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# Detection of hemicelluloses specific to the cell wall of tracheary elements and phloem cells by fluorescein-conjugated lectins

**T. Hogetsu** *

Department of Pure and Applied Sciences, College of Arts and Sciences, University of Tokyo, Tokyo

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**Summary.** Binding of fluorescein-conjugated wheat-germ agglutinin (F-WGA) and some other lectins to tissues from various plants were examined by epifluorescence microscopy. F-WGA bound specifically to the walls of tracheary elements (TEs) and phloem cells of pea roots. The binding sites in TEs were localized only in the secondary thickening and became evident at very early stages of differentiation. Fluorescein-conjugated derivatives of *Solanum tuberosum* lectin, *Lycopersicon esculentum* lectin, and *Datura stramonium* lectin, which bind N-acetylglucosamine residues as WGA, also bound to the secondary thickening of TEs of pea roots. The binding sites for F-WGA were not removed by extraction with hot EDTA and proteinase K, but removed by extraction with an alkali solution. The alkali-extracted binding sites from the roots were precipitated together with hemicelluloses by 80% ethanol. These results indicate that the binding sites are not present on pectins, proteins, or cellulose, but hemicelluloses. Localized distribution of the binding sites for F-WGA in TEs was found also in a variety of angiosperm plants.

**Keywords:** Cell wall; Fluorescein-conjugated wheat-germ agglutinin; Hemicellulose; Lectin; *Pisum sativum*; Tracheary element.

**Abbreviations:** BSL-II *Bandeiraea simplicifolia* lectin II; DSL *Datura stramonium* lectin; F- fluorescein-conjugated; LEL *Lycopersicon esculentum* lectin; MT microtubule; STL *Solanum tuberosum* lectin; TE tracheary element; WGA wheat-germ agglutinin.

## Introduction

The development of vascular cells in higher plants is a prominent example of cell differentiation. The ratio of wall components changes and specific components of the cell wall are synthesized in xylem cells during their development. Primary walls of cambial cells are composed of pectins, hemicelluloses and cellulose, whereas, during the formation of the secondary thickening of xylem cells, large amounts of cellulose, hemicelluloses and, at the final stage of development, lignin are deposited, but no pectins are deposited (Northcote 1963). Thornber and Northcote (1962) reported that the main components of the hemicelluloses deposited during the differentiation of the xylem are xylans, which are modified with 4-*O*-methylglucuronic acid residues in sycamore trees. Recently, deposition of a protein specific to primary xylem cells has also been detected (Keller et al. 1989).

Another peculiarity of the differentiation of the xylem is the fact that the secondary thickening is laid down in specific patterns, such as rings or spirals, and reticulate or pitted patterns. The synthesis of cellulose is highly localized and results in these specific patterns of secondary thickening in xylem cells. Lignins and the wall protein are also deposited exclusively in the secondary thickening of differentiated xylem cells. Recently, Northcote et al. (1989), using an antibody against xylans, found that large amounts of xylans are deposited in the secondary thickening, even though xylans are also found in the primary wall.

There are several reports on the mechanism of the localized deposition of the secondary thickening. The localized deposition of cellulose microfibrils seems to result from localized, cellulose-synthetic particles, which are called rosettes (Herth 1985, Schneider and Herth 1986). The localized distribution of rosettes is thought to be controlled by cortical microtubules (MTs) that run under the secondary thickening. However, the mechanism of the localized deposition of other, non-cellulosic components of the wall is un-

* Correspondence and reprints: Department of Forestry, Faculty of Agriculture, University of Tokyo, Yayoi, Tokyo 113, Japan.

known. In order to understand the mechanism involved in the localized deposition of these components, the process during which the components become localized in the secondary thickening of differentiating TEs must be first examined in more detail.

In the course of a study on the binding sites of lectins, it became apparent that fluorescein-conjugated wheat-germ agglutinin (F-WGA) binds specifically to hemicellulosic components of TEs and phloem cells. This reagent may become a powerful probe for exploration of the mechanism of localized deposition of non-cellulosic components of the cell wall during differentiation of vascular cells. In this paper, the localization and the timing of accumulation of the binding sites for F-WGA and some other related F-lectins in plant tissues are described.

## Material and methods

*Plants*

*Pisum sativum* L. cv. Alaska and *Avena sativa* L. cv. Victory II plants were grown on vermiculite at 26 °C in the dark. Epicotyls and roots of *Pisum sativum* were harvested at several times shortly after sowing. Coleoptiles and mesocotyls of *Avena sativa* were harvested a few days after sowing. Stems of *Aucuba japonica* Thunb. and *Ginkgo biloba* L. which had extended during the past year and present year, respectively, were taken from positions 5–10 cm under the shoot apices of trees grown on the campus of the Universdity of Tokyo at Komaba, from March through May. Stems of *Picea jezoensis* Carr., *Pinus Thunbergii* Parl., and *Equisetum arvense* L. were taken from the portions just below the shoot apices in May, those of *Lygodium japonicum* Swartz in August, and those of *Cornus controversa* Hemsl., *Firmiana platanifolia* Schott et Endl., *Mallotus japonicus* Muell. Arg., and *Prunus yedoensis* Matsum. in October.

*Staining with F-lectins and calcofluor*

The stems were sectioned at a thickness of 30 − 50 μm by cryo-sectioning after infiltration with a solution of 0.1% Nonidet P-40, 10% DMSO and 50 mM sodium phosphate (pH 7.0). The sections were washed thoroughly with 50 mM sodium phosphate (pH 7.0) that contained 0.01% Nonidet P-40, and suspended in about 50 μl of the buffer with the addition of 2 μl of 20 μg/ml F-WGA (Vector, Burlingame, CA, U.S.A. and EY Lab., San Mateo, CA) or 4 μl of 10 μg/ml other fluorescein-conjugated lectins (Vector). All solutions of lectins were prepared by dilution with a solution that contained 10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2$ and 0.08% $NaN_3$ (pH 7.5). After 15 min at room temperature, a drop of 0.01% Calcofluor M 2 RS New (American Cyanamid Co., Bound Brook, N.J., U.S.A.) was added. After 5 min the sections were washed several times with 50 mM sodium phosphate (pH 7.0) that contained 0.01% Nonidet P-40 and mounted in a solution of Mowiol (Weber and Osborn 1981) that contained 0.1% *p*-phenylenediamine dihydrochloride. Sections were then observed under an epifluorescence microscope (BHF-BH 2-RFL; Olympus Kougaku Co., Tokyo, Japan) in excitation mode B and V for F-lectins and Calcofluor, respectively. In order to bare strands of TEs from root tissues, pea roots were boiled in 70% lactic acid for more than 5 min and, after washing with 0.1 M sodium phosphate (pH 7.0) and water, squashed on a piece of slide glass with fingers only in the tip portion, becoming shaped like a broom. This treatment made lectins accessible to TEs. Storage of roots in 70% lactic acid for at least a week did not remove the binding sites of F-WGA from the walls of the TEs. The broom-shaped roots were stained with F-lectins and Calcofluor as mentioned above in 2.5 cm petri plates, which were inclined at a slight angle so that the roots were immersed in solutions at the corner of a plate. After washing with the buffer, they were mounted and observed under the epifluorescence, phase-contrast (DIAPHOTO equipped with ELWD 0.3; Nikon Co., Tokyo) and Nomarski differential-interference optics (IMT-2 equipped with IMT 2-NIC; Olympus Kougaku Co.).

*Treatments of sections with enzymes and some other reagents*

Squashed roots were treated with 200 μg/ml proteinase K (Merck Co., Darmstadt, Federal Republic of Germany), which was buffered by 9 mM Tris-HCl and 0.9 mM EDTA (pH 8.0), for 24 h at 37 °C. To examine the effect of triacetyl chitotriose, 2 μl of F-WGA were mixed with 2 μl of 50 mM triacetyl chitotriose (Sigma Chem. Co., St. Louis, MO, U.S.A.) in 10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2$ and 0.08% $NaN_3$ (pH 7.5), and, after standing for 30 min at room temperature, applied to cross-sections of pea roots. The binding of F-WGA to sections of pea roots was examined under the epifluorescence microscope as described above.

In competition experiments with unlabeled WGA, squashed roots were treated with the mixture of 25 μl of 10 mg/ml unlabeled WGA (Sigma) and 2 μl of 20 μg/ml F-WGA for 15 min at room temperature. The sections were washed three times with water, mounted in the Mowiol solution and observed under the epifluorescence microscope.

Eight squashed roots were extracted with 1 ml of 5% potassium hydroxide at 26 °C for 1 h, neutralized with acetic acid and added 4 volumes of ethanol. After standing at −20 °C for 1 h, the solution was centrifuged at about 1,500 **g** for 10 min. After twice washed with water, the precipitate was stained with F-WGA and observed under the epifluorescence microscope.

## Results

*Binding sites of F-WGA in pea roots*

When pea roots were treated with F-WGA, only the walls of TEs and phloem cells were fluorescent (Fig. 1). The appearance of binding sites for F-WGA in phloem cells began much later than that in TEs and preceded the deposition of lignin, as revealed by orange autofluorescence. There is a tendency for lignification to occur earlier in TEs whose positions in roots were outer. The binding sites in the walls of the TEs were localized exclusively in the secondary thickening.

To examine at which stage of the development of TEs the binding sites of F-WGA become detectable, a method was developed that involved the boiling of roots in 70% lactic acid and subsequent squashing by fingers. With this method strands of TEs could be bared from root tissues and stained with F-WGA and Calcofluor. The binding sites for F-WGA appeared in a

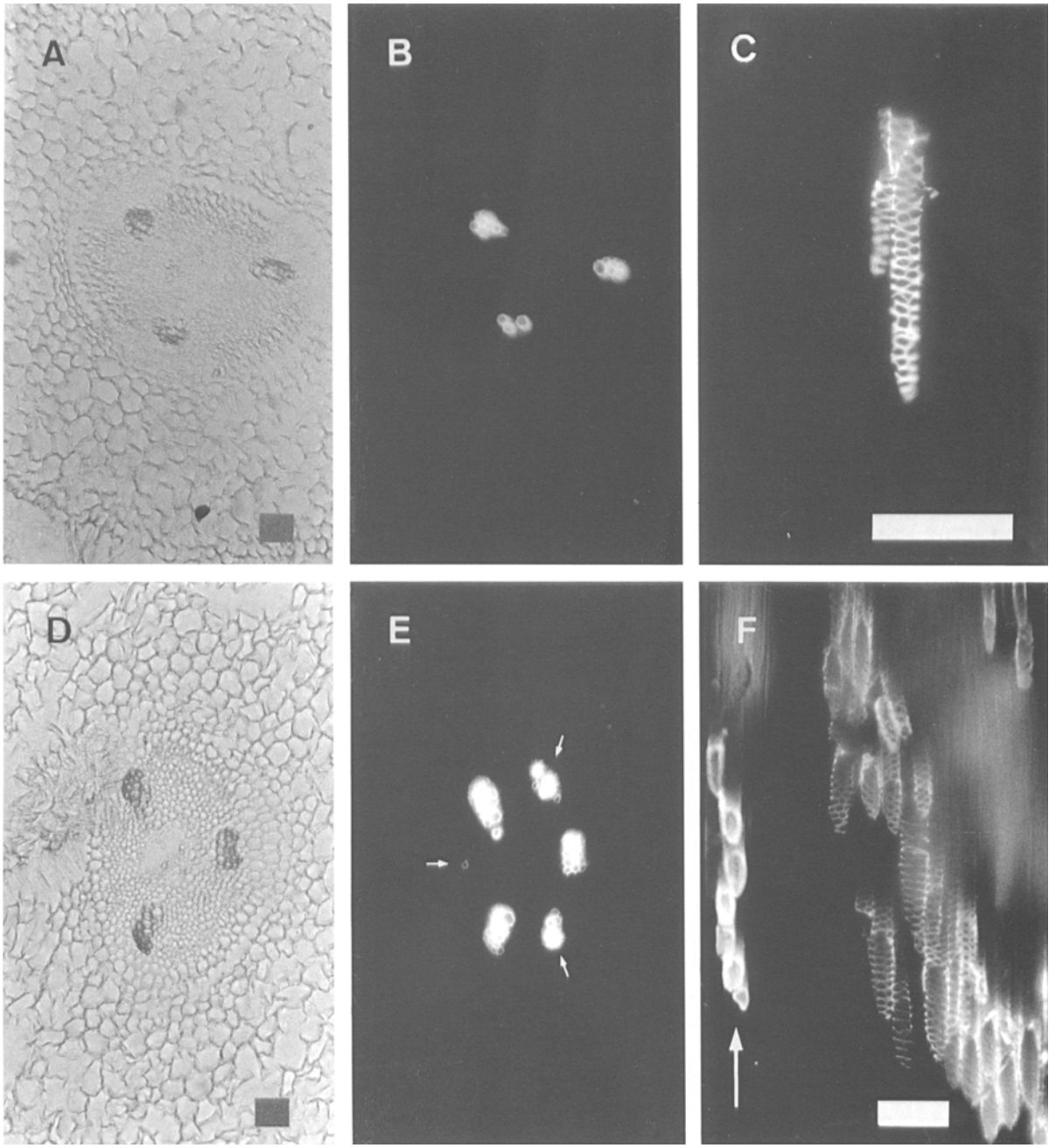


**Fig. 1 A–F.** Localization of the binding sites for F-WGA in pea roots. Cross- (**A, B**) and longitudinal (**C**) sections of lower portions (about 5 mm from the tip) of pea roots stained with F-WGA. **A** Bright-field image; **B, C** fluorescent images. **A, B** Images of the same sample. Cross- (**D, E**) and longitudinal (**F**) sections of upper portions (about 10 mm from the tip) of pea roots stained with F-WGA. **D** Bright-field image; **E, F** fluorescent images. **D, E** Images of the same sample. Arrows indicate phloem cells. Bars: 50 μm; A, B, D, E ×105; C ×420; F ×210

localized pattern in cells that were longitudinally adjacent to differentiated TEs at the tip of strands of TEs and that had not formed clearly any secondary thickening detectable even by either phase-contrast or Nomarski differential-interference microscopy (Fig. 2). This result indicates that the binding sites for F-WGA form at a very early stage of the differentiation of TEs. Localized deposition of cellulose also began before the secondary thickening was clearly detectable by phase-contrast or Nomarski differential-interference microscopy, as revealed by staining with Calcofluor (Fig. 2). Which of localized depositions of the binding

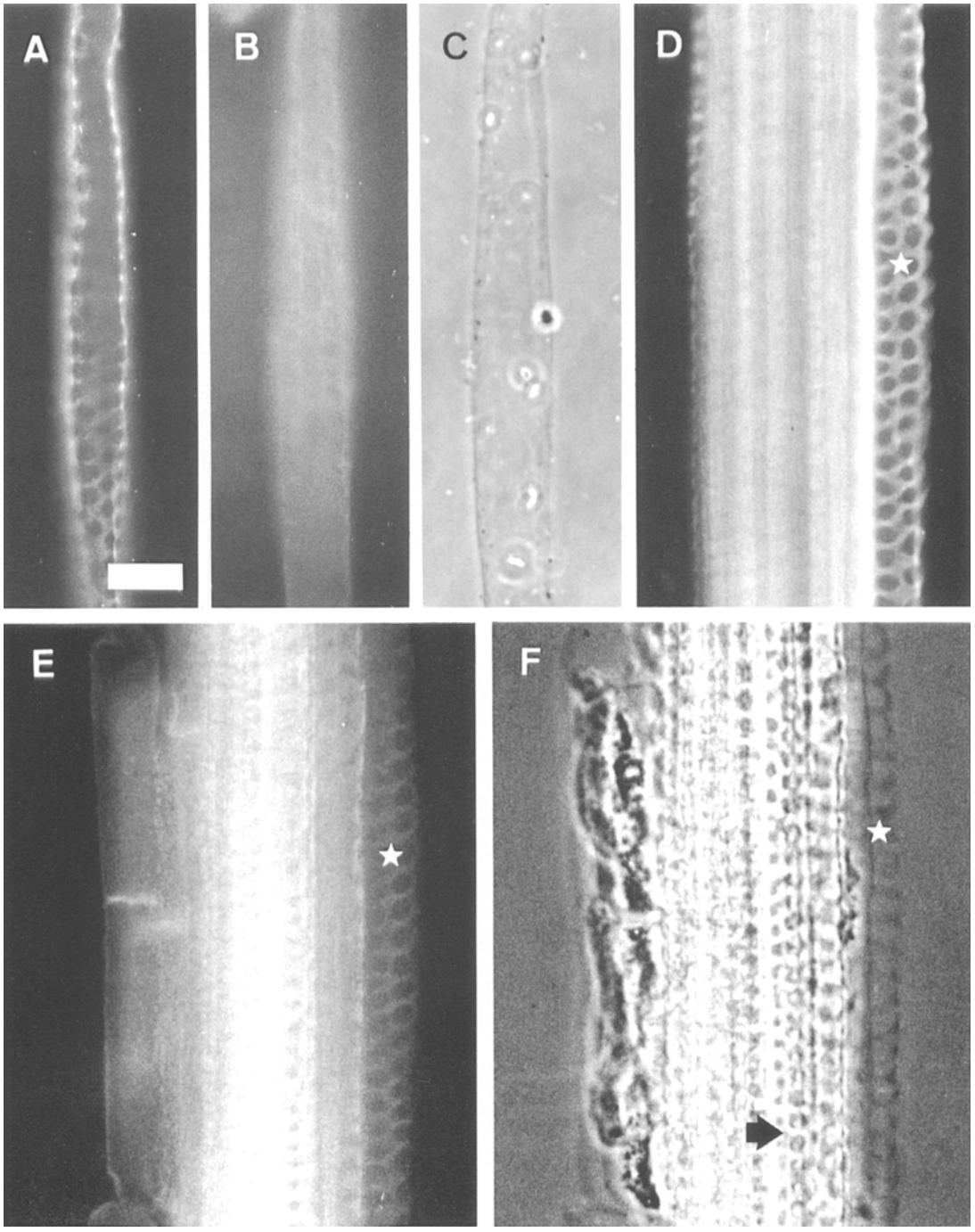


**Fig. 2 A–F.** Timing of appearance of the binding sites for F-WGA in TEs of pea roots. TE strands were mechanically bared from pea root tissues which were boiled in 70% lactic acid, and stained with F-WGA and Calcofluor. **A, D** Images of fluorescence from F-WGA; **B, E** images of fluorescence from Calcofluor; **C, F** phase-contrast images. **A–C** A cell at the tip of a TE strand; **D–F** the same portion of a TE strand at several hundred μm from the tip, at which many TEs were lignified. ☆ A non-lignified TE; lignified secondary thickening (arrow). Compare the cell in **C** and TEs in **F** in which the secondary thickening is hardly and clearly observed, respectively. Bar: 20 μm; × 560

sites for F-WGA and cellulose occurred earlier could not be determined. At a later stage of development, TEs began to emit orange auto-fluorescence under B-excitation, indicating the presence of lignin, and bound less F-WGA.

*Characteristics of the binding site of F-WGA*

The pretreatment of F-WGA with an excess amount of triacetyl chitotriose which binds to WGA, prevented F-WGA from binding to the walls of TEs and phloem cells in sections of pea roots (Table 1). This result

**Table 1.** The effects of various treatments on binding of F-WGA to cell walls of TEs

| Treatment | Binding |
|---|---|
| 50 mM EDTA in 50 mM sodium phosphate (pH 7.0), 120 °C, 2 h | + |
| Proteinase K, 200 μg/ml, 37 °C, 24 h | + |
| 5% KOH, 26 °C, 1 h | − |
| Mixing of F-WGA with an excess amount of unlabeled WGA | − |
| Preincubation of F-WGA with an excess amount of triacetyl chitotriose[a] for 30 min | − |

[a] The reagent was also present during the treatment of pea roots with F-WGA

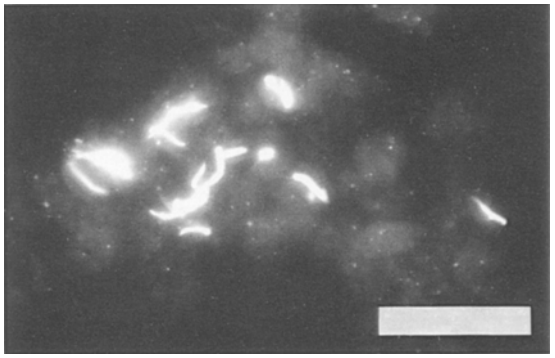

**Fig. 3.** The binding sites for F-WGA in alkali-soluble polymers of the walls of pea roots. Alkali-extracts of pea roots were neutralized with acetic acid and precipitated by 80% ethanol. The precipitate was collected and stained with F-WGA. Note fibrous and dotted aggregates of the binding sites for F-WGA. Bar: 100 μm; × 200

indicates that the active site in F-WGA for the binding to TEs and phloem cells is the same as that in unlabeled WGA for N-acetylglucosamine residues.

A competition experiment with unlabeled WGA revealed that an excess amount of WGA inhibits the binding of F-WGA to the walls of TEs or phloem cells. When squashed roots were treated with a mixture of F-WGA and an excess amount of unlabeled WGA, no cell walls of roots were bound with F-WGA. This result indicates that unlabeled WGA also bound to the walls of TEs and phloem cells.

The binding to the walls of pea roots of other F-lectins which have high affinity to N-acetylglucosamine residues as WGA (Allen et al. 1973) was examined. Sections of pea roots were stained with F-STL (Allen and Neuberger 1973), F-LEL (Kilpatrick 1980), F-DSL (Kilpatrick and Yeoman 1978) and F-BSL-II (Iyer et al. 1978), and observed under the epifluorescence microscope. F-STL and F-LEL bound the secondary thickening of TEs and the wall of phloem cells as F-WGA, F-DSL did weekly, and F-BSL-II did not.

The boiling in 50 mM EDTA in 50 mM sodium phosphate (pH 6.9) at 100 °C for 2 h did not eliminate but rather increased the number of binding sites in strands of TEs. Since this extraction usually removes pectins from the cell wall, this result indicates that the binding sites for F-WGA in TEs are not pectins. Some binding sites for F-WGA to which pectins prevent F-WGA from binding may be uncovered by the EDTA treatment, increasing the number of binding sites. The treatment with proteinase K did not remove the binding sites for F-WGA. The binding sites were, however, extracted completely from strands of TEs by the treatment with 5% potassium hydroxide for 1 h at 26 °C. When polymers were solubilized from eight squashed roots by the alkali treatment and precipitated by 80% ethanol, aggregates of the binding sites for F-WGA were found in the precipitate (Fig. 3). This result indicates that the binding sites were present on polymers that were solubilized by the alkali solution, i.e., hemicelluloses.

*Survey of the binding sites for F-WGA in other species*

The distribution of the binding sites for F-WGA in the cell wall was examined in various plant tissues. The binding sites for F-WGA were localized exclusively in the cell walls of TEs and phloem cells in epicotyls and leaves of *Pisum*, and in leaves, coleoptiles and mesocotyls of *Avena*. Localized sites of binding of F-WGA in the walls of TEs were also observed in stems of angiosperm plants, *Aucuba, Cornus, Firmiana, Mallotus*, and *Prunus*, but not in stems of gymnosperm and fern plants, *Picea, Pinus, Ginkgo, Equisetum*, and *Lygodium* stems. There may be an evolutional difference in components of the cell walls of TEs between angiosperms and others. The binding of F-WGA to the walls of phloem cells of the woody angiosperm plants was not detected.

## Discussion

In the present study it was found that F-WGA binds specifically to the cell walls of TEs and phloem cells with very little background fluorescence. Staining with Calcofluor did not provide any clearer images of the secondary thickening because Calcofluor stained also

the primary walls of TEs and neighboring, non-vascular cells, increasing background fluorescence. There is no specific marker for phloem sieve elements other than callose, which accumulates on the sieve plates and is detected by staining with aniline blue. Thus, F-WGA may be a powerful probe for studies of vascular differentiation.

The experiments involving chemical extraction and digestion by proteinase K indicate that the binding sites for F-WGA are not associated with pectins, proteins, lignins nor cellulose, but with hemicelluloses. The hemicellulosic components were deposited in the same localized pattern as the secondary thickening during the differentiation of TEs, just as cellulose, lignin and the glycine-rich protein are. There are two possibilities to explain how the binding sites are added to the wall in a localized manner. They may be added to hemicelluloses that have already been deposited on the walls of TEs or, alternatively, hemicelluloses that have already incorporated the binding sites may be deposited in a localized manner. The localized deposition of cellulose in the cell walls of TEs is brought about by the localized distribution of synthetic complexes of cellulose in the plasma membrane (Herth 1985, Schneider and Herth 1986). Irrespective of which of the alternative possibilities is correct, the localization of the binding sites of F-WGA may involve localized distribution of some proteins in the plasma membrane that control the addition of the binding sites to hemicelluloses or the deposition of hemicelluloses that carry the binding sites. The localized deposition of the binding sites for F-WGA and cellulose might be brought about by the same mechanism that involves the localized distribution of proteins in the plasma membrane. Baring xylem strands enables us to observe the process of differentiation of TEs and to determine the stage at which the binding sites for F-WGA appear. The binding sites became evident at very early stages of the differentiation of TEs, as did the localized deposition of cellulose. The temporal similarity between the localized depositions of the binding sites for F-WGA and cellulose also suggests that the localized depositions of the binding sites and cellulose are both subject to similar controls.

The localized distribution of synthetic complexes of cellulose is thought to be brought about under the control of the cortical MTs. Hepler and Newcomb (1964) found that cortical MTs are localized along the secondary thickening and aligned parallel to the microfibrils in the secondary thickening. Subsequently, Pickett-Heaps (1967) showed that disruption of MTs results in a deformed pattern of the secondary thickening, suggesting the involvement of MTs in the formation of the pattern of the deposition of cellulose. These studies were followed by many similar studies of the TEs of various plant species. The localized deposition of cellulose during semi-cell expansion of *Closterium*, a green alga, is another phenomenon that involves the localized distribution of synthetic complexes of cellulose (Hogetsu and Takeuchi 1982, Hogetsu 1983). In this case, disruption of MTs by colchicine also causes the loss of localized deposition of cellulose. MTs may be generally involved in localized deposition of cellulose. Therefore, the strict association of the timing of appearance and the distribution of the binding sites for F-WGA in TEs with those of the localized deposition of cellulose suggests that MTs are also involved in the control of the localized deposition of the binding sites for F-WGA. Actually, treatment of pea seedlings with amiprophosmethyl, a microtubule-disrupting reagent, caused the same disturbance of distribution of the binding sites for F-WGA in TEs as that of cellulose (Hogetsu, in prep.).

It is known that WGA binds to oligomers of N-acetylglucosamine and sialic acid residues. The present study demonstrated that several other lectins which bind to N-acetylglucosamine residues also bound to the walls of TEs, suggesting that the binding sites for F-WGA are analogous to N-acetylglucosamine residues. However, since the specificity of lectins for saccharide residues is not so strict, a possibility that the binding sites for F-WGA are quite different from N-acetylglucosamine residues should be also considered. Thornber and Northcote (1961) suggested that hemicelluloses of the walls of TEs of several woody angiosperms are xylans which have 4-*O*-methylglucuronic acid and acetic acid residues. Ingold et al. (1988) also reported that the hemicelluloses that accumulate in cultured mesophyll cells of *Zinnia* during the differentiation of TEs are xylans. Recently, Northcote et al. (1989) found, using an antibody against xylans, that large amounts of xylans are located in the secondary thickening, although some xylans are also present in the primary walls. Even though no attempt was made to determine whether the hemicelluloses in TEs of pea roots are xylans, it might be also possible that F-WGA binds to the 4-*O*-methylglucuronic acid or acetic acid residues of xylans. Molecular entity of the binding sites for F-WGA remains to be determined in future by strict chemical analysis.

In conclusion, the present study showed that F-WGA and some other F-lectins make it possible to detect

hemicellulosic components that are specifically formed in the secondary thickening of TEs and the wall of phloem cells. These lectins may be powerful tools for studies on vascular differentiation.

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