Regulation of *VrXTH1* Expression in Mungbean

Hye Sup Yun[†,¶], Chian Kwon[1†,¶], Tae-Wuk Kim, Se-Hwan Joo, Myeon Haeng Cho[1], Bin Goo Kang[1], Soo Chul Chang[2]*, and Seong-Ki Kim*

Department of Life Science, Chung-Ang University, Seoul 156-756, Korea
[1]Department of Biology, Yonsei University, Seoul 120-749, Korea
[2]University College, Yonsei University, Seoul 120-749, Korea

Xyloglucan endotransglucosylase/hydrolases (XTHs) play roles in plant development by rearranging xyloglucan cross-links. Previously, we isolated a cDNA of XTH, *VrXTH1*, from mungbean that is thought to be associated with auxin-related growth. Here we report that several factors regulate the expression of *VrXTH1*. This gene is predominantly expressed in the elongating regions of seedlings. Environmental stimuli, such as light and temperature, also affect its transcript levels. Because calcium acts as a second messenger in many signal transduction pathways, we examined its involvement in the regulation of *VrXTH1* expression. Interestingly, the application of ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; a specific calcium chelator) repressed transcription, regardless of the presence of auxin. Furthermore, treatment with either A23187 (a calcium ionophore) or calcium increased gene expression, suggesting that changes in the amount of intracellular Ca^{2+} is important to the modification of transcript levels. Taken together, our results demonstrate that the expression of *VrXTH1* is closely related to plant growth and may be modulated by the concentration of cytosolic calcium.

Keywords: auxin, calcium, cell wall, plant growth, VrXTH1

Plants modify and rearrange their cell wall components to control cell shape and, ultimately, to regulate growth (Cosgrove, 1993). Xyloglucans, one of the main components of the cell wall, binds adjacent cellulose microfibrils to form bridges between the microfibrils and the matrix (Hayashi, 1989; McCann et al., 1990). This interaction between cellulose and xyloglucan determines the plasticity of the cell wall (Chanliaud et al., 2002); the modification and rearrangement of these components, including xyloglucan, are required for cell elongation and division (Carpita and Gibeaut, 1993). Therefore, xyloglucan endotransglucosylase/hydrolases (XTHs) are considered key enzymes in plant development (Yokoyama and Nishitani, 2001).

The XTHs are a family of enzymes thought to reconstruct cell wall structure by cleaving and rejoining xyloglucans (Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992). They are encoded by multigene families (Nishitani, 1997; Rose et al., 2002; Yokoyama et al., 2004). To date, 33 XTH genes in *Arabidopsis* and 29 XTH genes in rice have been classified into two subfamilies based on sequence relationships (Rose et al., 2002; Yokoyama et al., 2004). However, despite their sequence similarities, differential regulation of XTH gene expression indicates that each protein has a specific function (Xu et al., 1996; Akamatsu et al., 1999; Yokoyama and Nishitani, 2001; Nakamura et al., 2003; Vissenberg et al., 2005). Furthermore, the gene expression of some XTHs is up-regulated by such growth-stimulating hormones as auxin, gibberellic acid (GA), and brassinosteroid (BR) (Zurek and Clouse, 1994; Xu et al., 1996; Catala et al., 1997; Schunmann et al., 1997; Yun et al., 2005).

Auxin plays a central role in cell division, expansion, and elongation; tropisms; abscission; and senescence, ripening, and flowering (Davies, 1995). We previously isolated an auxin-inducible XTH gene, *VrXTH1*, from mungbean and found that its expression is up-regulated by auxin and BRs, but down-regulated by abscisic acid, an antagonist of auxin (Yun et al., 2005). In the current study, we investigated the effects of light, temperature, and calcium on *VrXTH1* expression in whole seedlings and individual organs.

MATERIALS AND METHODS

Plant Materials

Seeds of mungbean (*Vigna radiata* L.) were soaked overnight in aerated tap water. Seedlings were grown on 0.5% (w/v) agar plates in the dark at 27°C with 100% relative humidity. For our chilling treatment, dark-grown seedlings were transferred to a cold room maintained at 4°C for 24 h. The illumination treatment included exposing the seedlings to continuous light or darkness. To study the effects of auxin, calcium, A23187 (a calcium ionophore), or EGTA (a calcium chelator), we excised hypocotyl segments (1-cm-long; 10 per treatment) at 0.5 cm below the seedling hooks, then incubated them for various time periods at 27°C under darkness in 5 mM potassium phosphate buffer (pH 6.8).

Total RNA Extraction

Total RNA was extracted by a modified phenol-SDS

*Corresponding author; fax +82-2-313-0328, +82-2-820-5206
e-mail schang@yonsei.ac.kr, skkimbio@cau.ac.kr
†These first two authors contributed equally to this work.
¶Current address: Department of Plant Microbe Interactions, Max Planck Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, D-50829, Cologne, Germany

Abbreviations: BR, brassinosteroid; CRK, calcium-dependent protein kinase-related kinase; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GA, gibberellic acid; IAA, indole-3-acetic acid; XTH, xyloglucan endotransglucosylase/hydrolase.

method (Sambrook et al., 1989). Samples were ground to a powder with a mortar and pestle under liquid nitrogen, and the powder was re-suspended with an RNA extraction buffer (0.1 M Tris-Cl pH 9.0, 0.1 M NaCl, and 1% SDS). An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added and vigorously mixed. After centrifugation at 4500 rpm for 15 min at 4°C, the supernatant was transferred to a new tube and total RNA was pelleted by mixing with 1/20 volume of 5 M NaCl and 2.5 volume of ethanol. The pellet was dissolved in TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA), and total RNA was pelleted again by mixing with 1/3 volume of 10 M LiCl. After the pellet was finally dissolved in TE buffer, the concentration of total RNA was measured spectrophotometrically.

Northern Blot Analysis

Total RNA, extracted as described above, was fractionated on a 1% (w/v) formaldehyde-agarose gel and blotted on Hybond N+ nylon membranes (Amersham Pharmacia Biotech, USA). Blots were hybridized with a ^{32}P-labeled *VrXTH1* or *VrCRK1* probe as previously described (Kwon et al., 2004; Yun et al., 2005). Signals on the blot were analyzed with BAS-2500 Film (Fuji, Japan).

RESULTS AND DISCUSSION

Expression of *VrXTH1* Is Closely Associated with Hypocotyl Elongation

To study the role of XTHs in plant growth and development, we have previously isolated an XTH1 gene, *VrXTH1*, from mungbean (Yun et al., 2005). *VrXTH1* is preferentially expressed in the elongating region of the hypocotyl (Fig. 1; Yun et al., 2005). Its expression is stimulated by auxin and brassinosteroids, but inhibited by abscisic acid, an auxin-antagonizing hormone (Yun et al., 2005). Here, we investigated the expression of *VrXTH1* in the growing roots and hypocotyls of mungbean, and found the same expression pattern in the hypocotyls (Fig. 1). In the roots, however, expression occurred only in the elongation zone (see region 'e' in Fig. 1) and was barely detectable in the root maturation region and at the junction between hypocotyl and root (respectively, 'c' and 'd' in Fig. 1). *VrXTH1* transcript levels were significantly lower in the root than in the hypocotyl, implying that VrXTH1 acts mainly on elongation of the latter.

We also examined *VrXTH1* expression under several conditions known to affect mungbean development. For example, seedlings grown continuously under darkness showed steady hypocotyl elongation and a constant level of *VrXTH1* transcripts (Fig. 2A). In contrast, when dark-grown seedlings were moved to an illuminated environment, expression as well as hypocotyl growth was diminished (Fig. 2A). Temperature also affected gene expression in dark-grown seedlings. Transfer to a 4°C dark chamber caused arrested hypocotyl development (data not shown) and repressed the expression of *VrXTH1* (Fig. 2B).

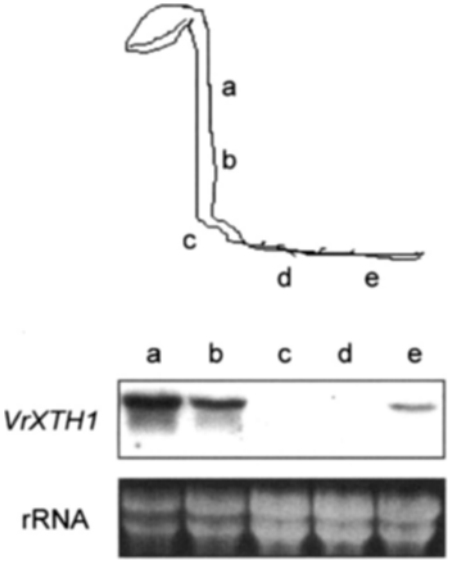

Figure 1. *VrXTH1* expression patterns from intact and segmented, dark-grown mungbean seedlings: a and e, elongating regions of hypocotyl and root, respectively; b and d, maturing regions of hypocotyl and root, respectively; c, junction between hypocotyl and root. Total RNA was extracted and subjected to Northern blot analysis.

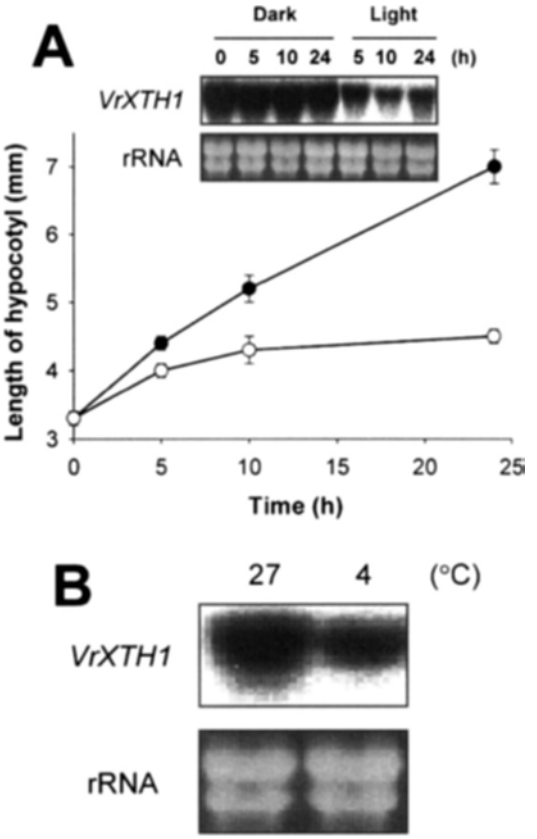

Figure 2. Effects of light and temperature on transcript levels of *VrXTH1*. **A.** Inhibition of hypocotyl growth by mungbean seedlings grown under darkness for 2 d, then transferred to continuous light (open circle) or dark conditions (closed circle) and incubated for indicated times. Hypocotyl lengths were measured from 10 to 15 seedlings. Total RNA was extracted from elongating region of hypocotyls at indicated times and subjected to Northern blot analysis with *VrXTH1* probe (inset). **B.** Dark-grown seedlings were transferred to dark cold room maintained at 4°C, and incubated for 24 h. Total RNA was extracted from elongating region of hypocotyls corresponding to 'a' in Figure 1, and subjected to Northern blot analysis.

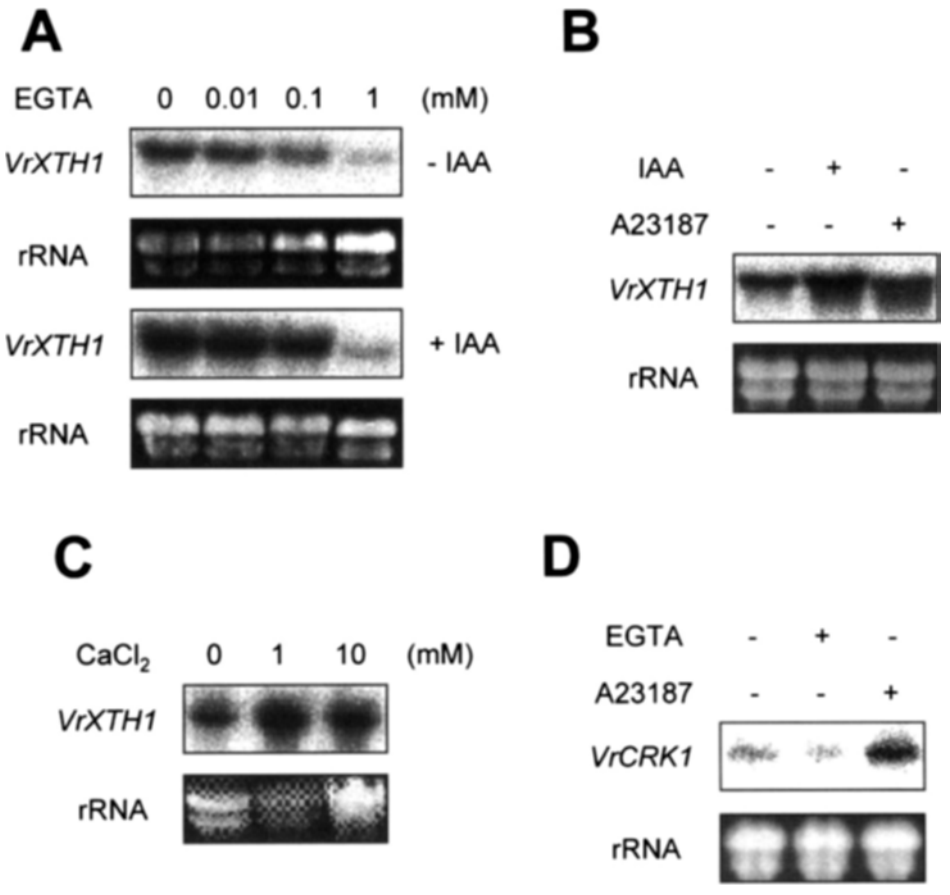

Figure 3. Effect of calcium modulators on *VrXTH1* expression. **A**, **B**, and **C**, Ten hypocotyl segments were incubated with various amounts of ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, EGTA (**A**), 50 mM A23187 (**B**), or $CaCl_2$ (**C**) in presence or absence of IAA for 16 h at 27°C under darkness. **D**, Ten hypocotyl segments were incubated with 10 mM EGTA or 5 mM A23187 for 16 h at 27°C under darkness. After incubation, total RNA was extracted and subjected to Northern blot analysis with *VrXTH1* or *VrCRK1* probe.

Auxin, gibberellic acid (GA), and BR enhance the gene expression of some XTHs (Zurek and Clouse, 1994; Xu et al., 1996; Catala et al., 1997; Schunmann et al., 1997; Nakamura et al., 2003; Romo et al., 2005; Vissenberg et al., 2005), while GA also stimulates the enzyme activity of some XTH proteins (Potter and Fry, 1994; Smith et al., 1996). Therefore, based on our current results (Fig. 1, 2), we propose that it is likely that VrXTH1 is associated with the regulation of hypocotyl elongation in mungbean, possibly by modifying cell wall structure through the cleaving and rejoining of cross-links between xyloglucan and microfibrils.

Calcium Modulates the Expression of *VrXTH1*

Calcium may be involved in auxin signaling as a second messenger. In corn, external auxin causes changes in the concentration of cytosolic calcium (Felle, 1988; Gehring et al., 1990) while, in pea, a calcium chelator, ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and a calcium channel blocker, D-600, inhibit auxin-induced segment elongation (Reddy et al., 1988). It was also reported that auxin-induced ethylene production was inhibited by verapamil, a calcium channel blocker (Lee and Kang, 1989). Because *VrXTH1* is an auxin-inducible gene (Yun et al., 2005), we tested whether the calcium ion is involved in regulating expression by exogenously applying EGTA to deplete the cellular calcium ions in our hypocotyl segments. Interestingly, EGTA decreased the transcript level of *VrXTH1* in a dose-dependent fashion, regardless of the presence of auxin (Fig. 3A). We speculated that EGTA may chelate the calcium present in the cell wall because it is well known that its concentration is much higher in the cell wall than in the cytoplasm. Therefore, we also tested whether the import of external calcium would drive the up-regulation of *VrXTH1* mRNAs. In hypocotyl segments treated with A23187, a calcium ionophore, the transcript level of *VrXTH1* increased even without auxin, with the degree of this enhancement being almost the same as that seen with our auxin treatment (Fig. 3B). We also examined the effect of exogenously applied calcium on *VrXTH1* expression. When Ca^{2+} was added to the incubation medium containing hypocotyl segments, transcripts increased without auxin (Fig. 3C), indicating that expression is regulated by the elevation in intracellular calcium concentration. To determine whether this observed calcium effect was limited to *VrXTH1*, we analyzed the transcript level of *VrCRK1*, another auxin-inducible gene for the calcium-dependent protein kinase-related kinase (Kwon et al., 2004). Treatment with either EGTA or A23187 had a similar effect on the expression of *VrCRK1* as

had been observed with *VrXTH1* (Fig. 3D). This indicates that changes in the level of intracellular calcium regulate the expression of both auxin-inducible genes. Nevertheless, although this increase up-regulated the expression of *VrXTH1* (Fig. 3A, B, C), its expression was down-regulated by our cold treatment, which is known to elevate cytosolic calcium (Fig. 2B) (Mahajan and Tuteja, 2005). The expression pattern of *VrXTH1* is also very similar to that of *At-XTH4* (previously named *EXGT-A1* or *EXT*) (Xu et al., 1996), thereby demonstrating that alterations in intracellular Ca^{2+} levels because of chilling may be differentially transduced, possibly by calmodulins or other calcium-binding proteins.

In conclusion, functioning of *VrXTH1* seems to be closely related to plant growth. This is supported by observations that its expression is regulated by growth-modifying stimuli, e.g., light, chilling, and hormones (Yun et al., 2005). In addition, changes in the mRNA level of *VrXTH1*, as induced by exogenous EGTA, A23187, or calcium, may prove that modifying the concentration of intracellular Ca^{2+} is important to the regulation of *VrXTH1*. Moreover, auxin may regulate its expression, possibly through modulation of the intracellular calcium level.

ACKNOWLEDGEMENT

This study was supported by a Korea Research Foundation Grant (KRF-2001-015-DP0483).

Received June 5, 2006; revised October 30, 2006; accepted January 17, 2007.

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