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Xylem Sap Lectin, XSP30, Recognizes GlcNAc Sugar Chains of Glycoproteins in Cucumber Leaves

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Abstract

The lectin activity of 30-kDa xylem sap protein (XSP30) found in cucumber (*Cucumis sativas* L.) xylem sap was analyzed by lectin blot coupled with immunological detection of XSP30. XSP30 is homologous to galactose-binding lectin, but it had only weak binding activity against asialofetuin, an animal glycoprotein with a terminal galactose. This reaction was not reduced by galactosidase treatement of asialofetuin. XSP30 bound strongly to soybean agglutinin, whose sugar chains consist solely of mannose and *N*-acetylglucosamine (GlcNAc), but bound only weakly to soybean peroxidase and γ-globulin, whose GlcNAc is fucosylated. The binding activity was inhibited by tri-*N*-acetylchitotriose $(GlcNAc)_3$. Leaf parenchyma cells were stained with XSP30 and aniti-XSP30 antibody; this staining was reduced by proteinase treatment of the sections, and XSP30 bound to proteins in leaf particulate fraction. These results suggest that XSP30 transported from root via xylem sap binds to chitobiose, GlcNAc-GlcNAc, in *N*-linked glycans of leaf glycoproteins.

Key words: cucumber, glycoprotein, lectin, *N*-acetylglucosamine, xylem sap protein.

Abbreviations

AGP, arabinogalactan protein; Con A, concanavalin A; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; PBS, phosphate-buffered saline; RCA, *Ricinus communis* agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; XSP30, 30-kDa xylem sap protein; Xyl, xylose.

Introduction

The roots and aboveground organs of higher plants are connected by the xylem and phloem of the vascular bundle. Vascular tissues not only provide a means of transporting nutrients and metabolites, but also facilitate whole-plant communication (Beveridge *et al.*, 1997; Kato *et al.*, 2002). Xylem vessels, which are continuous with the apoplastic space, permit the passage of inorganic nutrients and plant hormones such as cytokinin and abscisic acid from the roots via the xylem sap. Xylem sap is crucial for physiological and developmental phenomena in shoots and roots (Else *et al.*, 1995; Beveridge *et al.*, 1997; Liang *et al.*, 1997; Kato *et al.*, 2002; Kuroha *et al.*, 2002).

Recent reports have shown that xylem sap also contains macromolecules, including oligo- and polysaccharides (Satoh *et al.*, 1992; Campbell *et al.*, 1995) and proteins such as peroxidase (Biles and Abeles, 1991), chitinase (Masuda *et al.*, 2001), and glycine-rich proteins (Sakuta and Satoh, 2000). The physiological functions of xylem sap proteins are not well understood.

In a previous study (Masuda *et al.*, 1999), we found the exsistence of XSP30 in cucumber xylem sap. The *XSP30* cDNA sequence has significant homology to the sequence of the *Ricinus communis* seed ricin B chain, which has galactose-specific lectin activity (Lord *et al.*, 1994). Ricin superfamily lectins are characterized by a ribosome-inactivating domain (A chain); this A-chain is missing from XSP30. *XSP30* is expressed only in roots (Masuda *et al.*, 1999), and its rhythmic expression is controlled by the photoperiod perceived by aboveground organs and by the gibberellins produced mainly in mature young leaves (Oda *et al.*, 2001). These findings indicate the presence of some interractions between root and aboveground organs.

In this report, we analyze the sugar chains recognized by XSP30, using lectin blot coupled with immunological detection of XSP30. We show that XSP30 is transported from cucumber roots to aboveground organs via the xylem sap and that it

binds to core GlcNAc-GlcNAc groups in *N*-linked glycans of leaf glycoproteins. The XSP30 recognition site and possible functions are discussed.

Materials and Methods

Plant Materials

Cucumber (*Cucumis sativus* cv. Shimoshirazu-jibai) seeds were obtained from the Sakata Seed Co. (Kanagawa, Japan). Cucumber plants were grown in artificial soil (Kurehakagaku, Tokyo, Japan) under a 16-h photoperiod of white fluorescent light (40 μmol m^{-2} s^{-1}) at 28 ℃. Xylem sap was collected from cut stems of one-month-old cucumber plants as described (Sakuta *et al.*, 1998) and used for the lectin blot as described below.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of glycoproteins and transfer to nitrocellulose filter

Glycoproteins (ovalbumin, asialofetuin, soybean peroxidase, γ-globulin) were purchased from Sigma-Aldrich (St. Louis, USA) and soybean agglutinin was purchased from Seikagaku Corporation (Tokyo, Japan). Glycoproteins (1 μg per lane) were subjected to SDS-PAGE as described by Laemmli (1970). Proteins were transferred onto a nitrocellulose filter (ADVANTEC, Tokyo, Japan) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at 40 V for 2 h (Gershoni *et al.*, 1982). The filter was stained with amido-black or incubated in phosphate-buffered saline (PBS) with 2% (v/v) bovine serum albumin (BSA) overnight at 4 ℃, followed by reaction with xylem sap or lectin reagent as described below.

Lectin blot using XSP30 and commercial lectins

Filters were incubated at room temperature in the xylem sap containing XSP30 for 1 h, and then for 1 h in a 0.1% (v/v) solution of XSP30-specific antiserum from a rat immunized with an XSP30 fusion protein (Masuda *et al.*, 1999). Filters were washed with 0.1% Tween-20 in PBS and agitated for 1 h in PBS containing 1,000-fold diluted horseradish peroxidase-conjugated goat anti-rat IgG antibodies (Jackson Immunoresearch, Laboratories, Inc., West Grove, PA, U.S.A.). Proteins were visualized by incubating the filter in PBS containing 0.03% 3,3-diaminobenzidine and 0.003% (v/v) H_2O_2.

Ricinus communis agglutinin (RCA) and concanavalin A (Con A) conjugated with peroxidase were purchased from Seikagaku Corporation (Tokyo, Japan). The filter was incubated in a 1,000-fold dilution of peroxidase-conjugated lectins in PBS for 1 h. Proteins were visualized as described above.

Removal of galactose from asialofetuin

Galactosidase was purchased from Seikagaku Corporation (Tokyo, Japan). Asialofetuin (1 μg μl^{-1}) was treated with galactosidase (0.05 U μl^{-1}) in 50 mM sodium acetate buffer (pH 5.5) at 37 ℃ for 1 h. The resulting solution was mixed with an equal volume of buffer [120 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, 10% (v/v), -β-mercaptoethanol], boiled for 10 min, and then subjected to SDS-PAGE as described above.

Inhibition of XSP30 lectin activity by oligo-N-acetylglucosamine

Di-*N*-acetylchitobiose $(GlcNAc)_2$ and tri-*N*-acetylchitotriose $(GlcNAc)_3$, purchased from Seikagaku Corporation (Tokyo, Japan), were added to xylem sap to a final concentration of 0.1 or 1 mM. After incubation at room temperature for 1 h, the lectin activity of XSP30 to soybean agglutinin was analyzed as described above.

Histochemical analysis of XSP30 binding

Fully expanded leaves and mature stems of one-month-old cucumber plants were fixed in 3% paraformaldehyde in 50 mM 1,4-piperazinediethanesulfonic acid (pH 7.4) overnight at 4 ℃ with occasional mild degassing. The tissues were dehydrated by a graded ethanol series. The ethanol was replaced by *tert*-butyl alcohol and then by liquid paraffin, and the tissues were embedded in paraffin. Blocks containing the tissues were cut into thin (8-15 μm) sections. The sections were expanded in water, mounted on glass slides (APS-coated micro glass slides; Matsunami, Japan), and dried overnight at 45 ℃.

Slide-mounted sections were dewaxed with xylene and rehydrated by a graded ethanol series. Slides were rinsed with PBS, immersed in methanol containing 0.3% (v/v) H_2O_2 for 10 min to inactive endogenous peroxidases, and then subjected to XSP30 binding activity detection as described above or to protein removal as follows.

Protein removal from sections

Slides containing tissue sections were treated with proteinase K (0.4 mg ml^{-1}) (Takara, Tokyo Japan) in PBS at 37 ℃ for 15 min and immersed twice in 5 mM phenylmethylsulfonyl fluoride in PBS at room temperature for 30 min to inactivate proteinase K. XSP30 binding was then detected as described above.

Extraction of cucumber leaf proteins

Fully expanded leaves of one-month-old cucumber plants were crushed in liquid nitrogen using a mortar and pestle and then suspended in twice their volume of 100 mM Tris-HCl buffer (pH 8.0). The resulting homogenate was ground with 10 strokes of a glass-Teflon homogenizer and centrifuged at 100,000 *g* at 4 ℃ for 1 h. The resulting particulate precipitate was subjected to SDS-PAGE as described above.

Results

Lectin activity of XSP30

Two animal glycoproteins (ovalbumin, asialofetuin) with standard *N*-linked oligosaccharide chains (**Fig. 7**) were separated by SDS-PAGE and blotted onto nitrocellulose sheets. The sheets were treated with xylem sap from cucumber roots, and XSP30 bound to glycoproteins was detected by anti-XSP30 antiserum. XSP30 reacted with ovalbumin, but only weakly with asialofetuin (**Fig. 1C**). When the blot was treated with *Ricinus communis* agglutinin (RCA), which has galactose-specific lectin activity (Beanziger and Fiete, 1979), a much stronger signal was detected on asialofetuin than on ovalbumin (**Fig. 1D**).

Binding of XSP30 to galactose-removed asialofetuin

We examined the interaction of XSP30 to asialofetuin lacking its terminal galactose by treating the protein with galactosidase. XSP30 interacted with similar affinities to asialofetuin with and without galactose, whereas RCA reacted poorly with galactose-removed asialofetuin (**Fig. 2B, 2C**).

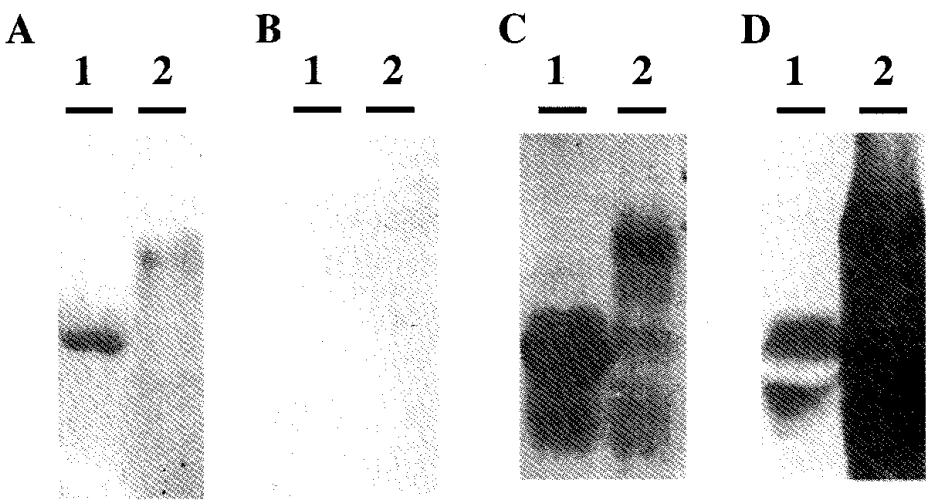

Fig. 1 Lectin activity of XSP30. Ovalbumin (lane 1) and asialofetuin (lane 2) (1 μg each) were separated by SDS-PAGE, blotted onto nitrocellulose sheets, and stained with amido black (A) or reacted with anti-XSP30 antiserum (B), xylem sap followed by anti-XSP30 antiserum (C), or RCA (D).

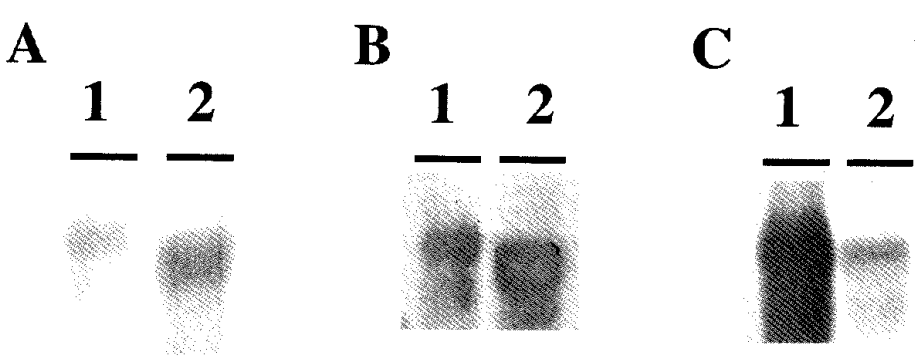

Fig. 2 Binding activity of XSP30 to asialofetuin lacking galactose. Asialofetuin (lane 1) and asialofetuin whose non reducing terminal galactose was removed by galactosidase treatment (lane 2) (1 μg each) were separated by SDS-PAGE, blotted onto nitrocellulose, and then stained with amido black (A) or analyzed by lectin blot with XSP30 (B) or RCA (C).

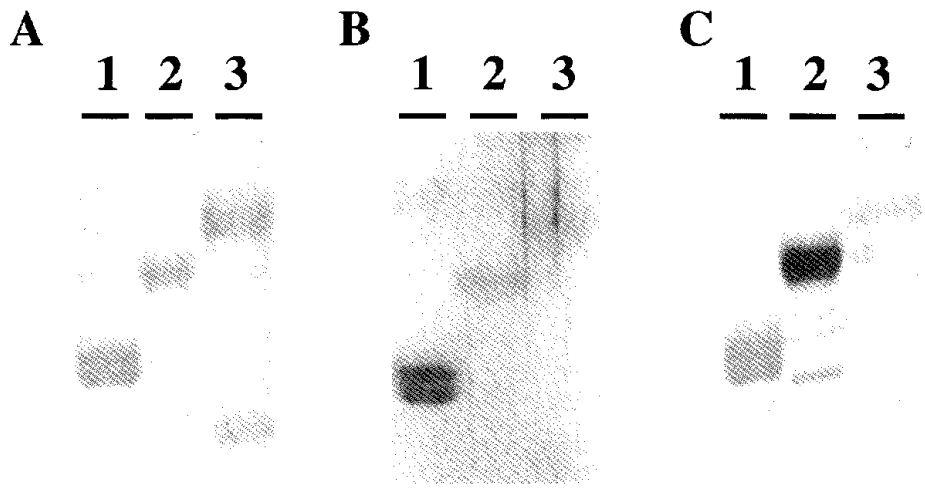

Fig. 3 Binding activity of XSP30 to high-mannose-type glycoproteins. Soybean agglutinin (lane 1), soybean peroxidase (lane 2) and γ-globulin (lane 3) (1 μg each) were separated by SDS-PAGE, blotted onto nitrocellulose, and stained with amido black (A) or analyzed by lectin blot with XSP30 (B) or Con A (C).

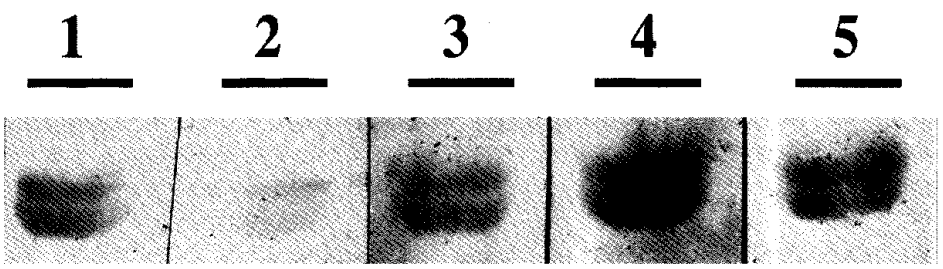

Fig. 4 Inhibition of XSP30 lectin activity by oligo-*N*-acetylglucosamine. Soybean agglutinin (1 μg) was separated by SDS-PAGE and blotted onto nitrocellulose, and XSP30 binding activity was examined in the absence (lane 1) or presence of 1 mM (lane 2) or 0.1 mM (lane 3) tri-*N*-acetylchitotriose or 1 mM (lane 4) or 0.1 mM (lane 5) di-*N*-acetylchitobiose.

Comparison of the lectin activities of XSP30 and concanavalin A

Binding capacity of XSP30 was also compared to that of concanavalin A (Con A), a mannose-specific lectin (Naismith *et al.*, 1996), using various proteins to identify the XSP30 recognition site. High mannose-type glycoproteins, soybean agglutinin (SBA), soybean peroxidase, and γ-globulin, were analyzed by lectin blot with XSP30 or Con A (**Fig. 3B, 7**). XSP30 interacted with SBA, but had little interaction with soybean peroxidase or γ-globulin, both of which have fucosylated core GlcNAc-GlcNAc region of the *N*-linked sugar chain (**Fig. 3B, 7**). Con A interacted with SBA and soybean peroxidase, but had little interaction with γ-globulin (**Fig. 3C**).

Inhibition of XSP30 binding with oligo-N-acetylglucosamine

XSP30 binding to SBA was partially inhibited by 0.1 mM and completely inhibited by 1 mM of tri-*N*-acetylchitotriose $(GlcNAc)_3$ (**Fig. 4**, lane 2 and 3). Di-*N*-acetylchitobiose $(GlcNAc)_2$ did not inhibit XSP30 (**Fig. 4**, lane 4 and 5).

Binding of XSP30 to cucumber leaf and stem tissue sections

We did not detect any XSP30 in either leaf or stem sections (**Fig. 5B, F**). When sections were first incubated in xylem sap, anti-XSP30 antiserum detected XSP30 on leaf parenchyma cells (**Fig. 5C**). Weak staining was also detected on stem parenchyma cells (**Fig. 5G**). When section proteins were removed by proteinase K treatment, most of the staining disappeared (**Fig. 5D, H**).

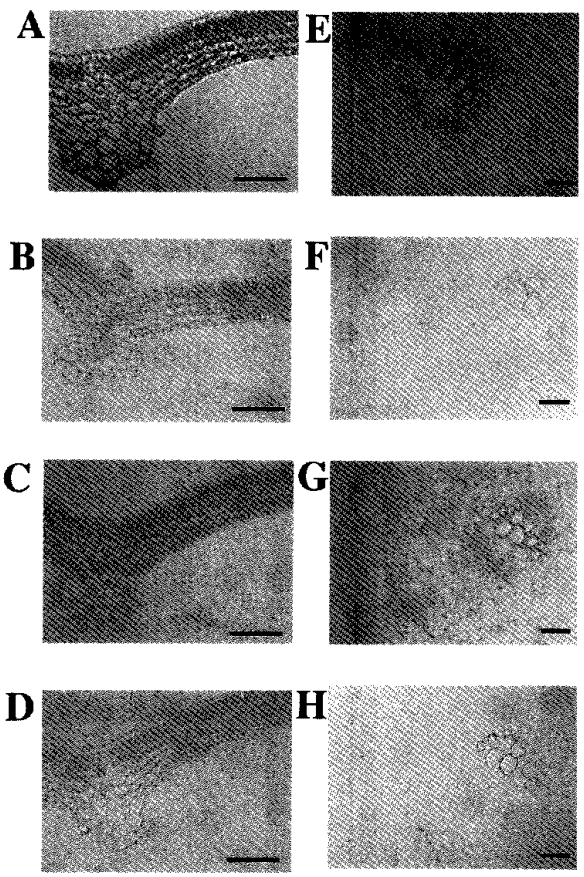

Fig. 5 Binding of XSP30 to cucumber leaf and stem tissue sections. Leaf (A-D) and stem (E-H) sections were stained with toluidine blue (A and E), or allowed to react with anti-XSP30 antiserum (B and F), or xylem sap followed by anti-XSP30 antiserum (C, D, G and H). Proteins in sections in D and H were digested by proteinase K before the xylem sap and anti-XSP30 treatments. Scale bars indicate 100 μm.

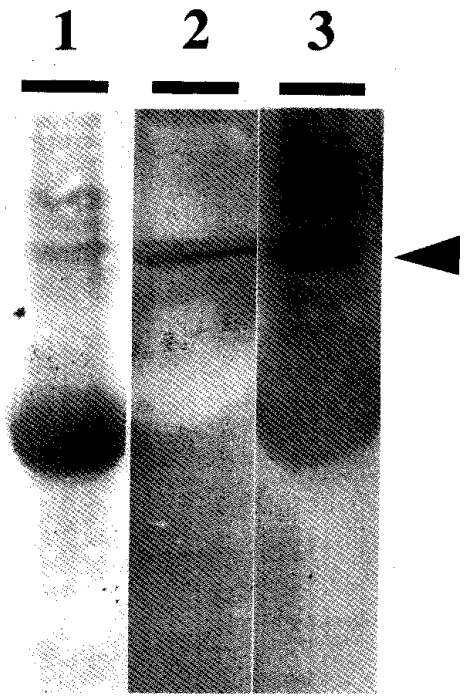

Fig. 6 XSP30 binding to proteins in particulate fraction of cucumber leaf cells. Particulate fraction proteins from cucumber leaves were separated by SDS-PAGE, blotted onto nitrocellulose, and stained with amido black (lane 1), or allowed to react with anti-XSP30 antiserum (lane 2) or xylem sap followed by anti-XSP30 antiserum (lane 3). Arrow indicates non-specific staining.

Binding activity of XSP30 to leaf proteins

The ability of XSP30 to bind to proteins in the particulate fraction of cucumber leaf cells was examined (**Fig. 6**). The proteins were analyzed by reaction with anti-XSP30 antiserum either with (lane 3) or without (lane 2) prior treatment with xylem sap. In both cases, non-specific high molecular weight bands (arrow) were detected. However, a smear of staining in the high molecular weight range was detected only after xylem sap treatment (lane 3).

Discussion

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. 1C**), 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. 2B, 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 (**Fig. 3B, 4**). From the result that the interaction to soybean agglutinin was not inhibited by chitobiose (**Fig. 4**), 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, we found that the core motif of the galactose-binding lectin was not completely conserved in XSP30 (Masuda *et*

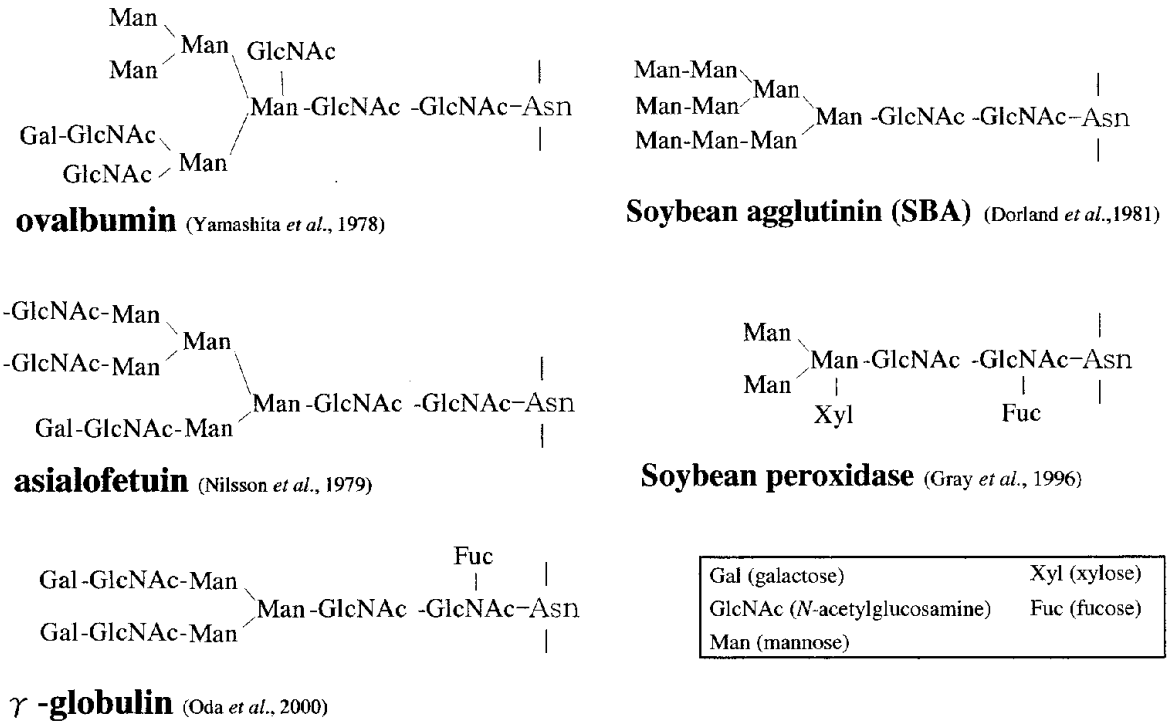

Fig. 7 Structure of *N*-linked oligosaccharide chain of glycoprotein

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 (**Fig. 3, 7**). 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. 1C**), it is supposed that the space structure of sugar chain or protein also affects the binding reaction of XSP30.

We 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. 5B, F**). We reasoned that XSP30 transported from the roots might be rapidly degraded in aboveground organs. We found strong lectin reaction of XSP30 on leaf parenchyma cells (**Fig. 5C**). We also found the lectin reaction of XSP30 to the high molecular weight glycoprotein in particulate fraction of cucumber leaf cells (**Fig. 6**), suggesting the abundance of XSP30 binding site 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. 5B, 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.*, 2001).

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 (**Fig. 5, 6**), 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.

Acknowledgments

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