Possible Involvement of Leaf Gibberellins in the Clock-Controlled Expression of *XSP30*, a Gene Encoding a Xylem Sap Lectin, in Cucumber Roots[1]

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Root-produced organic compounds in xylem sap, such as hormones and amino acids, are known to be important in plant development. Recently, biochemical approaches have revealed the identities of several xylem sap proteins, but the biological functions and the regulation of the production of these proteins are not fully understood. *XYLEM SAP PROTEIN 30 kD* (*XSP30*), which is specifically expressed in the roots of cucumber (*Cucumis sativus*), encodes a lectin and is hypothesized as affecting the development of above-ground organs. In this report, we demonstrate that *XSP30* gene expression and the level of XSP30 protein fluctuate in a diurnal rhythm in cucumber roots. The rhythmic gene expression continues for at least two or three cycles, even under continuous light or dark conditions, demonstrating that the expression of this gene is controlled by a circadian clock. Removal of mature leaves or treatment of shoots with uniconazole-P, an inhibitor of gibberellic acid (GA) biosynthesis, dampens the amplitude of the rhythmic expression; the application of GA negates these effects. These results suggest that light signals perceived by above-ground organs, as well as GA that is produced, possibly, in mature leaves, are important for the rhythmic expression of *XSP30* in roots. This is the first demonstration of the regulation of the expression of a clock-controlled gene by GA.

The higher plant body consists of functionally specialized organs such as the leaf, stem, flower, and root. Because plants grow in changing environments, it is essential for different organs to interact to ensure that the plant body develops and functions properly. Information transfer between organs is essential for synchronized plant development (Bernier, 1988; Kolek and Kozinka, 1991). A major route for the transfer of materials between organs consists of the vascular bundles, which are composed mainly of the xylem and phloem. Numerous materials are translocated over long distances through these bundles. The xylem is composed mainly of xylem vessels, which form a type of apoplastic space. Organic nutrients, such as amino acids, sugars, and organic acids, as well as water and inorganic nutrients, are transported through the xylem to the aboveground organs (Schurr and Schulze, 1995; Zornoza et al., 1996; Satoh et al., 1998). Interestingly, the roots control aspects of the development of aerial organs, possibly acting via growth-related compounds in the xylem sap (Kinet et al., 1993; Satoh, 1996). For example, cytokinin, abscisic acid, and other growth-related compounds that are synthesized in root tissues are involved in stomatal responses (Else et al., 1995; Liang et al., 1997), leaf senescence (Nooden et al., 1990; Soejima et al., 1992), lateral bud development (Bangerth, 1994; Beveridge et al., 1997), flower bud formation (Kinet et al., 1993), leaf greening (Kato et al., 2002), and adventitious root formation (Kuroha et al., 2002).

Recently, macromolecules have been found in xylem sap, 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), a cucumber (*Cucumis sativus*) root-specific Gly-rich protein (CRGRP; Sakuta et al., 1998; Sakuta and Satoh, 2000), pathogenesis-related (PR) proteins (Rep et al., 2002), a Cys-rich protein (Rep et al., 2003), and a novel 30-kD protein (XSP30; Masuda et al., 1999). However, the regulation of the production of these xylem sap proteins and their physiological functions are not well understood.

A major environmental factor for plants, the diurnal light cycle, is perceived mainly in leaves, but signal transduction resulting from this cue is controlled in many organs (McClung, 2001). In plants, photoreceptors that perceive light signals, such as phytochromes and cryptochromes, influence the expression of biological clock component genes, such as *TIMING OF CAB EXPRESSION 1* (Strayer et al., 2000), *LATE ELONGATED HYPOCOTYL* (Schaffer et al., 1998), and *CIRCADIAN CLOCK ASSOCIATED 1* (Wang and Tobin, 1998) via factors that interact with photoreceptors and adjust the biological clock in 24-h periods (Makino et al., 2002; Mizoguchi et al., 2002;

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Mouradov et al., 2002). The biological clock signals that are distributed to each organ regulate many aspects of development, such as flowering, leaf movement, and hypocotyl elongation (McClung, 2001). The expression of numerous genes is controlled by circadian rhythms (Somers, 1999; Harmer et al., 2000). In addition, the diurnal light cycle affects the levels of plant hormones produced in the shoot. The expression of the gene that encodes gibberellic acid (GA) 20-oxidase, a key regulatory enzyme in the gibberellin biosynthetic pathway in leaves, is controlled by the diurnal light cycle and is correlated to tuber formation in potato (*Solanum tuberosum*; Carrera et al., 1999). Endogenous levels of indole-3-acetic acid (IAA) fluctuate in a circadian rhythm and are involved in the growth of the internode (Jouve et al., 1999). However, the effects of circadian rhythms on the root have not been fully studied; these effects are less intuitive because roots are spatially separated from leaves, where the circadian rhythms are adjusted. Information on clock-controlled gene expression and protein accumulation in roots is quite limited.

In our previous study of cucumber xylem sap, a novel 30-kD protein (XSP30) was identified (Masuda et al., 1999). The sequence of the protein encoded by the *XSP30* cDNA has significant homology to the B chain of ricin, a toxic protein with Gal-specific lectin activity from the seeds of castor bean (*Ricinus communis*; Lord et al., 1994). Ricin superfamily lectins are characterized by a ribosome-inactivating domain (A chain) and a Gal-binding lectin domain (B chain; Lord et al., 1994). Although XSP30 lacks a ribosome-inactivating domain (A chain), it binds to the free (*N*-acetylglucosamine)$_2$ in *N*-linked glycans (Oda et al., 2003). *XSP30* is expressed only in roots, but the glycoprotein-conjugated *N*-linked glycans recognized by XSP30 are most abundant in leaves (Masuda et al., 1999; Oda et al., 2003), leading to speculation that XSP30 is a signaling molecule that is transported from the root to the shoot.

In this report, we show that *XSP30* expression in cucumber roots follows a diurnal pattern, and that the oscillation in the expression of its gene is controlled by a circadian clock, which is, in turn, possibly affected by leaf gibberellins. The specific expression of *XSP30* in root vascular tissues is also shown. This is the first report of a circadian control of a root-specific gene that is affected by GA.

RESULTS

Root-Specific Expression of the *XSP30* Gene

We have found that *XSP30* is expressed only in root tissue (Masuda et al., 1999), as no transcripts can be detected in the aerial organs of cucumber (Fig. 1A). To further analyze this expression pattern, we constructed a chimeric gene (P_{XSP30}::*GUS*), consisting of the *XSP30* promoter fused to a *GUS* reporter gene, and detected the distribution of the GUS activity driven by the *XSP30* promoter in transgenic cucumber plants (Fig. 1B). GUS activity was analyzed in the hairy roots that emerged from the cut surface of cotyledons transformed with P_{XSP30}::*GUS*. Strong GUS staining was observed in the central cylinder of mature roots (Fig. 1B), and the staining intensity gradually decreased in younger portions of the roots. No GUS staining was detected at the root tip or in emerging lateral roots. Cross sections revealed that *GUS* expression was restricted to the xylem parenchyma and pericycle cells in the central cylinder, and no GUS staining was observed in the phloem (Fig. 1, C and D).

Gel-blot analysis of total RNA prepared from the roots of cucumber seedlings of different ages showed that *XSP30* expression was barely detectable in the roots of 4-d-old seedlings, but increased gradually with maturity, concurrent with leaf development, after 6 d (Fig. 1E).

Diurnal Oscillation of *XSP30* Gene Expression and Protein Accumulation

To test whether environmental signals affect *XSP30* gene expression in cucumber roots, we examined *XSP30* mRNA levels in plants under various conditions. In the course of this study, we found that *XSP30* gene expression showed a diurnal pattern under a photoperiod of 16 h of light/8 h of dark (Fig. 2, A and B). *XSP30* gene expression peaked at time 16 and decreased to trough level at around time 4. Peaks in expression levels occurred at time 16, except for during the first cycle.

The amount of XSP30 protein in root xylem sap was evaluated by immunoblotting with anti-XSP30 serum (Masuda et al., 1999). A diurnal rhythm in XSP30 protein abundance occurred with a peak at dusk (time 16 in this experiment; Fig. 2, C–E). This diurnal pattern in protein abundance was quite similar to the fluctuation of the abundance of the *XSP30* mRNA (Fig. 2, A, B, and F).

Circadian Clock-Controlled Expression of the *XSP30* Gene

The *XSP30* mRNA level began to increase before dusk and to decrease before dawn (Fig. 2, A and B). This anticipation of the diurnal rhythm is a common feature in circadian rhythms. When seedlings grown under a 16-h light/8-h dark photoperiod for 13 days were transferred to continuous dark (DD) or continuous light (LL), the *XSP30* gene showed a rhythmic pattern of expression for at least two cycles under DD (Fig. 3, A and B) and three cycles under LL (Fig. 3, D and E). The data were Fourier transformed and the periods were estimated as 26.59 (±8.713) hours in DD and 20.31 (±1.547) hours in LL (Fig. 3, C and F). To confirm the strength of rhythms, the relative ampli-

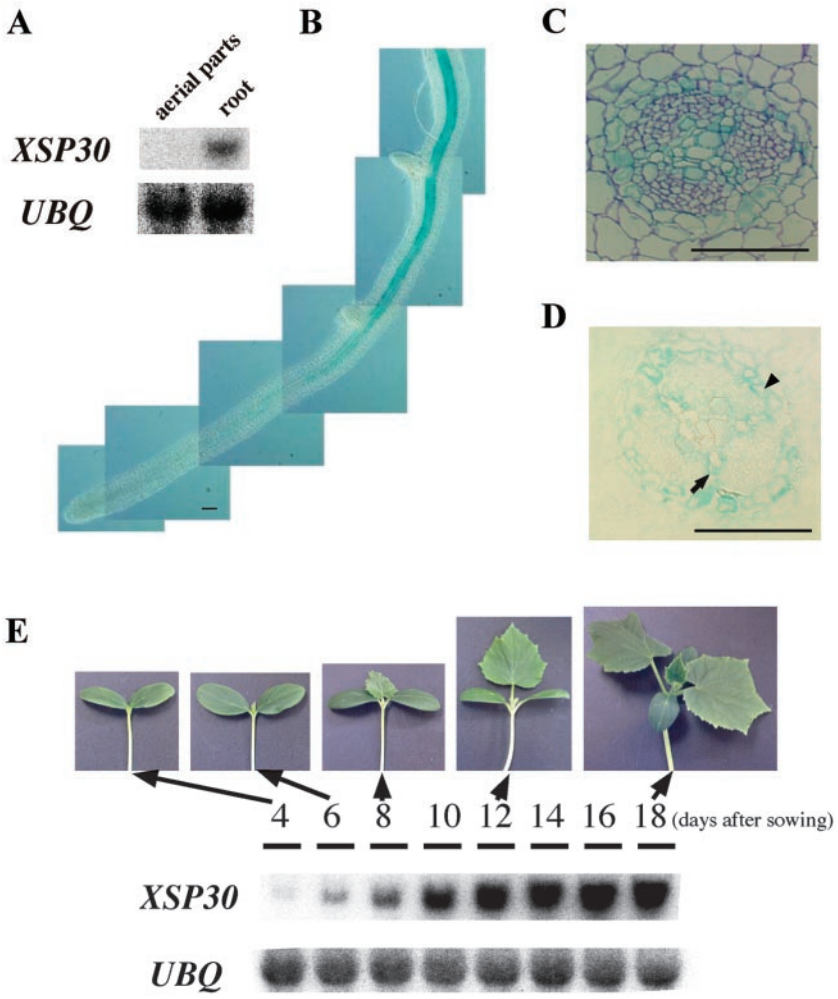

Figure 1. Root-vascular tissue-specific *XSP30* expression. A, Root-specific expression of *XSP30*. Total RNA (10 μg lane^{-1}) extracted from aboveground organs or roots of 30-d-old cucumber plants was subjected to RNA gel-blot analysis. The transcripts were probed using a full-length *XSP30* cDNA and ubiquitin fragments as probes. The ubiquitin cDNA was isolated from cucumber roots, where it is expressed constitutively. B through D, β-Glucuronidase (GUS) activity in P_{XSP30}*::GUS*-transgenic hairy roots. GUS activity was observed in the central cylinder of mature roots, but not in the root tip shown in B. GUS-stained transgenic roots were embedded in Technovit 7100 (Kulzer and Co., Werheim, Germany), and thin serial sections were stained with toluidine blue (C) or left unstained (D). The central cylinder is shown. Scale bars correspond to 100 μm. The arrow and arrowhead indicate GUS staining in the xylem parenchyma and pericycle cells, respectively. E, Seedling development-dependent expression of *XSP30* in roots. Total RNA (10 μg lane^{-1}) extracted at dusk from roots of seedlings at 4, 6, 8, 10, 12, 14, 16, and 18 d after sowing were subjected to RNA gel-blot analysis. The transcripts were probed using a full-length *XSP30* cDNA and a ubiquitin fragment as probes.

tude errors (RAE, see "Material and Methods") were calculated as 0.308 (0.6 > RAE) in DD and 0.731 (1 > RAE > 0.6) in LL. A perfect sine wave gives an RAE of 0 and RAE = 1 defines the limit of statistical significance of rhythm, suggesting substantial and weak rhythms of *XSP30* expression in DD and LL, respectively. The experiments of Figure 3 have been performed three times. Statistically significant rhythms were detected twice, and in the third replicate (data not shown), the time courses were suggestive, although not statistically significant. Therefore, we conclude that a circadian clock contributes to the regulation of expression of *XSP30*.

Decapitation Eliminates Rhythmic Expression of *XSP30* in Roots

Light signals are important for the entrainment of a circadian clock. Aboveground organs are thought to receive the light signals that control a variety of biological processes in plants. We have recently demonstrated that cotyledons are required for cell division during tissue reunion in the cortex of cut cucumber (Asahina et al., 2002). This led us to test whether the diurnal expression of *XSP30* in roots requires the aboveground organs. When the first leaf, the cotyledons, and the shoot apex were removed from cucumber seedlings at the middle of the hypocotyl, the *XSP30* mRNA abundance peaked at around dusk (time 12–16) after decapitation. However, this increase in *XSP30* expression at dusk was not observed on the 2nd d after organ removal (Fig. 4, B and C). In contrast, intact control plants showed a normal diurnal rhythm, and *XSP30* expression peaked at times 20 and 40 (Fig. 4, A and C). The results suggest that these aboveground organs are involved in the control of the rhythmic expression of *XSP30* in cucumber roots.

Involvement of the First Leaf in Diurnal Gene Expression

Gene expression in roots was examined after removal of the first leaf or the cotyledons and shoot apex from 13-d-old seedlings. Rhythmic expression of *XSP30* was observed during at least the 2nd, 3rd, and 4th d after removal of the cotyledon and shoot apex (Fig. 5, A and C). *XSP30* expression peaked at

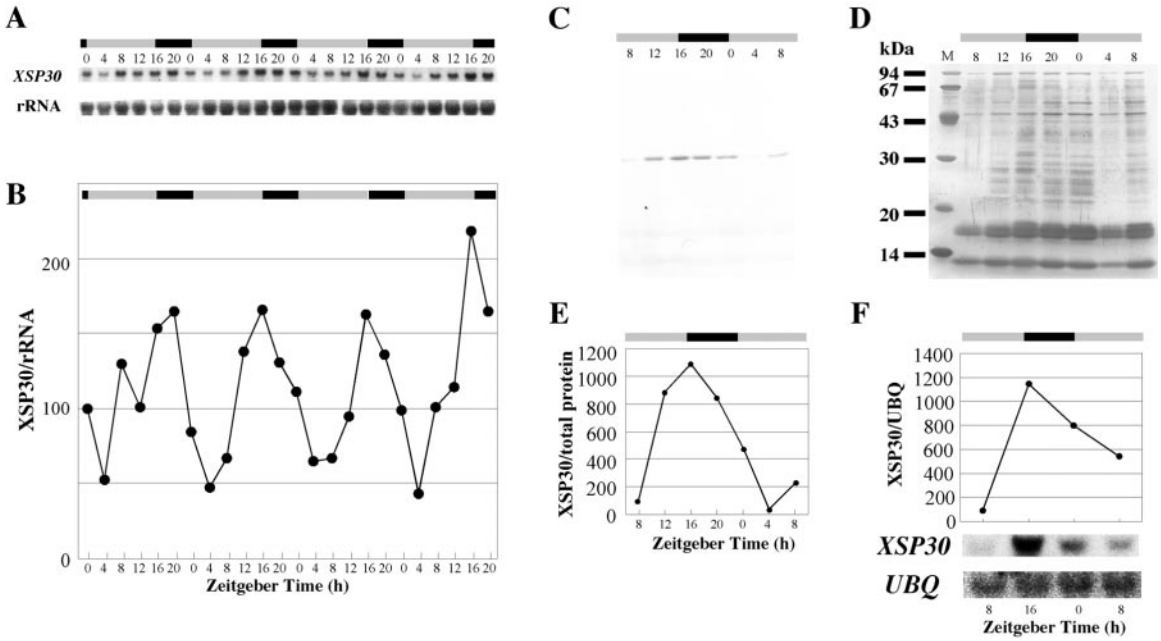

Figure 2. Diurnal regulation of *XSP30* gene expression and XSP30 content of xylem sap. A and B, Time course of *XSP30* expression. Cucumber plants were grown under a 16-h light/8-h dark photoperiod. Total RNA (10 μg lane^{-1}) was extracted from the roots of seedlings every 4 h, beginning on the 11th d after sowing. RNA samples were subjected to RNA gel-blot analysis with *XSP30* cDNA and rRNA probes. Shown are the original autoradiograph in A and the ratio of the intensity of hybridization of *XSP30* and rRNA in B. C through E, Detection of XSP30 protein in xylem sap. Xylem sap was collected at 4-h intervals from stems of cucumber plants 30 d after sowing. The proteins in equal volumes of sap (5 μL lane^{-1}) were separated by SDS-PAGE and detected with anti-XSP30 serum on nylon membranes (C) or silver stained (D). The ratio of the intensity of the *XSP30* signal and staining of the total proteins is shown in E. The *XSP30* gene expression in cucumber roots, 30 d after sowing, is shown in F. Total RNA (10 μg lane^{-1}) was extracted from the roots of seedlings every 8 h, beginning on d 30 after sowing. RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and a ubiquitin fragment as probes. The original autoradiograph and the ratio of the intensity of hybridization to the *XSP30* and ubiquitin transcripts are shown. Dawn was defined as zeitgeber time 0. The periods of light and dark are indicated as shaded and black bars, respectively.

times 40, 64, and 88 on the 2nd, 3rd, and 4th d after removal, respectively. In contrast, the *XSP30* mRNA level was significantly lower in plants from which only the first leaf was removed (Fig. 5, B and C). These results suggest that mature leaves are required for the high amplitude of the diurnal expression of *XSP30* in cucumber roots, and that translocatable signals, possibly including plant hormones such as GAs, auxins, and brassinosteroids may be involved in this process.

Oscillating *XSP30* Expression Is Possibly Related to Leaf Gibberellins

A gibberellin biosynthesis inhibitor (uniconazole-P), an auxin polar transport inhibitor (2,3,5-triiodobenzoic acid), or a brassinosteroid biosynthesis inhibitor (brassinazole; Asami et al., 2000) were applied to leaves, and their effects on *XSP30* expression in roots were analyzed. Only uniconazole-P significantly affected the expression of *XSP30* in roots (data not shown). Therefore, we examined the relationship between gibberellin and *XSP30* gene expression in roots. GA was sprayed on the cotyledons and shoot apices of cucumber seedlings after removal of the first leaf. Treatment with GA fully restored the high-amplitude rhythmic expression of *XSP30* in the roots of these seedlings (Fig. 6, A and C). In contrast, in control seedlings treated with water, *XSP30* expression failed to return to this expression pattern (Figs. 6, B and C, and 5, B and C). The data in Figure 6 were Fourier transformed and the periods and the RAE were estimated as 26.74 ($\pm$0.962) hours and 0.311 in plants treated with water and 27.32 ($\pm$0.564) hours and 0.256 in plants treated with GA.

To examine the involvement of endogenous gibberellins in activating *XSP30* expression, uniconazole-P was applied to the shoots of intact plants. This resulted in a dramatic decrease in *XSP30* expression within the 48 h after the uniconazole-P treatment (Fig. 7, A and C), but rhythmic expression with a lower amplitude was observed during the 3rd and 4th d. Simultaneous application of GA_3 with uniconazole-P resulted in no change in the oscillation pattern, with maximum expression at time 64 and 88 on the 3rd and 4th d, respectively (Fig. 7, B and C). The data in Figure 7 were Fourier transformed and the periods and the RAE were estimated as 22.82

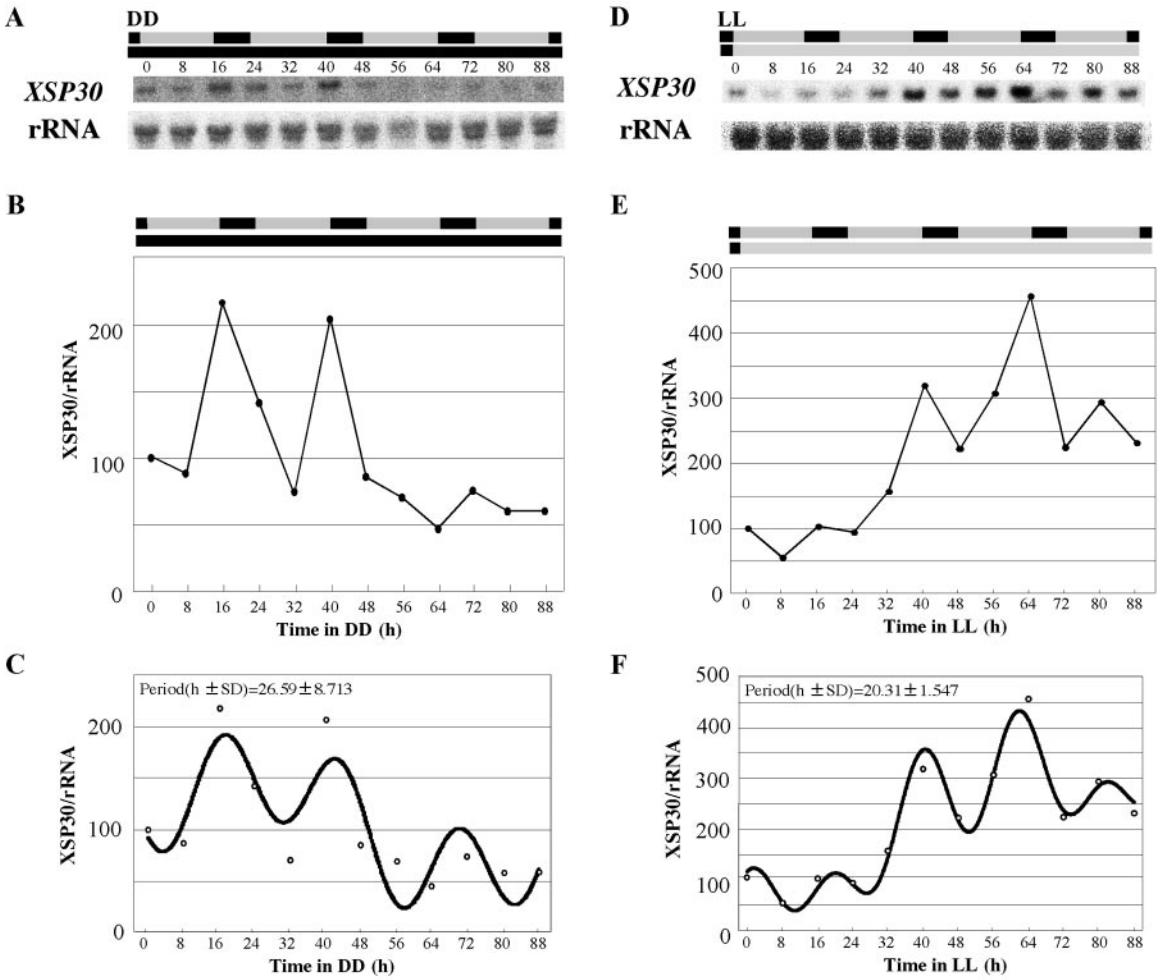

Figure 3. Expression of *XSP30* under DD or LL. Thirteen-day-old seedlings grown under a 16-h light/8-h dark photoperiod were transferred to DD (A–C) or LL (D–F). Total RNA was extracted every 8 h from roots of treated seedlings. RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and rRNA as probes. The original autoradiograph is shown (A and D), as well as the ratio of the intensity of hybridization to *XSP30* and rRNA (B and E). The data were Fourier transformed and the period were estimated using Fast Fourier Transform-Non-Linear Least Squares program as described (Plautz et al., 1997; C and F). The shaded and black bars indicate the original periods of light and dark, respectively.

(±0.909) hours and 0.366 in plants treated without GA and 23.79 (±1.224) hours and 0.341 in plants treated with GA. To elucidate whether gibberellin transported from the shoot affects the expression of *XSP30* in roots, 10^{-4} M GA_3 was supplied to the roots of uniconazole-P-treated plants. The application of GA_3 to roots did not reverse the effects of uniconazole-P (data not shown). These results suggest that gibberellins produced in leaves are involved in the control of the diurnally oscillating pattern of *XSP30* gene expression in cucumber roots.

DISCUSSION

Diurnal Control of *XSP30* Gene Expression in Roots

In plants, leaf movements, hypocotyl elongation, stomatal opening, and floral induction are controlled by a circadian clock (McClung, 2001). These effects are likely the result of the circadian rhythm-dependent expression of numerous plant genes (Somers, 1999). Among 8,200 genes examined using microarrays of Arabidopsis genes, approximately 6% exhibited circadian expression patterns in steady-state mRNA levels (Harmer et al., 2000). Clock-controlled expression of these genes was tested using young seedlings 7 to 10 d after germination. Because roots are spatially separated from leaves, where the circadian rhythms are adjusted, the effects of circadian rhythms on roots have not been fully studied. However, a few examples of diurnally oscillating gene expression in roots have been reported. The genes that encode certain enzymes involved in nitrogen fixation are expressed in a diurnal pattern (Lejay et al., 1999; Abd-el Baki et al., 2000), as are the histone H1 and chalcone synthase transcripts (Corlett et al., 1998; Thain et al., 2002). Apart from the report that

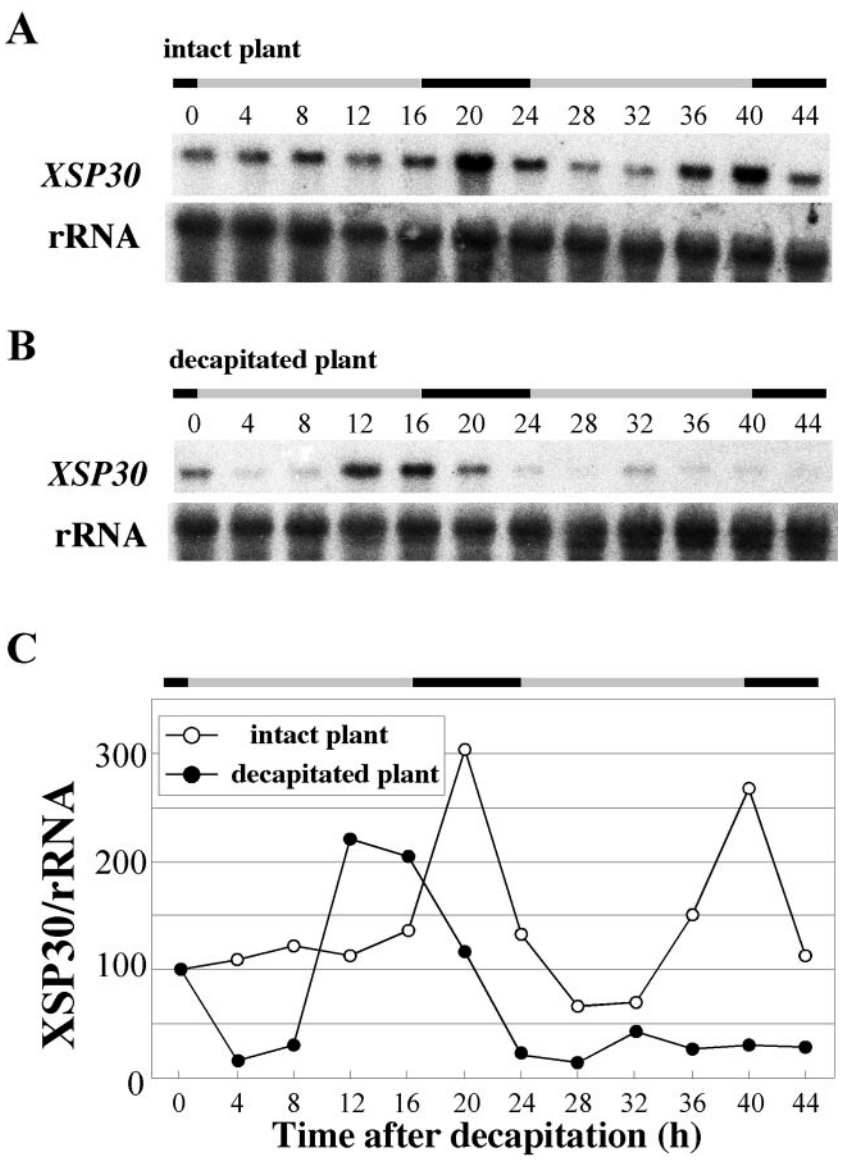

Figure 4. Effect of decapitation of aboveground organs on *XSP30* expression in roots. Total RNA (10 μg lane^{-1}) was extracted every 4 h from the roots of 15-d-old cucumber plants grown under a 16-h light/8-h dark photoperiod from intact (A) or decapitated plants (B). Root RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and rRNA as probes. The original autoradiograph is shown (A and B), as well as the ratio of the intensity of hybridization to *XSP30* and rRNA (C). The periods of light and dark are indicated as shaded and black bars, respectively.

the diurnal expression pattern of the nitrate transporter gene *Nrt* in roots is caused by photoassimilates (Lejay et al., 1999), there has been no report on diurnal control mechanisms in roots. In this report, we have demonstrated diurnal oscillations in the expression of *XSP30* in cucumber roots (Fig. 2). XSP30 protein levels also oscillated after the expression pattern of the gene (Fig. 2). The oscillation in gene expression was sustained for two or three cycles under continuous DD and LL conditions (Fig. 3). This result suggests that the expression of *XSP30* in roots is controlled by a circadian clock.

Possible Fine Tuning of *XSP30* Expression by GA

The regulation of the period, which is the time after which a definite phase of the oscillation reoccurs, and the amplitude, which is the difference between maximum value and mean value in a sinusoidal oscillation, is quite important for the proper functioning of clock-controlled genes in various biological processes. For example, the expression of the floral activator gene *CONSTANS* (*CO*) is controlled by a circadian clock. The diurnal pattern of *CO* mRNA accumulation is different in inductive (long days) versus noninductive (short days) photoperiods (Suarez-Lopez et al., 2001). Several circadian rhythm mutants affect *CO* expression in ways that correlate with their early- or late-flowering phenotypes. For example, in short day-grown *elf3* and *elf4* mutants, the level of *CO* mRNA is increased at all time points as compared with wild-type plants (Suarez-Lopez et al., 2001; Doyle et al., 2002). Increased *CO* expression is consistent with the early flowering phenotypes of *elf3* and *elf4*.

We report evidence that gibberellin is involved in the expression of *XSP30* in roots (Figs. 6 and 7). One

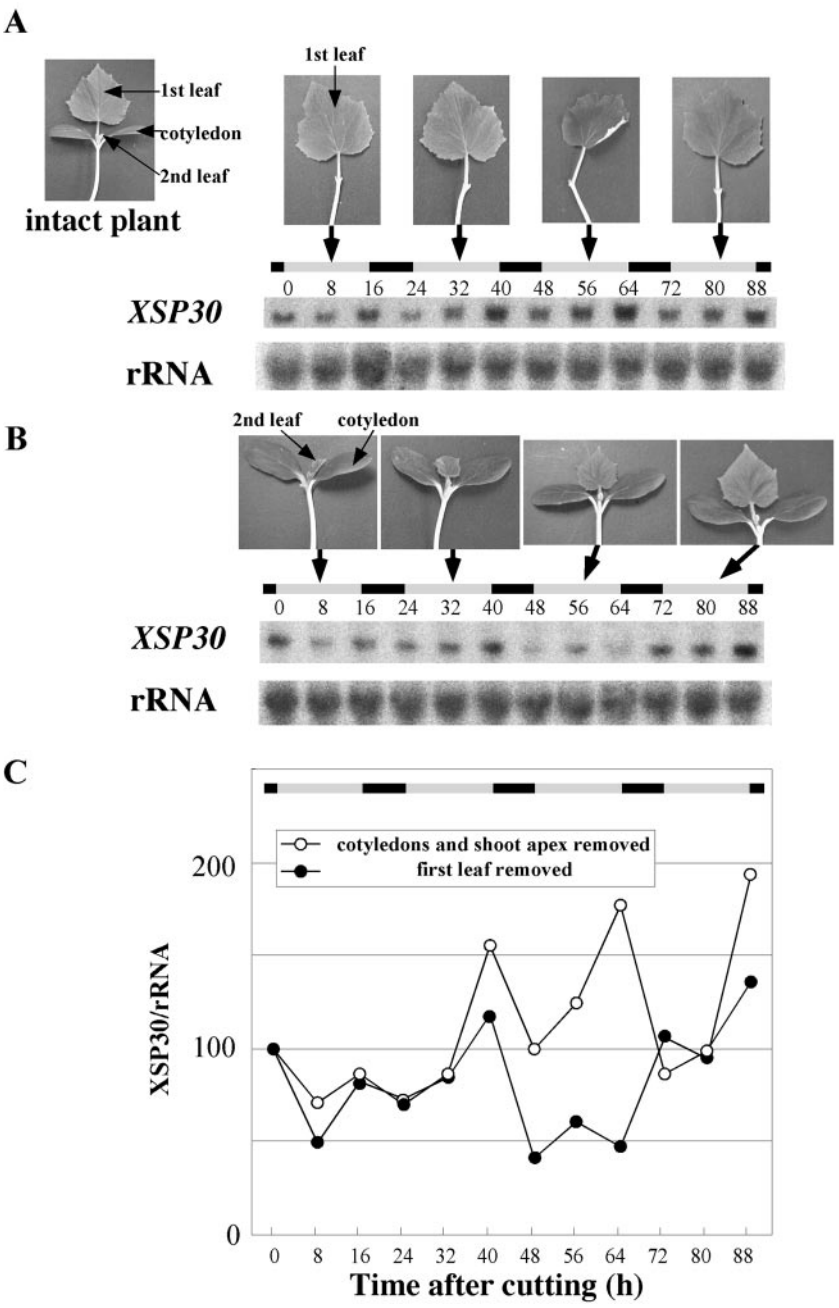

Figure 5. Effect of leaf removal on *XSP30* expression in roots. The cotyledons plus the shoot apex (A), or the mature first leaf (B), were removed from 13-d-old seedlings, and the roots were collected every 8 h for RNA extraction. Root RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and rRNA as probes. The original autoradiograph is shown (A and B), as well as the ratio of the intensity of hybridization to *XSP30* and rRNA (C). The periods of light and dark are indicated as shaded and black bars, respectively.

of the major sites of production of gibberellin, which controls various aspects of plant development, is the leaf (Yamaguchi and Kamiya, 2000). Gibberellin production in pea (*Pisum sativum*) and pumpkin (*Cucurbita pepo*) is thought to be greatest in young expanded leaves (Smith et al., 1992, 1998). It is possible that this may also be true in cucumber seedlings, and that the decrease in *XSP30* expression after removal of the first leaf is caused by the decrease in the endogenous levels of gibberellin (Figs. 4 and 5).

The effect of gibberellin on the regulation of *XSP30* gene expression appears to be independent of the possible circadian regulation. In other words, the diurnal oscillation pattern of *XSP30* expression does not appear to be controlled by the oscillating level of endogenous gibberellin, because application of high concentrations of GA did not disrupt the diurnal expression pattern of *XSP30* (Figs. 6 and 7). GA is likely involved in the regulation of the amplitude or maximum expression level of *XSP30*, but not the periodicity, which might be controlled by a circadian clock (Figs. 2 and 3).

It is possible to interpret our results based on this hypothesis. The *XSP30* expression cycles because the clock, if it is involved, may be gating the responsiveness of *XSP30* expression to GA, because GA (or

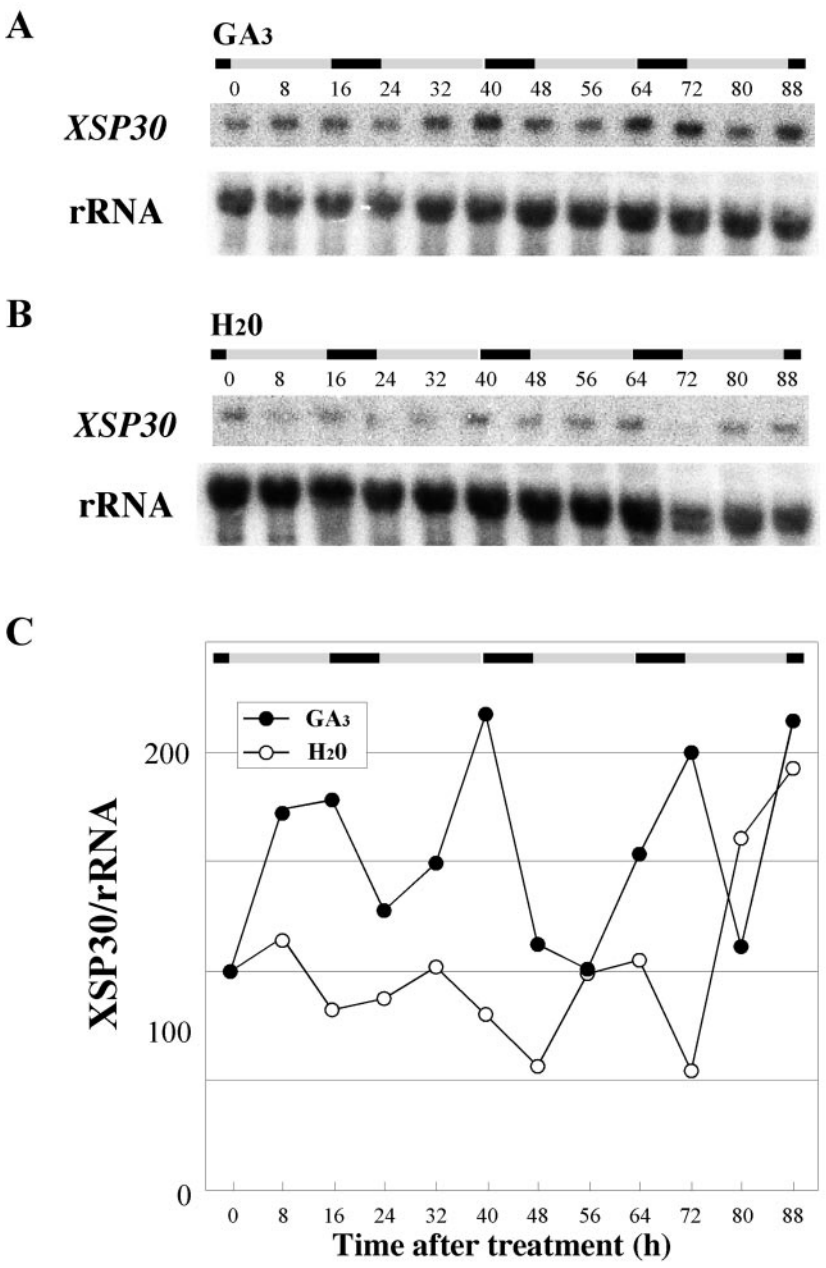

Figure 6. Effects of gibberellin on *XSP30* expression in roots. Mature first leaves were removed from 13-d-old seedlings, and 2×10^{-4} M GA_3 (A) or water (B) was applied every 2 d to the cotyledons and shoot apex. Roots were collected every 8 h for RNA extraction. Root RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and rRNA as probes. The original autoradiograph is shown (A and B), as well as the ratio of the intensity of hybridization to *XSP30* and rRNA (C). The periods of light and dark are indicated as shaded and black bars, respectively.

some signal downstream of GA) transport to the roots may oscillate over the diurnal cycle, or both. Diurnal GA synthesis in sorghum (*Sorghum bicolor*) partly supports this idea (Foster and Morgan, 1995). The overall expression pattern of *XSP30* seems to be fine-tuned by GA and also, possibly, by a circadian rhythm.

We tested whether inhibitors of gibberellin, auxin, and brassinosteroid biosynthesis or transport affected the diurnal oscillation pattern of *XSP30* gene expression, and we found that only the GA biosynthesis inhibitor, uniconazole-P, had a significant effect in our experimental conditions (Fig. 7 and A. Odah and S. Satoh, unpublished data). However, there is a possibility that signals other than gibberellin also participate in the diurnal expression pattern of *XSP30* in roots. One signaling molecule candidate is IAA, which controls the rate of internodal elongation, and the endogenous levels of which fluctuate in a circadian rhythm in Arabidopsis (Jouve et al., 1999). Although no circadian rhythm in IAA levels has been demonstrated in cucumber, we postulate that such a rhythm might occur. In pea, removal of the apical bud inhibits certain GA-controlled processes in the remaining stem tissue, such as elongation, and the level of GA_1 is also dramatically reduced. The effect of the apical bud can be replaced by applying auxin to the cut stump of the decapitated stem; this procedure restores the endogenous GA_1 level and the elongation (Ross et al., 2000). It is possible that auxin may

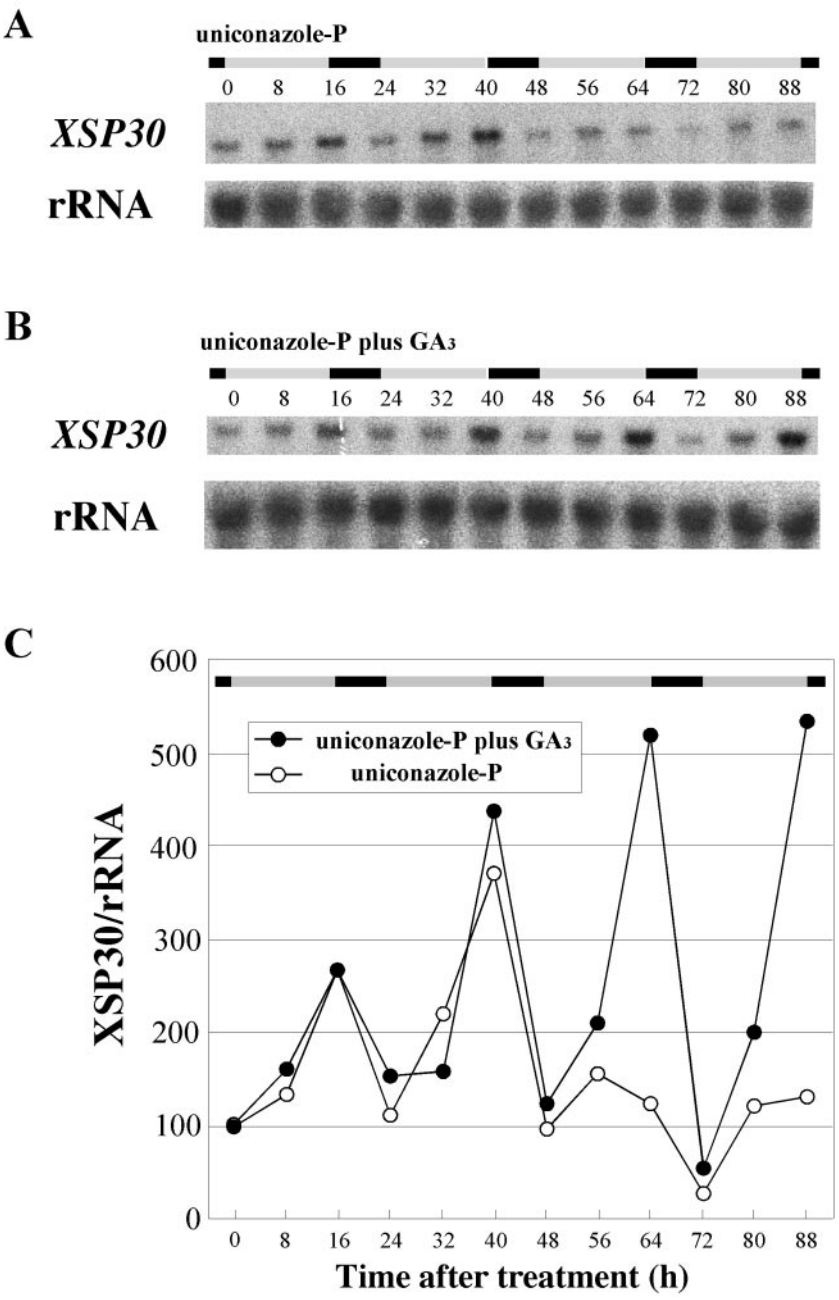

Figure 7. Effect of uniconazole-P and gibberellin on *XSP30* expression in roots. Uniconazole-P (10^{-4} M; A) or 10^{-4} M uniconazole-P plus 2×10^{-4} M GA_3 (B) was applied to the shoot, including the cotyledons, first leaf, and shoot apex, of 13-d-old seedlings. Roots were collected every 8 h for RNA extraction. Root RNA samples were subjected to RNA gel-blot analysis with an *XSP30* cDNA and rRNA as probes. The original autoradiograph is shown (A and B), as well as the ratio of the intensity of hybridization to *XSP30* and rRNA (C). The periods of light and dark are indicated as shaded and black bars, respectively.

act similarly in cucumber seedlings, with the effects of GA replaced by applying auxin. IAA, or other signals produced in the shoot in a diurnal pattern, may be translocated to roots to establish a diurnal rhythm in underground tissues. Further analysis using inhibitors of IAA and other plant hormones at different concentrations will give us more information on this process.

The overall expression pattern of *XSP30* may be fine-tuned by GA and by the circadian clock. This is quite similar to the growth pattern of the Arabidopsis inflorescence stem in which the circadian clock modulates auxin transport, auxin sensitivity, or both, to yield a rhythm in the rate of elongation (Jouve et al., 1998, 1999). The *Lhc* (also called *CAB*) mRNA levels exhibit a robust circadian pattern under free-running conditions in LL, despite the continuous presence of phytochrome in the active form (Millar and Kay, 1996). This observation led to the conclusion that the circadian clock must negatively regulate (or "gate") the light-induced transcription of the *Lhc* and other clock-regulated plant genes, and this is called the gating hypothesis (Millar and Kay, 1996). Rhythmic alteration in the sensitivity of stomata to extracellular signals are also reported (Webb, 1998). The response of the stomata to these signals (the acute response)

depends on the phase of the circadian cycle at which the signal is applied. Thus, the circadian clock can modulate (or gate) the acute responses of stomata (Webb, 1998).

Possible Physiological Function(s) of *XSP30*

Several examples of the effects of gibberellins applied directly to roots have been reported (Tanimoto, 1994; Yaxley et al., 2001). In the present study, gibberellins applied to the shoot affected *XSP30* gene expression in roots (Figs. 6 and 7), suggesting that gibberellins are translocated to the roots or that gibberellins induce the production of a mediator in the shoot that causes a response in the roots. Supplying 10^{-4} M GA directly to the roots of uniconazole-P-treated plants did not affect *XSP30* expression (data not shown), suggesting that mediators are involved in the transmission of gibberellin signals to roots. An alternative possibility is that GA applied directly to roots does not produce a physiological reaction detectable under our experimental conditions. However, in vegetative pea plants, grafting studies have indicated that endogenous GA_1 is essentially immobile. In contrast, applied GA_1 is very mobile, moving readily throughout the plant. In this sense, GA_1 applied to pea plants behaves quite differently from endogenous GA_1 (Proebsting et al., 1992). A detailed analysis to assess whether the effect(s) of the endogenous and applied GAs are similar will be performed using genetic and transgenic approaches.

The *XSP30* promoter directed *GUS* expression specifically in the xylem parenchyma and pericycle cells in the central cylinder of mature transgenic hairy roots (Fig. 1). Thus, XSP30 is likely to be produced in the vascular tissues of mature roots. *CRGRP-1* and *CRGRP-2*, genes that encode other xylem sap proteins, are also expressed in xylem parenchyma cells in the central cylinder of cucumber roots, and these proteins are transported to aboveground organs via xylem vessels (Sakuta et al., 2000). Production of proteins in the central cylinder of the root is probably necessary for the loading of macromolecules into xylem vessels. For example, the gene encoding the 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, the gene for a transporter involved in the xylem loading of boron was reported to be expressed in the pericycle cells of Arabidopsis roots (Takano et al., 2002).

In summary, our results suggest that GA originating in the leaf, or some signal downstream of GA, provides important inducer(s) to generate high amplitude diurnal oscillations of the *XSP30* gene expression. A circadian clock might be involved in the rhythmic expression of the gene, and both of these factors may optimize and fine-tune *XSP30* gene expression in xylem parenchyma and pericycle cells of mature cucumber roots (Fig. 2). Any reciprocal regulation between circadian clocks and GA functions is still unclear. Possible clues include the expression of the GA20 oxidase gene being controlled by daily light/dark cycles, and the involvement of PHOR1, a GA-signaling mediator, in the photoperiodic control of tuber formation in potatoes (Carrera et al., 1999). Red light controls GA turnover by up-regulating the gene expression of GA2ox2, which converts GA_1 to GA_8 in pea (Reid et al., 2002). Therefore, there is an alternative possibility that the clock might affect GA turnover as well as synthesis. Analysis of endogenous GA level and *XSP30* expression under the light cycle condition will elucidate the possible regulation between circadian clocks and GA functions. Recently, we have found that XSP30 has lectin activity and recognizes the *N*-linked glycans of glycoproteins in cucumber leaves (Oda et al., 2003). One attractive possibility is that the XSP30 protein is produced in underground roots and is delivered to leaves, where it could regulate biological processes through direct binding to the *N*-acetylglucosamine sugar chains of glycoproteins. In this sense, XSP30 may play a role in the communication between roots and shoots.

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) under white fluorescent light (40 μmol m^{-2} s^{-1}) with a 16-h light/8-h dark photoperiod at 28°C.

RNA Gel-Blot Analysis

Full-length cDNA probes for *XSP30* (DDBJ; AB025717), ubiquitin, and rDNA were prepared using the PCR. Total RNA (10 μg $lane^{-1}$) was isolated as described previously (Sakuta et al., 1998) from the combined roots of 10 treated plants. RNA gel-blot analysis was performed using a ^{32}P-labeled probes and a BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo). Signal intensity was quantified using Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film). Values were represented relative to the values of samples at time 0 after normalization to the rRNA controls. Values at time 0 were shown as 100. All RNA gel blots were performed at least twice and usually with independent samples (see Supplemental Figs. 2 and 3). Fourier transforms, period, and RAE estimates were obtained using the Fast Fourier Transform-Non-Linear Least Squares program as described (Plautz et al., 1997). The RAE is the value of the amplitude error estimate divided by the value of the most probable amplitude estimate. RAE can range from a value of zero for an infinitely well-determined rhythmic component (zero error) to a value of one, theoretically, for a minimally determined rhythmic component (error in the amplitude equals the amplitude value itself).

Cloning of the *XSP30* Promoter

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

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 was cloned upstream of, and in frame with, the GUS coding sequence in a modified pBI121 vector, which contains the cauliflower mosaic virus 35S promoter fused 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 excised surface of 7-d-old cucumber plant cotyledons with *A. tumefaciens* harboring the above plasmid. 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 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-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 et al., 1987). Stained samples were fixed in 2% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.0) at room temperature for 4 h and were then dehydrated by passage through an ethanol series. Dehydrated samples were embedded in Technovit 7100. Thin serial sections were cut with a tungsten knife using a microtome (RM-2415; Leica, Wetzlar, Germany). The sections were expanded in drops of water on glass slides (APS-coated microglass slides; Matsunami Glass Industries, Kishiwada, Japan) and were dried at 50°C.

SDS-PAGE of Xylem Sap Proteins

Xylem sap was collected, as described previously (Sakuta et al., 1998), at 4-h intervals from the cut stems of 1-month-old cucumber plants, at 4 h before dusk, at dusk, at midnight, at dawn, at 4 h after dawn, and at midday. The samples were immediately frozen in liquid nitrogen and stored at −20°C. Xylem sap samples (5 μL $lane^{-1}$) 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] bromphenol blue, and 10% [v/v] β-mercaptoethanol), boiled for 10 min in a water bath, and then centrifuged at 15,000*g* for 5 min. The resulting supernatants were subjected to SDS-PAGE, as described by Laemmli (1970). After electrophoresis, the gels were stained with silver (Sil-Best stain; Nacalai Tesque, Kyoto) or subjected to immunological detection (Sakuta et al., 1998). To determine the amount of total protein, the entire intensity of each lane staining was quantified using Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film).

Immunological Detection of *XSP30*

Proteins were transferred from SDS-PAGE gels to nitrocellulose filters (ADVANTEC, Tokyo) in 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol at 40 V for 2 h (Gershoni and Palade, 1982). The filters were incubated in phosphate-buffered saline (PBS) with 2% (w/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% (v/v) Tween 20 in PBS and was agitated in a solution of 1,000-fold diluted goat anti-rat immunoglobulin G horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 1 h. Proteins were visualized by incubating the filter in PBS containing 0.03% (w/v) 3,3-diaminobenzidine and 0.003% (v/v) H_2O_2. The intensity of the signals was quantified using Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film).

Decapitation and Elimination of Aboveground Organs

The shoots of seedlings were removed at dawn with a razor blade at the middle of the hypocotyl, 15 d after sowing. Roots were collected from these seedlings every 4 h for RNA preparation. The first leaf or the cotyledons plus the shoot apex of the cucumber plants was cut off, 13 d after sowing, at dawn with a razor blade, and the roots were collected every 8 h for RNA preparation.

Gibberellin and Uniconazole-P Treatments

GA_3 and uniconazole-P were dissolved in ethanol and the concentration of the solution was diluted 1,000-fold in 0.1% (v/v) Tween 20. The leaves and the shoot apex of 13-d-old plants were sprayed with 2×10^{-4} M GA_3, 10^{-4} M uniconazole-P, or, as a control, 0.1% (v/v) ethanol, each with 0.1% (v/v) Tween 20. Roots were collected every 8 h for RNA preparation.

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