Discussion

Part I

Aboveground organ-dependent production of XSP30 in cucumber roots

The expression of many genes follows a pattern of diurnal oscillation (Somers 1999). Out of 8,200 genes, approximately 6% exhibited circadian expression patterns in steady-state messenger RNA when compared with *Arabidopsis* microarrays (Harmer *et al.* 2000). A circadian clock in the leaf tissue may directly regulate the genes expressed in leaves. Fewer examples of diurnally oscillating gene expression in roots have been reported, and because roots are spatially separated from leaves, it is less likely that circadian regulation of the leaf directly affects root activities. However, expression of some nitrogen fixation enzymes follows a diurnal oscillation in roots (Lejay *et al.* 1999; Abd-el Baki *et al.* 2000), and the expression of histone H1 exhibits a diurnal pattern in roots (Corlett *et al.* 1998).

In this study, I clearly showed concurrent, diurnal oscillations of gene expression and xylem sap protein production in cucumber roots (Figs. 2 and 3). The oscillation amplitude declined under continuous dark, and showed pattern abnormalities in continuous light (Fig. 4), suggesting that the gene expression in roots is not directly regulated by a leaf oscillator, but that a root-based oscillator controls the oscillation of gene expression. Alternatively, the *XSP30* gene expression in the root might be regulated by some signaling molecule from the shoot. One likely signaling molecule is indole-3-acetic acid, IAA, which controls the rate of internode growth and whose endogenous level exhibits a circadian rhythm (Jouve *et al.* 1999). IAA or some assimilates produced in the shoot according to a circadian pattern might be translocated to roots to establish a similar rhythm in the subterranean tissues. The transient increase in the expression of *XSP30* at 12 hours after elimination of the aboveground tissues

(Fig. 5) could be caused by a loss of appropriate signaling molecules; alternatively, this result could reflect a response to injury.

The regulation of *XSP30* expression seems to consist of two components: one that determines the phase of expression and another that determines the amplitude. The elimination of leaf tissue and the application of gibberellin and its biosynthesis inhibitor affected mainly the amplitude of the diurnal oscillation in *XSP30* expression (Figs. 6-8). Furthermore, application of a high level of GA did not increase the minimum level of gene expression (Figs. 7 and 8).

Many kinds of gibberellin are produced in leaves, and they control various and specific aspects of plant development (Yamaguchi and Kamiya 2000; Asahina *et al.* 2002). Since gibberellin production is greater in young expanded leaves (Smith *et al.* 1992, 1998), the first leaf of 13-day-old cucumber plants is the primary source of gibberellin. The positive correlation between the expression of *XSP30* and 1st and 2nd leaf development during the early growth of the seedlings (Fig.1) is therefore supporting evidence for the role of gibberellin.

The effects of direct application of gibberellin on roots have rarely been reported (Tanimoto 1994; Yaxley *et al.* 2001). In this study, application of GA to the shoot caused an increase in the amplitude of root gene expression, suggesting that either GA itself is translocated to the roots, or that GA induces the production of a mediator in the shoot, which caused a response in the roots. When 10^{-4} M GA_3 was supplied directly to the roots of uniconazole-P-treated plants, the amplitude of *XSP30* expression did not increase (Fig. 9A); this supports the hypothesis that there is a leaf-

produced signal. Under continuous light, the mediator might be produced at a high level in the shoot, increasing the amplitude of *XSP30* expression in the root on 2 days after treatment (Fig. 4A).

The combined results reported here show that the diurnal oscillation of *XSP30* expression in cucumber roots is controlled both by circadian clock-controlled factor(s) and shoot-produced gibberellin, and these factors optimize the gene expression of *XSP30* in the roots. Consequently, supply of XSP30 protein to the shoot via xylem sap oscillates (Fig. 3), while other xylem sap proteins, such as CRGRP-2, are constitutively produced. XSP30 is likely involved in regulating shoot functions.

Part II

Promoter activity of *XSP30* in transgenic cucumber root

The expression of *XSP30* in roots follows a diurnal pattern, but the diurnal expression is not directly regulated by the circadian rhythm (Fig. 4A). Although I found GTCONSENSUS and CIACADIANLELHC motifs, which are related to circadian expression, in the promoter sequence of XSP30 (Terzaghi *et al.* 1995, Piechulla *et al.* 1998) (Fig. 10), the motifs are not thought to be essential for the diurnal expression of *XSP30* in roots for several reasons. First, the amplitude of the oscillating expression of *XSP30* in roots is upregulated by gibberellin produced in the leaves (Fig. 7A). The gibberellin effect on *XSP30* expression is not direct, because gibberellin supplied directly to the roots did not affect *XSP30* expression (Fig. 9A). For the same reasons, the MYBGAHY motif related to the gibberellin response (Gubler *et al.* 1995) is also thought inessential for *XSP30* expression in the root (Fig. 10). Conversely, the ARFT motif related to the auxin response might control the diurnal expression of *XSP30* in roots (Ulmasov *et al.* 1999), because the endogenous level of indole-3-acetic acid (IAA) exhibits a circadian rhythm (Jouve *et al.* 1999). The ERE motif related to the ethylene response might also be involved in *XSP30* expression (Montgomery *et al.* 1993), because elimination of the aboveground organs caused a transient increase in *XSP30* expression in roots (Fig. 5A).

The *XSP30* promoter directed *GUS* expression specifically in the xylem parenchyma and pericycle cells in the central cylinder of mature transgenic hairy roots (Figs. 12, A and C). Thus, XSP30 is likely to be produced in the vascular tissues of mature roots. *CRGRP-1* and *CRGRP-2*, genes encoding other xylem sap proteins, are also expressed in xylem parenchyma cells in the central cylinder of cucumber roots,

and the products are transported to aboveground organs via xylem vessels (Sakuta *et al.* 2000). Production of proteins in the central cylinder of the root is likely to be necessary for the loading of macromolecules into xylem vessels. Indeed, the gene encoding a putative phosphate channel (PHO1), which is hypothesized to load phosphate into the xylem, is expressed preferentially in parenchyma cells in the central cylinder of *Arabidopsis* roots (Hamburger *et al.* 2002). Recently, a boron transporter gene for xylem loading was reported to be expressed in pericycle cells of *Arabidopsis* root (Takano *et al.* 2002) There are reports on genes that are expressed in the central cylinder of the root, but no one has identified the controls that direct their specific spatial expression. Therefore, it is thought that novel motifs in their promoter sequence direct their specific expression. Their production in the central cylinder of the root appears necessary to load macromolecules into the xylem vessels. A system for loading material into these vessels might have evolved in vascular plants.

Part III

Lectin activity of XSP30 with *N*-linked glycans of glycoproteins

XSP30 is homologous to ricin, a galactose-specific lectin (Masuda *et al.* 1999). Thus, it has been proposed that XSP30 also has galactose-binding activity. However, XSP30 showed only weak interaction with asialofetuin (Fig. 14C), which has a terminal galactose at non-reducing end. Further, XSP30 showed interaction to asialofetuin lacking its terminal galactose by treating the protein with galactosidase (Fig. 15, B and C). Because the biding activity of XSP30 and *Ricinus communis* agglutinin were completely different, XSP30 does not bind to the same site as *Ricinus communis* agglutinin. These results clearly showed that XSP30 does not recognize the terminal galactose in the sugar chain.

XSP30 showed interaction with soybean agglutinin and its interaction was inhibited by chitotriose, thus, it was shown that the XSP30 does not recognize mannose but core structure of chitobiose, GlcNAc-GlcNAc, of *N*-linked sugar chains (Figs. 16B and 17). From the result that the interaction to soybean agglutinin was not inhibited by chitobiose (Fig. 17), it is supposed that added sugar linkage is necessary for the binding activity of XSP30 to core structure of chitobiose, GlcNAc-GlcNAc, of *N*-linked sugar chains. In the previous study, I found that the core motif of the galactose-binding lectin was not completely conserved in XSP30 (Masuda *et al.* 1999). Possibly, the slight changes in this binding motif altered its sugar chain recognition from galactose to *N*-acetylglucosamine.

The binding reaction of XSP30 was inhibited by fucose in the glycoprotein sugar chain (Figs. 13 and 16). If sugar chain modification did not affect lectin activity, XSP30 would recognize all glycoproteins, because the core GlcNAc-GlcNAc is

conserved in all *N*-linked sugar chains. Thus, XSP30 may use fucose to differentiate glycoprotein recognition. Because XSP30 showed weaker interaction with asialofetuin than ovalbumin (Fig. 14C), it is supposed that the space structure of sugar chain or protein also affects the binding reaction of XSP30.

It was previously reported that XSP30 did not accumulate in any cucumber organs (Masuda *et al.* 1999). In support of these findings, we did not detect XSP30 in either leaf or stem sections (Fig. 18, B and F). I reasoned that XSP30 transported from the roots might be rapidly degraded in aboveground organs. I found strong lectin reaction of XSP30 on leaf parenchyma cells (Fig. 18C). I also found the lectin reaction of XSP30 to the high molecular weight glycoprotein in particulate fraction of cucumber leaf cells (Fig. 19), suggesting the abundance of XSP30 binding sites in the membranes of leaf parenchyma cells. Because the solutes of xylem sap are mainly transported to leaf parenchyma cells due to the transpiration, it is supposed that XSP30 affects the physiological condition of leaf parenchyma cells.

Most plant species have lectins that bind to specific mono- or oligosaccharides. Lectins function to protect the plant from higher animals, insects, or fungi (Peumans *et al.* 1995; Nahalkova *et al.* 2001). For such functions, toxic lectins should accumulate in accessible regions of the plant. XSP30 is transported in the xylem to aboveground organs, but does not accumulate there (Fig. 18, B and F; Masuda *et al.* 1999), indicating that XSP30 is rapidly degraded in aboveground organs. Ricin, a homologuos lectin of XSP30, has a toxic ribosome-inactivating domain, but XSP30 lacks it. Therefore it is difficult to imagine that XSP30 contributes to plant

defense.

PP1 and PP2 (Cucurbitaceae phloem lectins) recognize chitin oligomer, which is composed of 1,4-linked *N*-acetylglucosamine (GlcNAc), and are transported via sieve elements (Allen 1979; Golecki *et al.* 1999). Phloem lectins have also been found in cucumber and in melon, and agglutinating activity is effectively inhibited by chitin oligomer (Allen 1979). These lectins are thought to protect the plant from parasites by attacking chitin oligomer that is necessary for the synthesis of fungal cell walls (Allen 1979). XSP30 is similar to phloem lectins in the recognition of 1,4-linked GlcNAc sugar chains and their transportation via the vascular bundle. However, XSP30 is transported in the nutrient-poor xylem, where there is little attraction for pathogens.

It has also been reported that some lectins have functions other than plant defense. Pea root lectin and pea seed lectin (PSA) are involved in the recognition of rhizobium and the initiation of nitrogen-fixing nodule formation (Diaz *et al.* 2000; Raijn *et al.* 2001). Although wheat germ agglutinin (WGA), whose recognition site is the core 1,4-linked GlcNAc-GlcNAc in *N*-linked glycans, is one of the most effective antibiotic factor against insect (Murdock *et al.* 1990), it has also been reported to be involved in rhizobium recognition, and *N*-acetylglucosamine inhibits the deformation of root-hair in wheat (Yeorenkova *et al.* 2001). The recognition site of XSP30 is similar to that of WGA; thus, XSP30 may be involved in the initiation of developmental processes in aboveground organs, as XSP30 production in roots is controlled by light and gibberellins in the shoot (Oda *et al.* 2003a).

The importance of arabinogalactan proteins (AGPs) in development has also been reported. Some AGPs with *N*-acetylglucosamine moieties control somatic embryogenesis in carrot (Hengel *et al.* 2001) and tracheary element differentiation in zinnia (Motose *et al.* 2001). Yieldin, a protein homologous to endochitinase and Con A, controls cell-wall elongation (Okamoto-Nakazato *et al.* 2001). XSP30 may control similar developmental processes, given that it binds the GlcNAc-GlcNAc groups of glycoprotein *N*-linked sugar chains. Although many proteins interact with XSP30 (Figs. 17 and 18), the spatiotemporal distribution of XSP30 in cucumber plants is probably limited, as XSP30 is supplied only by the xylem sap and is rapidly degraded. This may provide a convenient system for the coordination of plant processes, if AGPs or glycoproteins are produced in response to the status of aboveground organs and XSP30 interacts with them to control development.