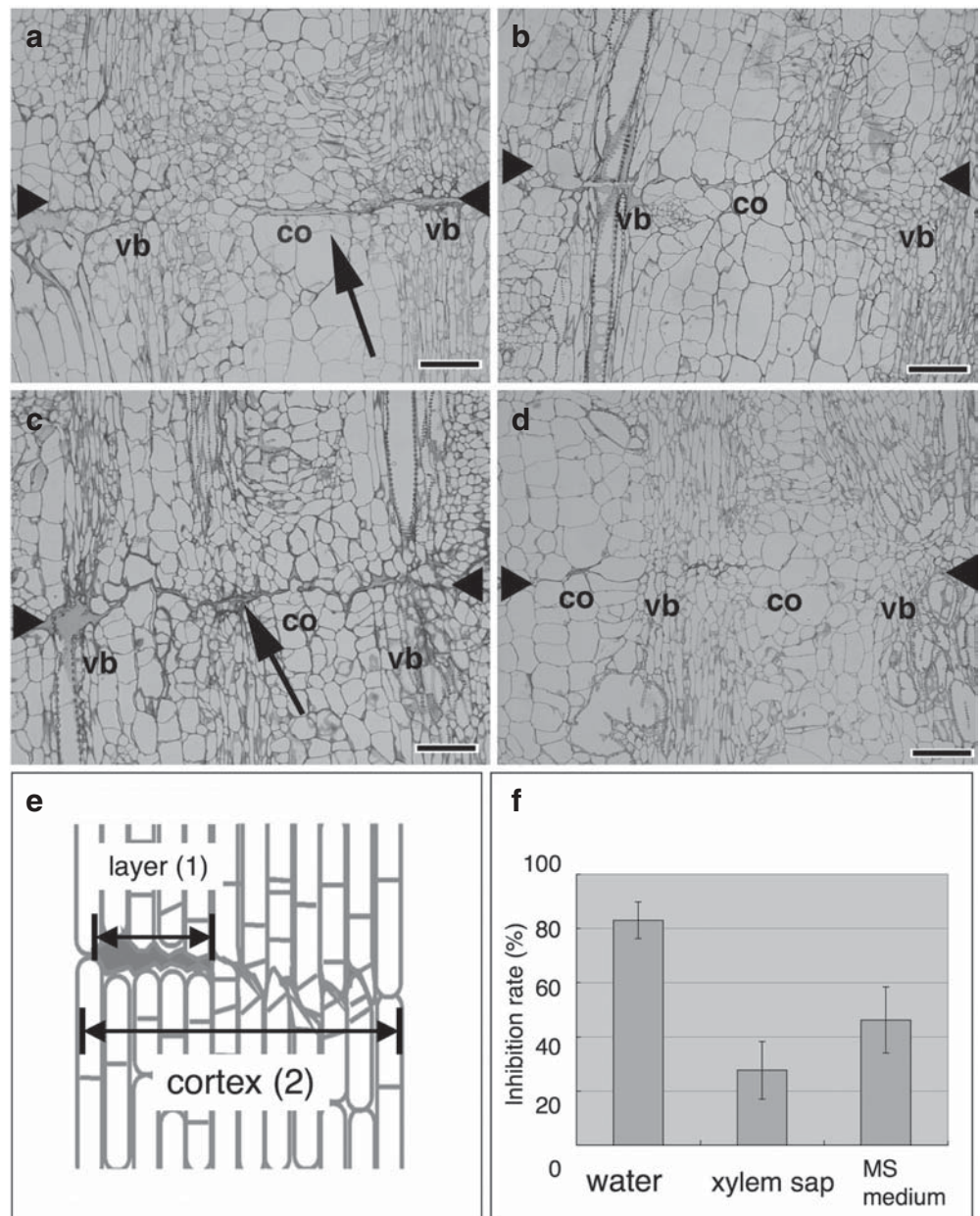
Interdigitation of cells was quantitatively determined by measuring the length of the thick-wall layer in longitudinal sections (Fig. 2e); interdigitation of cells was observed under supplementation with MS medium or xylem sap (Fig. 2f). We also examined which factors in the MS medium were involved in interdigitation of cells. Interdigitation of cells was almost restored by supplementation with MS microelements alone, in a mixture of inorganic compounds (Fig. 3). Similar but higher efficiency was observed by application of MS medium (Fig. 3), suggesting that the effect of MS medium on the interdigitation of cell during tissue-reunion were mainly due to microelements contained in MS medium. The other MS components may have negative effects. The exclusion of boron (B) from the microelements resulted in less interdigitation, although interdigitation was not restored with the application of B alone (Fig. 3).

MS microelements can be divided into three relatively major elements, B, Mn, and Zn ( $0.31 \text{ mg l}^{-1} \text{ H}_3\text{BO}_3$ ,  $1.115 \text{ mg l}^{-1} \text{ MnSO}_4 \cdot 4\text{H}_2\text{O}$ ,  $0.43 \text{ mg l}^{-1} \text{ ZnSO}_4 \cdot \text{H}_2\text{O}$ , respectively) and four relatively minor elements, I, Mo, Cu, and Co ( $41.5 \text{ }\mu\text{g l}^{-1} \text{ KI}$ ,  $12.5 \text{ }\mu\text{g l}^{-1} \text{ Na}_2\text{MoO}_4 \cdot 5\text{H}_2\text{O}$ ,  $1.25 \text{ }\mu\text{g l}^{-1} \text{ CoCl}_2 \cdot 6\text{H}_2\text{O}$  and  $1.25 \text{ }\mu\text{g l}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ). When the relatively major elements were simultaneously supplied at the same concentrations, the interdigitation of the cells was significantly restored (Fig. 4). The application of only one of these components (data not shown) or one of each compound in combination with B showed lower efficiency compared to the co-application of B, Zn, and Mn (Fig. 4).

## Discussion

In plants from which the roots were removed, cell division occurred during tissue reunion, but a thick-wall layer formed in the cut region and intrusive cell elongation and interdigitation of cortical cells on the cut surface did not occur, even after 7 days (Figs. 1, 2a). There are two explanations for the relationship between the formation of the thick-wall layer and the inhibition of the reunion process. First, because the thick-wall layer that formed at the reunion region was not degraded during the reunion process, the interdigitation of cells may have been inhibited by the thick-wall layer. Alternatively, because intrusive cell elongation did not occur during the reunion process, some compound such as callose, pectin, or protein, may have

**Fig. 2.** Effects of culture solutions on tissue reunion in shoots from which the roots were removed. The shoots were cultured in **a** water; **b** squash xylem sap; **c**  $10^{-5}$  M *t*-zeatin riboside; or **d** 1/20-strength MS medium. **e** Schematic illustration of thick-wall layer measurements: (1) length of the thick-wall layer; (2) length of the cortex between two vascular bundles. **f** Rate of interdigitation of cells during tissue reunion in shoots that had the roots removed, calculated as (1)/(2). All micrographs were taken 7 days after cutting the hypocotyls. An arrow indicates the thick-wall layer; *co* cortex, *vb* vascular bundle. Scale bars indicate 100  $\mu$ m (**a–d**). The bars indicate the means of results obtained in five measurements; error bars indicate standard error (**f**)



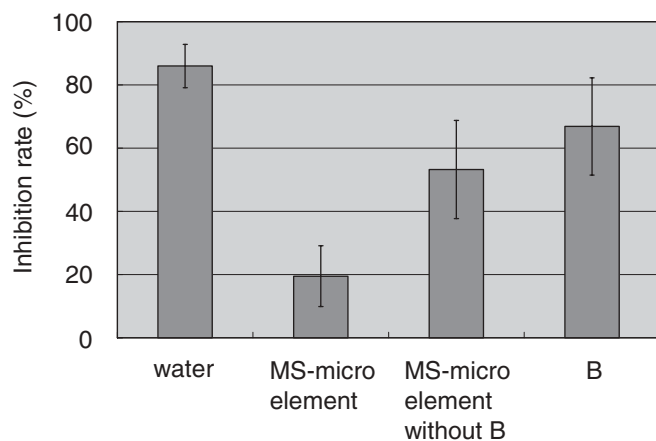
accumulated in the space between the upper and lower regions, resulting in the formation of the thick-wall layer.

Histochemical analyses indicated that callose is abundant in the thick-wall layer (data not shown). Callose accumulates at an injured region just after cutting or after pathogen attacks (Kauss 1985; Donofrio and Delaney 2001; Ostergaard et al. 2002). In addition, pathogen-related substances are added to the plant cell wall as a localized and rapid response to fungal invasion or wounding. These substances may include phenolic compounds (e.g., lignin), which are products of the phenylpropanoid pathway, carbohydrates (e.g., pectin and callose), and several types of proteins (e.g., enzymes such as peroxidase; reviewed by Denny 2002).

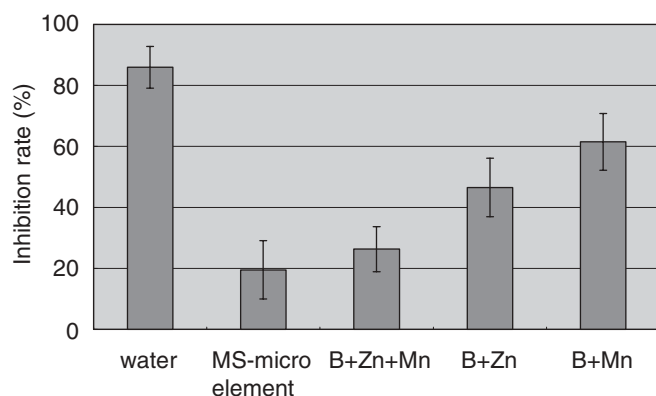
Localized elevation of  $\text{Ca}^{2+}$  levels results in the localized deposition of callose (Kauss 1985, 1987), which may func-

tion in blocking wound sites against pathogen invasions. After the defense response, the accumulated callose may be degraded, and a new cell wall is formed during the healing process. Similar replacement of callose is also observed during cytokinesis (Smith 2001) and protoplast regeneration (Klein et al. 1981; Hayashi et al. 1986). The newly formed cell wall contains mainly callose, rather than cellulose. During maturation of the cell wall, flattening and rigidification of the cell wall may be associated with the replacement of callose by cellulose.

Our results show that the co-application of B, Mn, and Zn is required to induce the interdigitation of cells during tissue reunion in the cortex. It is suggested that these minerals might be transported to hypocotyls from root via xylem sap because xylem sap of squash contains these inorganic elements (Iwai et al. 2003).



**Fig. 3.** Effects of the components of MS medium on the interdigitation of cells during tissue reunion in shoots from which the roots were removed. Interdigitation of cells was restored with the addition of MS microelements; the addition of MS microelements without B, or the addition of B alone had little effect. The concentrations of all elements were adjusted to those in 1/20-strength MS medium. The bars indicate the means of results obtained in four measurements; error bars indicate standard error



**Fig. 4.** Effects of the components of MS microelements on the interdigitation of cells during tissue reunion in shoots that had their roots removed. When B, Zn, and Mn were simultaneously supplied, interdigitation of the cells was restored; the addition of either Zn or Mn in combination with B showed lower efficiency. The concentrations of all elements were adjusted to those in 1/20-strength MS medium. The bars indicate the means of results obtained in four measurements; error bars indicate standard error

Boron is involved in a number of essential processes in plants, including cell division (Matoh et al. 1992; Dell and Huang 1997) and cell wall functions such as intercellular attachment via pectin RG-II (Iwai et al. 2002). Our previous study suggests that active cell wall metabolism including biosynthesis and accumulation of pectic substances occurs at the attached cells in the reunion region of the cortex (Asahina et al. 2002). Boron may be involved in the interdigitation of cells as a regulator of cell wall function.

Manganese has an important role in the activity of certain enzyme (Mn-enzyme) reactions, including photosynthesis (Fox and Gueriot 1998) and lignin biodegradation in fungi (Mn-peroxidase; Hildén et al. 2000). Zn also plays

a critical structural role in many proteins and is an essential catalytic component of over 300 enzymes including alkali phosphatase, protease, and arabinofuranosidase (Sozzi et al. 2002). Mn and Zn may be required for enzyme activity related to cell wall degradation during the tissue reunion process.

The modification of cell wall components was thought to be important for the reunion process. Histochemical analyses indicated that pectin, callose and protein are abundant in the thick-wall layer (Asahina et al. 2002, M. Asahina and S. Satoh, personal observations). In addition, active cell elongation and cell division were observed during reunion-process. These results suggest that cell wall synthesis and modification might occur in this process.

Since there is no evidence that these microelements were transported to the reunion region and/or accumulated at this region, it is still unclear whether these microelements have directly affected interdigitation of cells or not in this study. However, the current study strongly suggests that these elements are necessary for interdigitation of cells during tissue-reunion.

Here, we showed that the microelements B, Zn, and Mn supplied by the root via the xylem sap are involved in the interdigitation of cells during tissue reunion in the cut hypocotyls of cucumber by promoting the degradation of the thick-wall layer and/or interdigitation of the cells. Concentrations of these elements may be adjusted by the roots to an appropriate level for the tissues (Denny 2002). Further detailed analyses of the transportation and the functions of these elements including molecular and chemical analyses of cell wall biosynthesis are required.

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