DISCUSSION

Satoh et al. (1992) reported the detection of proteins in the xylem sap of squash after collection and analysis of a large volume of xylem sap. However, squash is not a good plant material for studies of genetic transformation. So I used another cucurbitaceous plant, cucumber, in this study.

The bands in cucumber xylem sap after fractionation by SDS-PAGE and staining with amido black (Figure 1, lane 3) were similar to those obtained from squash xylem sap. The antiserum against XSPs reacted with a wide range of proteins of low to high molecular mass, but the proteins with lower molecular mass lower immuno reactivity. Because the pattern of the proteins in SDS-PAGE of the xylem sap was quite different from that of the whole root, and the composition of the sugar in the xylem sap was different from that of cytoplasm (Satoh et al., 1992), the possibility of contamination of the cytoplasmic proteins should be little.

In this study, I prepared a cDNA library from roots because some of the organic compounds in xylem sap are thought to be produced in the central cylinder of roots and transported *via* xylem vessels (Kolek et al., 1991). I selected 24 cDNA clones using the antiserum against XSPs. Five clones corresponding to transcripts that were expressed predominantly in roots (Figure 2) were finally selected as candidates for cDNAs for XSPs and all were subjected to sequence analysis. Two of the cDNAs, *CRGRP-1* and -2, encoded a glycine-rich domain within their deduced amino acid sequences and the corresponding transcripts were expressed predominantly in roots. Moreover, strong expression of each mRNA was detected in the root-hair zone of tap roots.

It has been suggested that glycine-rich protein (GRP) is structural

protein of the plant cell wall and is fixed in the cell wall by an unknown cross-linking (Ye et al., 1991). GRP synthesis is induced by various enviroment, for example, wound in petunia and *Daucus carota* (Condit and Meagher, 1987, Strum 1992), stress of heavy metals in maize (Didierjean et al., 1992), stress of low temperature in barley (Hughes et al., 1996), tratment with certain plant hormones (Gomez et al., 1998) and the formation of nodules in *Vicia faba*(Schroder et al., 1997). Although GRPs have been found in various organs and species, their biological functions have not been established. Some extremely glycine-rich GRPs (with about 70% of their amino acid residues being glycine), such as GRP1.8 of kidney bean (Keller et al., 1991), might be involved in maintaining the structure and function of cell walls, and the glycine-rich domain might be associated with the solubility and specific functions of the proteins.

In CRGRP-1 and -2, the contents of glycine in the glycine-rich domain is relatively low (CRGRP-1, 40%; CRGRP-2, 58%) and both CRGRP-1 and CRGRP-2 have non-glycine-rich domains adjacent to the putative signal sequences at their amino termini. CRGRP-1 is about 160 amino acids shorter than CRGRP-2, with the major difference being associated with the length of the glycine-rich domain. In the glycine-rich domain, glycine is present in a G_{2-3}-X motif, while G_{5-6}-X motif has been reported in other GRPs, such as GRP1.8 (Keller et al., 1991). These characters indicate that *CRGRP-1* and *-2* products differ from previously reported GRPs.

The high-level expression of mRNAs for CRGRP-1 and CRGRP-2 coincided with the development of vascular tissues during the formation of adventitious roots (Figure 7) and was evident in the root-hair zone of tap

roots (Figure 6). This pattern of expression coincides with the movement of water in roots. Water is absorbed mainly in the root-hair zone and transported into newly differentiated xylem through the endodermis. Because the apoplastic movement of materials is blocked by the casparian strip of endodermis, no materials can move through the apoplastic space of the endodermis into the central cylinder (Kolek et al., 1991). Therefore, it is likely that the production of the organic materials that are present in the xylem sap is closely associated with the vascular tissues in the central cylinder. The synthesized proteins might be secreted into the cell wall or apoplastic space of the cells in the central cylinder according to their signal sequences, and then apoplastically transported to the xylem vessels with the movement of water.

It has been reported that some xylem sap-related proteins expressed specifically in the cells of the central cylinder of root. Scopolamine is synthesized by hyoscyamine 6-β-hydroxylase and is translocated *via* the xylem sap to shoots. The gene for the hydroxylase is expressed in the pericycle of branch roots of plants in Solanaceae (Kanegae et al., 1994). The *SKOR* gene (for a K^+ channel identified in *Arabidopsis*) is involved in the release of K^+ ions into the xylem sap and is expressed in the pericycle and stelar parenchyma cells of roots (Gaymard et al., 1998). *In situ* hybridization and expression analyses revealed that these expression patterns resembles to CRGRP genes (Figures 5 and 9; xylem parenchyma of central cylinder). The newly synthesized CRGRPs might be secreted into the apoplastic space in the central cylinder, in a process mediated by their signal sequences, and then loaded into xylem vessels with the water stream, since the expression of *CRGRP* genes was predominant in the root-

hair zone, a major site of the absorption of water (Figure 16) (Sakuta et al., 1998).

In immunoblotting of the xylem sap, extracts of cucumber organs and of GRP1.8 protein with the CRGRPs- and GRP1.8-antisera yielded totally different patterns of bands (Figure 10). This result clearly indicated that the CRGRP-1 antiserum never reacted with other GRPs, such as GRP1.8. Although a faint 18-kDa band, which might be a degradate of CRGRPs, were detected only in expanded leaf (Figure 10). Immunohistochemical staining revealed the accumulation of CRGRPs in the walls of metaxylem vessels of all examined organs and in the cell walls of perivascular fibers in the stem (Figures 11, 12 and 13). This discrepancy might have resulted from the insolubility of accumulated CRGRPs. Since the signals on the walls were somewhat stronger when treated the tissues by sonication in hot SDS-containing solution. CRGRPs might also have an affinity for lignified walls because all the sites at which CRGRPs accumulated were lignified (Figures 11, 12 and 13).

The 18-kDa band detected in expanded leaves by immunoblotting (Figure 10) might have been free and partially degradated CRGRPs, since large amounts of free CRGRPs in the xylem sap should be supplied to expanded leaves *via* the water flow that is due to transpiration. The present data reveal the production of novel GRPs in cucumber roots and their delivery to the lignified walls of aboveground organs *via* the xylem sap. CRGRPs should be provided to such walls after tracheary cells have differentiated and autolyzed, not during the formation of xylem vessels.

The reason of the supply of CRGRPs from the root to the aboveground organs can be explained as follows. (Figure 17). CRGRPs

are involved in strengthening walls by acting as repair materials for xylem vessels. Xylem vessels may be exposed to slight physical injury, for example, when stems are bent by the wind or by animals. Since xylem vessels are composed of dead cells, and are impossible for them to repair themselves. Moreover, because the water current in the shoot is directed to the outside from the inside of xylem vessels, it is also difficult for xylem vessels to be supplied with materials from the neighbor cells beyond them, against the current of water. Therefore, the flow of xylem sap may be a convenient system for the maintenance of the walls of xylem vessel. The perivascular fibers, in which CRGRPs is also accumulated, might be supplied with xylem sap from xylem vessels in stem, and be involved in the hardening of the stem and in limiting water flow. Such a system might be universal, at least in dicotyledonous plants, because, in the hypocotyl of kidney bean, a CRGRP-like protein also accumulates in the walls of metaxylem vessels and in the cell walls of sclerenchyma (Figure 15).

In this study, I showed that novel GRPs are produced in the vascular tissue of cucumber root, transported systemically over a long distance *via* the xylem sap, and immobilized in the walls of metaxylem vessels and in the cell walls of perivascular fibers in aboveground organs. This is, to my knowledge, the first report of the molecular characterization and behavior of xylem sap proteins. The physiological functions of these xylem sap proteins should become clearer in further molecular biological studies.