Materials and Methods

Plant materials

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

Elimination of aboveground organs

The shoots of 15-day-old seedlings were cut off with a razor blade at the middle of the hypocotyl at dawn; roots were collected every 4 h for RNA preparation.

Alternatively, either the first leaf or the cotyledons plus shoot apex of 13-day-old cucumber plants were cut off with a razor blade at dawn and the roots were collected every 8 h for RNA preparation.

Gibberellin and uniconazole-P treatments

The leaves, including the shoot apex of 13-day-old plants, were sprayed with 2 $\times$ 10^{-4} M GA_3 or 10^{-4} M uniconazole-P dissolved in 0.1% Tween 20 in water; the roots were collected every 8 h for RNA preparation.

Gibberellin-treatment of root in Uniconazole-P treated plant

The leaves and the shoot apex of 13-day-old plants were sprayed with 10^{-4} M

Uniconazole-P dissolved in 0.1% Tween 20 in water. After two days, 10^{-4} M GA_3 was supplied to roots with soil and the roots were collected every 8 h for RNA preparation

Northern blot analysis

A full-length cDNA probe of *XSP30* (DDBJ; AB025717) and a partial cDNA probe of *CRGRP-2* (DDBJ; AB015174, from base 680 to 959) were prepared using the polymerase chain reaction (PCR). Total RNA was isolated as described previously (Sakuta *et al.*, 1998) from the mixed roots of 10 treated plants and the RNA (10 μg/lane) was loaded on a 1.5% agarosegel and electrophoresed. RNA gel blot analysis was performed using a ^{32}P-labeled probe and a BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan). The amount of RNA loaded was confirmed by hybridization with an rDNA probe, and the data were quantified using Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film, Tokyo, Japan). All experiments were repeated at least twice with essentially similar results.

Cloning of the *XSP30* promoter

To analyze the *XSP30* promoter, *XSP30* genomic DNA was cloned by thermal asymmetric interlaced (TAIL)-PCR (Liu *et al.* 1995). The primers used (from the 3' to the 5' end) were: (1) 5'-CTTTCCGTGACTTCCCAACTTTGTG-3', (2) 5'-TCTTCTGGGTCCATTTCTTGTTAGG-3' and (3) 5'-CGTCACATGGTGATAATCGAGTTGG-3'. Short arbitrary degenerate (AD) primers were designed to have melting points (T_m) of 63 to 68 and 44 to 46°C (Liu *et*

al. 1995).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of xylem sap proteins

Xylem sap was collected from the cut stems of one-month-old cucumber plants as described previously (Sakuta *et al.*, 1998) at 4-h intervals, starting 4 h before dusk, with other samples taken at dusk, midnight, dawn, 4 h after dawn, and midday; the samples were immediately frozen in liquid nitrogen and stored at –20 °C. The xylem sap samples were 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] and boiled for 10 min in a water bath, then centrifuged at 15,000 × g for 5 min. The resulting supernatants (10 μl / lane) were subjected to SDS-PAGE (Sakuta *et al.* 1998) as described by Laemmli (1970). After electrophoresis, the gels were either stained with silver stain (Sil-best stain; Nacarai Tesque Inc, Kyoto, Japan) or subjected to immunological detection.

Immunological detection of XSP30

Proteins on the SDS-PAGE gel were transferred to 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 incubated in phosphate-buffered saline (PBS) with 2% (v/v) bovine serum albumin overnight at 4 °C, and then for 1 h in a 0.1% (v/v) solution of the XSP30-specific antiserum, prepared from a rat

immunized with an XSP30 fusion protein (Masuda *et al.* 1999). The filter was washed with 0.1% Tween 20 in PBS and agitated in a solution of 1,000-fold diluted horseradish peroxidase-conjugated antibodies in PBS for 1 h; antibodies were raised in a goat against rat IgG (Jackson Immunoresearch, Laboratories, Inc., West Grove, PA, USA). Proteins were visualized by incubating the filter in PBS containing 0.03% 3,3-diaminobenzidine and 0.003% (v/v) H_2O_2.

Lectin blot using XSP30 and commercial lectins

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 / lane) were subjected to SDS-PAGE and transferred onto a nitrocellulose filter as described above. 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°C, followed by reaction with xylem sap or lectin reagent as described below.

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, USA). 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) was treated with galactosidase (0.05 U / μl) in 50 mM sodium acetate buffer (pH 5.5) at 37°C 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.

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°C for 1 h. The resulting particulate precipitate was subjected to SDS-PAGE as described above.

Plant transformation and induction of hairy roots

An 820-bp DNA fragment of the *XSP30* genomic sequence upstream of the putative initiation codon (P_{XSP30}) was amplified by PCR. The product (promoter region of *XSP30*) was cloned into upstream of the ß-glucuronidase (GUS) coding sequence of a modified pBI121 vector that includes the cauliflower mosaic virus *35S* promoter (*35S*) cloned upstream of the hygromycin phosphotransferase gene (Iwai *et al.* 2001). This plasmid was introduced into *Agrobacterium tumefaciens R1000* (Kamada *et al.* 1995) by the freeze-thaw method (Zahm *et al.* 1984). Transgenic hairy roots were obtained by inoculating the *Agrobacterium* to the excised surface of 7-day-old cucumber plant cotyledons. The cotyledons were cultured on Murashige and Skoog agar-solidified medium under continuous light at 28°C.

Histochemical analysis of GUS activity

For GUS staining, hairy roots that emerged from the cotyledons were immersed in X-

Glu solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide in 50 mM sodium phosphate pH 7.0). Samples were then subjected to a vacuum for 5 min and incubated at 37°C for 8 h (Jefferson 1987). Stained samples were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.0) at room temperature for 4 h and then dehydrated by passage through ethanol series. Dehydrated samples were embedded in Technovit 7100 (Kulzer and Co., GmbH, Werheim, Germany). Thin serial sections were cut with a tungsten knife on a microtome (RM-2415; Leica, Wetzlar, Germany). The sections were expanded in drops of water on glass slides (APS-coated micro glass slides; Matsunami Glass Ind. Ltd., Kishiwada, Japan) and dried at 50°C.

Histochemical analysis of XSP30 binding

Fully expanded leaves and mature stems of one-month-old cucumber plants were fixed and dehydrated as described above. 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°C.

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) (Takara, Tokyo Japan) in PBS at 37°C 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.