Two Azuki Bean XTH Genes, VaXTH1 and VaXTH2, with Similar Tissue-Specific Expression Profiles, are Differently Regulated by Auxin

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To gain insight into the functional diversity of the XTH (xyloglucan endotransglucosylase/hydrolase) gene family, we analyzed the expression profiles of two azuki bean genes, VaXTH1 and VaXTH2, which share a striking resemblance in their amino acid sequences. The two XTH genes exhibit essentially similar tissue-specific expression profiles, in that both mRNAs are found predominantly in the phloem fibers of growing internodes. However, their expression profiles are not identical. Whereas VaXTH1 is expressed nearer to the top of the internode than VaXTH2, which is found in the xylem, little or no expression of VaXTH1 is found in the xylem. Furthermore, they exhibit spatially divergent RNA distribution profiles along the internode, VaXTH1 being expressed nearer to the top of the internode than VaXTH2. This indicates their temporally divergent expression profiles during development of the phloem fiber. Indole-3-acetic acid (IAA) up-regulates both of the mRNA levels. However, this effect of IAA on the VaXTH1 gene is nullified in 0.25 M mannitol, which prevents cell expansion without affecting auxin action per se. In contrast, the IAA-induced up-regulation of the VaXTH2 gene is not affected by mannitol. Furthermore, fusicoccin, which promotes acidification and growth, up-regulates VaXTH1 expression, but not VaXTH2 expression. Thus, the two XTH genes are committed to different steps of the cell wall dynamics in the same cell type at different stages of phloem fiber development, and are regulated by IAA in different ways.

Keywords: Auxin — Azuki bean — Cell wall — EXGT/XET — Phloem fiber — XTH.

Abbreviations: EXGT, endoxylolucan transferase; FC, fusicoccin; VaXTH, Vigna angularis XTH; XET, xyloglucan endotransglycosylase; XTH, xyloglucan endotransglucosylase/hydrolase.

The nucleotide sequence of the VaXTH1 and VaXTH2 cDNAs have been deposited in GenBank under accession numbers AB086395 and AB086396, respectively.

Introduction

The plant cell wall is a dynamic structure composed of cellulose microfibrils and matrix polymers. In most flowering plants, including the azuki bean, the cell wall microfibrils are cross-linked by xyloglucans to form a cellulose–xyloglucan framework, in which xyloglucans function as tension-bearing bridges between microfibrils (Hayashi 1989, Carpita and Gibeaut 1993). This architecture is continually changing during cell differentiation, from cell-plate formation (Yokoyama and Nishitani 2001a) through to expansion of the primary cell wall (Nishitani and Masuda 1981, Nishitani and Masuda 1983, Tabuchi et al. 2001) and differentiation of the secondary cell wall (Nakashima et al. 2000, Shinohara et al. 2000). Despite good documentation of genes encoding proteins responsible for cell wall dynamics in plants, little is known about the regulatory mechanism by which the wall architecture is constructed, maintained, and modified during the cell expansion and differentiation processes.

Endoxylolucan transferases (EXGTs) or xyloglucan endotransglycosylases (XETs) are a class of enzyme capable of mediating the splitting and reconnection of xyloglucan cross-links. They are considered essential for cell wall dynamics, which encompass construction, modification, and maintenance of the cell wall architecture (Fry et al. 1992, Nishitani and Tominaga 1992). In various plant species, EXGT/XET proteins are encoded by a group of genes (Okazawa et al. 1993, Xu et al. 1996, Campbell and Braam 1999, Yokoyama and Nishitani 2000), which are currently termed the XTH (xyloglucan endotransglycosylase/hydrolase) gene family (Rose et al. 2002). In Arabidopsis thaliana, the XTH gene family consists of 33 members that are classified into three subfamilies based on gene structure (Xu et al. 1996, Nishitani 1997, Yokoyama and Nishitani 2000, Yokoyama and Nishitani 2001b). Expression profiles of XTH genes have been analyzed in several plant species. In Arabidopsis, Xu et al. (1996) have shown that seven XTH genes, AtXTH4, -15, -22, -24, -25, -28, and -30, respond more or less differently to different environmental stimuli such as light, touch, and temperature. Akamatsu et al. (1999) have shown that AtXTH4, -5, -25, -27, and -28 exhibit organ- and growth-dependent expression patterns. In the soybean, an XTH gene, BRUI, is expressed in phloem and paratracheary parenchyma cells (Oh et al. 1998), whereas a tomato XTH gene, LeEEXT, is expressed in the epidermis (Catala et al. 1997). Recent comprehensive studies on the 33 Arabidopsis XTH genes have revealed that individual genes essentially exhibit their own organ-specific expression patterns, and respond dif-

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Auxin-up-regulation of XTH genes in phloem differently to different sets of plant hormones (Yokoyama and Nishitani 2000, Yokoyama and Nishitani 2001b). Despite many expression studies on the XTH genes, the biological implications of diversification in the expression profiles of the XTH gene family remain elusive.

The VaXTH1 gene was identified a decade ago as a cDNA clone encoding an XTH protein that had been purified from the apoplastic fluid of azuki bean internodes (Nishitani and Tominaga 1992, Okazawa et al. 1993). The VaXTH2 gene was subsequently isolated as a VaXTH1 homologue. The proteins encoded by the VaXTH1 and VaXTH2 genes share 84% identity in their deduced amino acid sequences and belong to the class I subfamily (Fig. 1, Yokoyama and Nishitani 2000), indicating similar structures and enzymatic activities. This raises the question of whether they are, or are not, functionally redundant or play individual roles. To address this question, we have analyzed the expression patterns of the two XTH genes under various developmental, as well as physiological, conditions.

Fig. 1 Cladogram indicating structural similarity of VaXTH1 and VaXTH2. Alignment analysis was performed using the CLUSTAL W software accessible at the DDBJ web site (http://www.ddbj.nig.ac.jp/). Thirty-three Arabidopsis XTH genes (AtXTH1 to AtXTH33) (Yokoyama and Nishitani 2001b), a soy bean XTH gene (BRU1) (Aducci 1995), and a tomato XTH gene (LeEXT) (Okazawa et al. 1993) are used as reference genes. The VaXTH1 and VaXTH2 genes belong to the class I subfamily of the gene family, whereas BRU1 belongs to the class II subfamily. The members of the class I subfamily are boxed.

Fig. 2 Expression pattern of VaXTH1 and VaXTH2 mRNA in azuki bean plants (A). RNA blot analyses of 20-μg samples of RNA derived from shoot apexes, petioles, leaves, the first internode, cotyledons, and roots harvested from 7-day-old azuki bean plants. The filter was hybridized with gene-specific probes for VaXTH1 and VaXTH2. Equal loading was confirmed by ethidium bromide staining of rRNA. Growth stage-dependent expression of VaXTH1 and VaXTH2 in the first internode of azuki bean plants (B). RNA blot analysis of 20 μg each of total RNA derived from the internode of the azuki bean harvested at days 4, 7, 10, 15, and 20 after germination. Equal loading was confirmed by ethidium bromide staining of rRNA. The growth curve for the first internode as a function of the day after germination is shown at the bottom. Mean internode lengths with standard errors as vertical lines are shown (n = 25).
and have found that the two genes are expressed essentially in the same cell types, but that the timing of their expression and mode of response to indole-3-acetic acid (IAA) differ significantly.

**Results**

*Expression profiles of the VaXTH1 and VaXTH2 mRNAs in azuki bean seedlings*

Fig. 2A shows expression levels of the *VaXTH1* mRNA and *VaXTH2* mRNA in different organs of 7-day-old azuki bean seedlings. High levels of the two mRNAs were found in the first internode of the seedlings. Lower levels of the *VaXTH1* mRNA were expressed in the shoot apex and petioles than in the internodes. In the root, a much lower level of the *VaXTH1* mRNA was detected. On the other hand, a lower but significant expression of the *VaXTH2* mRNA was detected in xylem cells (cf. Fig. 4D and 4E). In roots of 6-day-old plants, the *VaXTH1* and *VaXTH2* mRNAs were also located in phloem fibers of leaves and petioles, but could not be detected in shoot apical meristem (data not shown). Unlike *VaXTH1*, little or no *VaXTH2* mRNA was not detected in xylem cells (cf. Fig. 4D and 4E). In roots of 6-day-old plants, the *VaXTH1* mRNAs were expressed both in the phloem and xylem, whereas the *VaXTH2* mRNA was predomi-
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...nantly expressed in phloem cells (Fig. 6).

To characterize the cell wall type of the phloem fiber cells and the outer cell layer of the xylem, in which the VaXTH1 mRNA was extensively expressed, transverse sections of the sub-apical part (20 mm from the apex) of the 9-day-old internode were subjected to staining with Calcofluor and phloroglucinol-HCl. In the 9-day-old seedling, the sub-apical region is no longer elongating and corresponds to the region 60 mm from the apex in the 7-day-old seedling. The cell walls in these cell piles were clearly stained with Calcofluor (Hayashi et al. 1986), indicating massive deposition of glucans (Fig. 5C, D). By contrast, the cell pile was not stained by phloroglucinol-HCl, which instead stained the inner layer of xylem cells (Fig. 5E, F). The data indicate clearly that the cell walls in the phloem fiber and the outer layer of the xylem contained glucans, such as cellulose and xyloglucans, but were not lignified.

Divergent responses of the VaXTH1 and VaXTH2 genes to IAA and FC

The effects of plant hormones on expressions of the two genes were investigated using 10-mm tissue segments excised from sub-apical regions of elongating internodes of 7-day-old seedlings. RNA blot analysis showed that levels of both the VaXTH1 and VaXTH2 mRNAs were up-regulated substantially by the application of IAA. On the other hand, gibberellic acid (GA) slightly up-regulated both genes, whereas brassinolide slightly up-regulated only the VaXTH2 gene (Fig. 7). While ACC did not affect the expression of the two genes, abscisic acid significantly down-regulated only the VaXTH2 gene. IAA application caused elongation of the internodal segments, as reported previously (Nishitani and Masuda 1981, Nishitani and Masuda 1983), whereas other hormones did not cause apparent...
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morphological change in the internode segments under the conditions examined (data not shown).

To dissect the mode of action of IAA on the two genes, we analyzed the effects of fusicoccin (FC) on both, and compared its effect quantitatively with that of IAA using real-time reverse transcription-polymerase chain reaction (RT-PCR). Fig. 8 shows that IAA application up-regulated both of the VaXTH1 and VaXTH2 mRNAs in excised segments. On the other hand, FC up-regulated only the VaXTH1 gene, but did not affect VaXTH2 mRNA expression. Application of IAA to the internode segment did not cause any apparent change in the distribution patterns of the mRNAs as shown by in situ RNA hybridization analysis (data not shown).

Finally, we examined the effects of IAA and FC on the two XTH genes under conditions where cell expansion was inhibited in the presence of 0.25 M mannitol solution. Under the conditions where water uptake was inhibited by mannitol, neither FC nor IAA up-regulated the VaXTH1 mRNA level. On the other hand, IAA up-regulated the VaXTH2 gene significantly even in the presence of mannitol, whereas FC did not. Thus, the up-regulation of the VaXTH1 gene was closely correlated with turgor pressure-dependent cell expansion induced either by IAA or by FC, whereas expression of the VaXTH2 gene was up-regulated by IAA, but not by FC, independent of turgor pressure.

Discussion

Expression sites of VaXTH1 and VaXTH2 genes
Previous biochemical studies have demonstrated that the azuki bean VaXTH1 protein exclusively catalyzes the molecular grafting of xylolglucan molecules, but does not exhibit any hydrolytic activity toward xylolglucans (Nishitani and Tominaga 1992). Based on its mode of action, this type of enzyme has been postulated to take part in some construction-directed cell wall modifications rather than a simple disassembly of the cell wall architecture (Nishitani 1997, Nishitani 1998). The VaXTH1 and VaXTH2 proteins are strikingly alike in that 84% of amino acid residues are identical, and they belong to proteins encoded by the same subfamily of the XTH gene family (Fig. 1). Judging from their structural similarity, it is quite likely that the two proteins exhibit similar transferase activities, and mediate similar processes in the cell wall modification processes.

The present study has disclosed that the two azuki bean genes, VaXTH1 and VaXTH2, with structural resemblance, exhibit overlapping expression patterns in the phloem fiber cells in the growing first internode. Histochemical staining analyses revealed that the two genes are expressed extensively in non-lignified cell walls. These expression patterns suggest their common roles in the construction process of the cellulose/xylolglucan framework in the phloem-fiber cell walls during the early stages of the fiber-cell development before the lignification process commences.

In Arabidopsis, several groups of XTH genes with similar gene structures exhibit similar expression profiles, particularly in terms of organ specificity (Yokoyama and Nishitani 2000, Yokoyama and Nishitani 2001b). For example, AtXTH12, -13 and -14 are closely related phylogenetically and exhibit root-specific expression profiles. Thus, it seems to be a common trait among plant species that sister members of the XTH gene family, such as VaXTH1 and -2, with similar genome structure, exhibit similar organ-specific expression patterns.
**Divergent auxin actions on the VaXTH1 and VaXTH2 genes**

One of the primary actions of auxin at the cellular level is to activate plasma membrane-localized proton ATPase, thereby causing acidification of the apoplast, which in turn triggers cell wall alteration, including those processes that lead to cell wall expansion (Luehnen et al. 1990, Hager et al. 1991). This activation process is mediated by the 14-3-3 protein (Svennelid et al. 1999, Fuglsang et al. 1999). Fusicoccin has been shown to mimic auxin action on the H⁺ pump by interacting directly with this protein to activate the H⁺ pump (Aducci et al. 1995, Fullone et al. 1998).

Our present analyses disclosed that the VaXTH1 and VaXTH2 genes responded to IAA and FC in quite different ways. The VaXTH1 gene was up-regulated by FC, indicating that the auxin action is mediated via activation of the proton ATPase that is located in the plasma membrane. Furthermore, the promotive effects of both IAA and FC on the VaXTH1 mRNA levels depended on the turgor pressure in the plant tissue, and were nullified in the presence of 0.25 M mannitol solution (Fig. 8). Because the addition of mannitol does not suppress proton ATPase activity (Marre et al. 1973), the up-regulation of the VaXTH1 gene by auxin may require a turgor-dependent process in addition to the hydrogen ion produced by auxin or FC. These facts imply involvement of membrane integrity or mechanosensor localized on the plasma membrane in the regulatory process for the VaXTH1 gene expression.

An acidic buffer solution at pH 4 induces a decrease in average molecular size of xyloglucans in the internode sections of azuki bean (Nishitani and Masuda 1982). This acid effect on xyloglucans is completely reversed by subsequent treatment with neutral pH solution. Taken in the light of the current idea of XTH-mediated xyloglucan modifications, the acid effect on xyloglucans has been considered to be caused by pH-dependent changes in the enzyme activity or catalytic rate of VaXTH1 (Nishitani and Tominaga 1991, Nishitani and Tominaga 1992). It is known that apoplastic pH can be controlled between 4.5 and 6.0 by the actions of hydrogen ion pumps as activated by IAA and FC. Because the VaXTH1 exhibits a steep pH dependency curve at pH 5.8, it has been commonly accepted that the activity of the enzyme is altered drastically by a subtle change in the apoplastic pH, which is precisely regulated by the action of IAA and FC.

Whereas our present results do not contradict the commonly accepted view of the acid-pH-induced xyloglucan change, the turgor dependency of the FC up-regulation of the VaXTH1 gene suggests an alternative signaling pathway from FC to the VaXTH1 gene, a pathway that is not mediated by enzyme activity changes caused by pH changes. Because the effect of the acid buffer on VaXTH1 gene expression was not examined, we cannot exclude the possibility that hydrogen ions secreted into the apoplast space might function as signals to up-regulate the VaXTH1 gene. An examination of the effects of acidic buffer solutions on expression of the VaXTH1 gene is required to address this issue.

Unlike VaXTH1, the expression of VaXTH2 is not regulated by acid pH. Furthermore, its expression was up-regulated by IAA application, regardless of the presence or absence of the mannitol solution (Fig. 8), and the promotive effect of IAA was not mimicked by FC. This indicates that the cell wall acidification via proton ATPase is not required for the IAA-induced up-regulation of the VaXTH2 gene. These findings imply the presence of two divergent modes of action of auxin on the two similar XTH genes: a “direct action” on the VaXTH2 gene and a “turgor-pressure-dependent action” on the VaXTH1 gene, with both acting in the same cell type.

Fig. 8  Effects of IAA and FC on the VaXTH1 and VaXTH2 mRNA levels under different growth conditions. Excised internodal sections (10 mm long) were pre-incubated in 10 mM potassium phosphate buffer solution with or without 0.25 M mannitol solution for 1 h. The pre-incubated segments were designated as initial segments. The initial segments were incubated in the buffer solution (control), or the buffer solution containing 10⁻⁴ M IAA or 10⁻⁶ M fusicoccin (FC), in the presence or absence of 0.25 M mannitol for 3 h. The lengths of the segments were measured before and after the 3 h of incubation. The segments were frozen and subjected to RNA extraction. The copy numbers of the VaXTH1 and VaXTH2 mRNAs were analyzed by real-time RT-PCR.
Temporal regulations of VaXTH1 and VaXTH2

In addition to the responsiveness to auxin, the timings of the expression of the two genes have diverged with respect to developmental stages of the phloem fiber during shoot maturation. The VaXTH1 gene is expressed in the relatively more juvenile stages of the phloem fiber, whereas VaXTH2 is expressed at later stages. The temporally divergent expression patterns of the two genes during development of the phloem fiber imply that VaXTH1 takes part in cell wall construction during the early stages of phloem fiber development, whereas VaXTH2 acts in the later stages.

In the first internode of the 7-day-old seedlings, the VaXTH1 mRNA level peaked at the middle part of the internode, where little or no elongation growth occurred. On the other hand, in the apical elongating region, lower levels of the VaXTH1 mRNA were found. The relatively lower expression levels in the apical region might be partially due to smaller numbers of phloem-cells in the apical region than to those in the middle and basal regions (see Fig. 4A–D). Even though the numbers of phloem-fiber cells are taken into account, the expression of the VaXTH1 mRNA is clearly predominant during developing stages of phloem fibers in the middle of the internode, where the cell elongation has just stopped. Thus, we conclude that the VaXTH1 gene is involved in phloem fiber development from the beginning of its differentiation through to the early stage of maturation, which lasts even after cell elongation has terminated. This idea is consistent with the fact that the up-regulation of the VaXTH1 gene by auxin is pressure dependent, and is closely related to the cell elongation process. It is quite likely that auxin up-regulates VaXTH1 gene expression as a secondary event, triggered via primary actions of auxin, such as auxin-induced cell elongation. On the other hand, the accumulation of VaXTH2 in the non-growing region of the internode suggests a role for this gene in secondary cell wall thickening of the phloem fiber, particularly after the elongation growth has stopped. It should be noted that this is achieved through deposition of non-lignified cell wall.

Shoot phloem fibers with thickened cell walls are considered to function as a major supporting tissue in mature plant shoots (Esau 1977), whereas epidermal cells have been demonstrated to function as tension-bearing cells of the plant shoot, which mechanically constrain the extension of the whole stem (Tanimoto and Masuda 1971, Kutschera 1994). That neither VaXTH1 nor VaXTH2 was expressed in the epidermal tissue excludes the possibility of their commitment to the epidermis-mediated regulatory system of internode elongation. Instead, the result hints at the possibility that phloem fibers with non-lignified primary cell walls formed during the elongation period of the first internode might function as tension-bearing cell piles, as does the epidermis. After internode elongation stops, phloem fibers with thickened cell walls will be lignified and will function as a major supporting tissue in mature internodes. Construction-directed cell wall modifications by VaXTH1 and VaXTH2 products might be essential for developing the tension-bearing properties of phloem fibers that give mechanical strength. The divergent expression patterns of the VaXTH1 and VaXTH2 genes in the phloem fiber cells imply that they have cooperative actions in the sequential construction processes of this complex architecture during a long period of development.

Functional divergence of sister genes in cell-wall-related genes

The most important part of this argument is that the two structurally related genes, VaXTH1 and VaXTH2, exhibit temporally different expression profiles in the phloem fibers of the internode and respond differently to auxin. A few XTH genes expressed predominantly in growing internodes have been reported in other plant species. BRU1 is a soybean XTH, and is expressed preferentially in growing epicotyls (Zurek and Clouse 1994). The tissue-specific expression profile of the BRU1 transcript is similar to that of VaXTH1 and VaXTH2, in that it is localized in phloem and paratracheary parenchyma cells (Oh et al. 1998). However, BRU1 does not seem to be the ortholog for VaXTH1 or VaXTH2, because it belongs to the class II subfamily in terms of protein structure (cf. Fig. 1) and is not at all up-regulated by auxin, but is prominently up-regulated by brassinosteroid. A tomato XTH gene, LeEXT, which is structurally similar to VaXTH1 and VaXTH2, belongs to the class I subfamily of the XTH gene family (cf. Fig. 1), and is expressed preferentially in the growing region of etiolated tomato seedlings. The LeEXT gene is up-regulated specifically by 2,4-D, a synthetic auxin (Catala et al. 1997). However, in situ hybridization analysis has shown that the LeEXT mRNA is localized in the epidermal regions (Catala et al. 1997), and the tomato LeEXT gene seems to be different from the VaXTH1 and VaXTH2 genes in terms of its tissue-specific expression pattern. In these plant species, detailed expression analysis on a sister-gene pair such as VaXTH1 and VaXTH2 has not been carried out, and no partner either for the tomato LeEXT or soybean BRU1 has thus far been identified in the respective plant species.

In Arabidopsis, several sets of structurally related XTH genes have been shown to exhibit similar organ-specific expression patterns (Yokoyama and Nishitani 2001b: AtXTH6, -7, -9, -10, and -11 are expressed preferentially in siliques and flower, while AtXTH12, -13 and -14 are expressed in the root. AtXTH27 and -28 are expressed in all organs. Comprehensive analyses have shown that some AtXTH genes with similar organ-specific expression patterns exhibit similar responses to plant hormones and that others respond differently to plant hormones (Xu et al. 1996, Yokoyama and Nishitani 2001b). Considering the expression patterns of the 33 Arabidopsis XTH genes, it seems probable that sister-gene pairs such as the VaXTH1 and VaXTH2 pair might be found upon detailed analysis of the gene family, focusing on both expression cell-types and mode of hormonal regulation. Molecular dissection of individual roles for such sister-genes affords a clue to elucidate the functional diversity of the cell-wall-related gene family as represented by the XTH gene family in the construction and maintenance of
individual cell-wall types, and hence cell types in plants.

Materials and Methods

Plant material

Seeds of azuki bean (*Vigna angularis* Ohwi and Ohashi cv. Takara-wase), obtained from Watanabe Seed Co., Ltd. (Miyagi, Japan), were allowed to germinate in running tap water at 25–28°C for 36 h, and were then sown in a moistened vermiculite bed in plastic trays. They were grown at 25°C under continuous light (about 70 μmol s⁻¹ m⁻²) under conditions described previously (Nishitani and Masuda 1981).

Growth experiments

From the first internodes of 7-day-old seedlings, 10-mm segments were excised between 5 and 15 mm below the shoot apex, washed, and pre-incubated in 10 mM potassium phosphate buffer (pH 6.0) containing 0.1 mM IAA or 1 mM IAA for 15 min at 65°C in a solution containing 2% mannitol. After hybridization, the membrane was washed in a solution containing 0.1 M SSC and 0.1% SDS twice for 10 min at room temperature, and twice for 20 min at 65°C. The color reaction was performed in AP buffer containing 5-bromo-4-chloro-3-indolyl-phosphate. Sections were dehydrated in a graded ethanol series, acetonic, and 3.7% formaldehyde, then dehydrated in a graded series of ethanol, permeated in xylene, and embedded in Paraplast Plus. Microtome sections (6–8 μm thick) fixed on polys-l-lysine-coated slide glass were deparaffinized with xylene, and rehydrated through a graded ethanol series. The dehydrated sections were subsequently washed in 2× SSPE solution (pH 7.4) at 60°C for 10 min, treated with 10 μg ml⁻¹ proteinase K at 37°C for 30 min, and fixed in 4% (v/v) paraformaldehyde in PBS (pH 7.4) at 4°C for 20 min. Sections were then incubated in the hybridization buffer containing 50% (v/v) formamide, 2× SSPE, 1 mM EDTA, 50 mM sodium phosphate buffer at pH 7.4, and 1% (w/v) blocking reagent (Roche Diagnostics) with 1 μg ml⁻¹ of the sense or antisense RNA probes at 42°C for 16–18 h. After hybridization, sections were washed successively twice in 2× SSPE containing 50% formamide for 10 min at 37°C, twice in 2× SSPE containing 50% formamide for 10 min, twice in 0.5× SSPE containing 50% formamide for 10 min, and finally, once in PBS containing 0.1% Triton X-100 for 15 min at room temperature.

For immunological detection of the DIG-labeled probes, the sections were treated with 1% blocking reagent in a maleic acid buffer (100 mM maleic acid-NaOH and 150 mM NaCl, pH 7.5) for 30 min, then transferred to a maleic acid buffer containing anti-digoxigenin-alkaline phosphatase and Fab fragments (Roche Diagnostics, 1: 500 dilution). Sections were incubated at room temperature for 30 min, washed three times with maleic acid buffer for 10 min, and rinsed with detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM MgCl₂). The color reaction was performed in AP buffer containing 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. Sections were dehydrated in a graded ethanol series, dipped in xylene, and mounted in Canada balsam. Tissue sections were observed and photographed using a differential interference contrast (DIC) microscope (DMRXP, Leica, Heerbrugg, Switzerland).

**Quantitative real-time RT-PCR**

The oligonucleotide primers used for the RT-PCR analysis were designed based on the nucleotide sequences in the 3' UTR of the two genes, according to the procedure described previously (Yokoyama and Nishitani 2001b). The primer sets used are as follows. For VaXTH1, V1F (5'-CACTCCCATAAAGCTCCACCT-3') and V1R (5'-GCTAGCCTCAACAGTACAGC-3'). For VaXTH2, V2F (5'-GTCTTACCAACACATTTCATACGACATT-3') and V2R (5'-GGCCTTAAATTACACATTCACATCAACAAA-3'). Quantitative one-step RT-PCR was performed using an SYBR Green RT-PCR Reagents kit in an ABI Prism™ 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems) according to the protocol provided by the supplier. The reaction mixture contained 100 ng of total RNA, 1× SYBR® Green PCR Master Mix, 0.4 units RNase inhibitor, 0.25 units MultiScript™ reverse transcriptase, 50 nM forward primer, and 300 nM reverse primer in a 25 μl solution. The first-strand cDNA fragments were generated by reverse transcription at 48°C for 30 min, followed by inactivation of the reverse transcriptase at 95°C for 10 min. Subsequently, double-stranded cDNA fragments were amplified by PCR (95°C for 15 s, 60°C for 1 min) for 40 cycles. The RT-PCR experiments were conducted in triplicate, and the mean copy numbers of individual mRNA species were estimated using the cDNA preparations of known molar concentrations as standards.

**In situ mRNA hybridization**

Antisense and sense RNA probes were synthesized and labeled in vitro with a DIG RNA labeling mix (Roche Diagnostics) using T3 or T7 polymerase according to the manufacturer’s protocol. Tissue samples were fixed in FAA solution containing 50% (v/v) ethanol, 5% acetic acid, and 3.7% formaldehyde, then dehydrated in a graded series of ethanol, permeated in xylene, and embedded in Paraplast Plus. Microtome sections (6–8 μm thick) fixed on polys-l-lysine-coated slide glass were deparaffinized with xylene, and rehydrated through a graded ethanol series. The dehydrated sections were subsequently washed in 2× SSPE solution (pH 7.4) at 60°C for 10 min, treated with 10 μg ml⁻¹ proteinase K at 37°C for 30 min, and fixed in 4% (v/v) paraformaldehyde in PDB (pH 7.4) at 4°C for 20 min. Sections were then incubated in the hybridization buffer containing 50% (v/v) formamide, 2× SSPE, 1 mM EDTA, 50 mM sodium phosphate buffer at pH 7.4, and 1% (w/v) blocking reagent (Roche Diagnostics) with 1 μg ml⁻¹ of the sense or antisense RNA probes at 42°C for 16–18 h. After hybridization, sections were washed successively twice in 2× SSPE containing 50% formamide for 10 min at 37°C, twice in 2× SSPE containing 50% formamide for 10 min, twice in 0.5× SSPE containing 50% formamide for 10 min, and finally, once in PBS containing 0.1% Triton X-100 for 15 min at room temperature.

RNA isolation and RNA gel blot analysis

Total RNA was isolated from frozen tissue samples that had been excised from azuki bean plants according to an SDS–phenol method. Twenty-microgram samples of each of the total RNA were separated by electrophoresis in 1.2% agarose gel containing 17% formaldehyde. The RNA was blotted onto Hybond-N+ nylon membranes (Amersham Biosciences) according to an alkaline blotting procedure. Equal loading for individual blots was confirmed by ethidium bromide staining of ribosomal RNAs. DNA probes were labeled with [α-32P]dCTP (Amersham Biosciences, 110 TBq mmol⁻¹) using the EcoBEST™ labeling kit (Takara Shuzo, Kyoto, Japan) according to the supplier’s protocol. Hybridization was performed at 65°C in a solution containing 5× SSC buffer, 5× Denhardt’s solution, 0.5% (w/v) SDS, 10 μg ml⁻¹ denatured salmon sperm DNA, and the labeled gene-specific probe. After hybridization, the membrane was washed in a solution containing 2× SSC and 0.1% SDS twice for 10 min at room temperature, and twice for 15 min at 65°C in a solution containing 0.2× SSC and 0.1% SDS. The radioactivity on the nylon membrane was disclosed and quantified with the aid of a Bio-imaging analyzer (LAS-1800, Fuji Film, Tokyo, Japan).
Histochemistry

Tissue samples were fixed, paraffin-embedded, sectioned (6–8 μm thick) and deparaffinized in the same procedure as for the in situ mRNA hybridization analysis. β-Glucans such as cellulose, xyloglucan, (1→3,1→4)-β-glucan, and callose, were stained in a 0.001% aqueous solution of Calcofluor (Fluorescent brightener 28, Sigma-Aldrich). Fluorescent images of Calcofluor-stained glucans were recorded under an epifluorescence microscope (DMRXPS, Leica, Heerbrugg, Switzerland) equipped with a UV fluorescence filter set. Lignins were stained with a saturated solution of phloroglucinol in 18% HCl. Stained sections were observed and photographed under a differential interference contrast microscope, as above.

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