ORIGINAL ARTICLE

Developmental expression of the cucumber *Cs-XTH1* and *Cs-XTH3* genes, encoding xyloglucan endotransglucosylase/hydrolases, can be influenced by mechanical stimuli

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Abstract

The expression of two genes encoding xyloglucan endotransglucosylase/hydrolases (XTHs), *Cs-XTH1* and *Cs-XTH3*, was upregulated during the onset of cucumber somatic embryogenesis. As a means of characterising the developmental regulation of these genes, the activity of the respective upstream regulatory regions was investigated in seedlings and somatic embryos of *Arabidopsis thaliana* and *Cucumis sativus*. GUS assays revealed that both genes are under developmental control. In addition, elevated promoter activity was found in the tension-bearing regions of the plant and in response to touch and wounding, which is consistent with the existence of numerous stress-related *cis* elements in the 5′-regulatory regions. In vivo xyloglucan endotransglucosylase (XET) action assays were performed to gain an overview on the role of XTHs during somatic embryogenesis. The highest XET action was observed in the external cell layers of somatic embryos in the cotyledonary region and in the presumptive region of peg formation. Based on the results, we propose a dual mechanism (one developmental and the second adaptive) for the regulation of *Cs-XTH1* and *Cs-XTH3* activity wherein the developmental pattern can be modified by mechanical stimuli.

Keywords Xyloglucan endotransglucosylase/hydrolase (XTH) · Somatic embryogenesis · Cucumber · *Cucumis sativus* · Developmental and adaptive gene regulation · Xyloglucan–oligosaccharide–sulphorhodamine conjugate (XGO-SR)

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Introduction

The *XTH*s are a family of plant genes encoding the xyloglucan transglucosylase/hydrolase enzymes (XTH; EC 2.4.1.207). These enzymes are capable of modulating the chemistry of the primary cell wall of plants, which consists

of cellulose microfibrils embedded in a matrix of hemicellulosic and pectic polysaccharides, by remodelling the hemicellulose xyloglucan, the major component of the matrix in dicotyledonous plants. XTHs exhibit one or both of two enzymatic activities: the catalysis of the cleavage of a xyloglucan chain and subsequent religation to a different acceptor chain (xyloglucan endotransglucosylase activity—XET) and the catalysis of the cleavage of a xyloglucan chain with religation to water as an acceptor molecule (xyloglucan endohydrolase activity—XEH) (Eklöf and Brumer 2010; Franková and Fry 2013).

The enzymatic XET and XEH activities have been shown to lead to enhanced viscoelasticity or increased strength when applied to cellulose/xyloglucan biocomposite material mimicking a cell wall structure (Chanliaud et al. 2004), and exogenously applied XTHs have been shown to modify the extensibility of real plant cell wall material (Maris et al. 2009). These results have provided strong support for previous demonstrations of the ability of these proteins to integrate new xyloglucan molecules (Thompson et al. 1997) and to remodel existing xyloglucan molecules (Thompson and Fry 2001) of the cell wall. Based on these results, it would appear that by changing the mechanical properties of the cell wall, XTHs significantly influence the structural phenotype of the cell, thereby contributing to the regulation of the resulting plant phenotype. XTH enzymes are believed to be capable of strongly influencing the arrangement of cellular and tissue tensions in the plant. This type of morphological change, especially when it occurs locally, may be important for plant morphogenesis (Hejnowicz et al. 1977; Dumais et al. 2004).

The morphogenetic potential of XTH proteins should also be discussed in the light of the extensive number of genes that make up this family and the possible functional redundancy that may result from this. There are 33 XTH genes in the *Arabidopsis* genome, most of which exhibit individual tissue-specific and growth stage-dependent expression profiles (Yokoyama and Nishitani 2001). Similar family characteristics have been reported for other plants such as rice (29 members; Yokoyama et al. 2004), *Populus trichocarpa* (37 members; Ye et al. 2012) and the moss *Physcomitrella patens* (32 members; Yokoyama et al. 2010). Assuming that each cell type possesses its own specific "cell wall type", the diversity of expression patterns of XTH genes is consistent with the hypothesis that each "cell wall type" is characterised by a specific set of cell wall-modifying enzymes (Nishitani 2002).

There are several reports in the literature suggesting specific roles of XTH proteins in the regulation of intercellular relationships during cell differentiation. Antosiewicz et al. (1997) determined that the XTH22 protein is involved in intercellular space formation during xylem and phloem development in *Arabidopsis*. The transcription of

the *VaXTH1* and *VaXTH*2 genes during phloem formation in the azuki bean also supports the involvement of XTHs in the regulation of cell mechanical properties during plant development (Nakamura et al. 2003). Bourquin et al. (2002) observed that an XTH protein is involved in secondary cell wall formation in poplar (*Populus tremula* var. *tremuloides*) vascular tissue. Vissenberg et al. (2001) observed XET action [i.e. co-localisation of XET-active XTH(s) with the accessible endogenous donor substrate xyloglucan] in *Arabidopsis* roots at the site of root hair differentiation. Similar engagement of XTH proteins during root hair differentiation was shown in numerous evolutionarily distinct plants (Vissenberg et al. 2003). Similarly, testing promoter activities of root-specific *Arabidopsis XTH* genes confirmed previously observed expression profiles and the correlation of XET actions with different localizations and pH optima (Vissenberg et al. 2001, 2005).

Plant development is strongly modified by environmental factors. External stimuli often trigger the plant's adaptive response by regulating the mechanisms requiring specific growth modifications, of which cell wall remodelling is of great importance. XET enzyme activity seems to be no exception to this and appears to be an important element of the plant's response to mechanical stimuli or other stress factors (Potters et al. 2007, 2009). This phenomenon was first described for the *TCH4* gene (*XTH22*) of *Arabidopsis thaliana* (Braam and Davis 1990) which was activated by environmental factors such as wind, rain or touch. The XTH22 protein accumulated in cells exhibiting adaptive changes to mechanical stress—i.e. in vascular and epidermis tissues (Antosiewicz et al. 1997). Moreover *XTH22* gene transcription was activated in the dark or in response to low (0 °C) or high (35 °C) temperatures (Braam and Davis 1990; Xu et al. 1995; Iliev et al. 2002). Xu et al. (1996) suggested that depending on the adaptive needs of the plant the XTH22 protein can cause local cell wall remodelling, leading to either temporary loosening or rapid reinforcement of the cell wall. However, extrapolations from gene transcription (or protein accumulation) to protein action can be misleading; for example, various factors such as an incompletely characterised heat-stable plant polymer (Sharples et al. 2017) can influence the activity of XTH proteins. A full understanding of the role of the XTH proteins in these processes requires assays for their activity and/or action (Vissenberg et al. 2000).

Linkiewicz et al. (2004), Malinowski et al. (2004) and Wiśniewska et al. (2012) reported an increase in the activity of two cucumber (*Cucumis sativus* L.) *XTH* genes, *Cs-XTH1* and *Cs-XTH3*, during somatic embryo development. The localisation of their mRNAs in presumptive cotyledon regions suggested that these genes may play a role in embryo pattern formation. In continuation of this research, we report

here our investigation of the mechanisms regulating the expression of these genes and XET action.

Materials and methods

Plant material

Cucumber (*C. sativus* L.) cv. Borszczagowski plants used for the analysis of promoter activity and for the induction of an embryogenic suspension culture were grown for 20–30 days under a 17/7-h (light, 25 °C/dark, 21 °C) photoperiod regime. The cucumber cell suspension culture was established and the induction of somatic embryogenesis was carried out according to the cytokinin model established by Burza and Malepszy (1998).

A. thaliana ecotype 'Columbia' plants were grown under a 10/14-h (light, 21 °C/dark, 19 °C) photoperiod regime. Flowering was induced by switching to a 14-h light period.

Promoter::GUS (ß-glucuronidase) fusion constructs and plant transformation

The PLACE (Higo et al. 1999) and PlantProm DB (Shahmuradov et al. 2003) databases were used to predict the *cis* elements. Predicted elements were arranged into developmentally- and stress-related groups according to database

description for particular element (Fig. 1; for a detailed list of the *cis* elements predicted, see Table 1 of the supplementary data). Based on the distribution of predicted regulatory regions in the 5′-upstream sequence, we generated three promoter::GUS fusions each for *Cs-XTH1* and *Cs-XTH3* (Fig. 1) in the binary pCAMBIA 1381Z vector (Hajdukiewicz et al. 1994). The main criterion applied to the construct design was the frequency of putative *cis* elements observed in particular region of the promoter analysed.

All constructs were used for the *Arabidopsis* transformation according to the Clough and Bent (1998) floral dip method. Transgenic plant selection was performed on GRODAN (GRODANIA A/S Doense, Hobro, Denmark) according to Hadi et al. (2002) with minor modifications. In brief, approximately 1000 seeds were sown on $15 \times 10 \times 2$-cm 'rockwool mate' watered with 1/4-strength MS medium (Murashige and Skoog 1962) containing 5 mg l^{-1} hygromycin B (Duchefa, Haarlem, the Netherlands). Following matriconditioning at 4 °C for 3 days, the seeds were replaced in the growth chamber under vegetative growth conditions (see Materials and methods, plant material). After 3 weeks, resistant seedlings that had developed leaves were transferred to soil. For construct -928pCs-XTH1, we obtained 8 independent transformation events, -467pCs-XTH1—8, -275pCs-XTH1—26, -610pCs-XTH3—6, -430pCs-XTH3—2 and -244pCs-XTH3—43.

Fig. 1 Schematic representation of generated promoter::GUS fusions and the localisation of predicted *cis* elements in 5′ upstream regions of *Cs-XTH1* (**a**) and *Cs-XTH3* (**b**). The *cis* elements shown on the upper part of each scheme (S) represent stress-related elements; those on the lower part are developmentally-related elements (**d**). For more detailed information on the localisation of the *cis* elements localization, see SupplementaryTable2

Two constructs (-928pCs-XTH1 and -610pCs-XTH3; Fig. 1) were introduced into the cucumber genome. The *Agrobacterium*-mediated transformation of the cucumber embryogenic suspension culture was performed according to the method of Burza et al. (2006). For construct -928pCs-XTH1, we obtained eight independent hygromycin-resistant embryogenic cell lines and regenerated five transgenic plants (independent transformation events), whereas transformation with -610pCs-XTH3 gave six embryogenic cell lines and no transgenic plants were regenerated. PCR analysis verified the presence of the transgene in T0 *Arabidopsis* and cucumber plants.

GUS assay

GUS activity was determined basically according to the method of De Block and Debrouwer (1992). Subsequent modifications to this method were designed on the basis of the experimental configuration.

To check the influence of wounding on the activity of the *Cs-XTH1* and *Cs-XTH3* promoters, we cut leaves three times with scissors at 30-min intervals (1 h between the first and last incision). Immediately after the last incision, the leaves were excised and placed in 1 mg ml^{-1} X-Gluc (Duchefa) working solution overnight (approximately 10–12 h). Wild-type cucumber and *Arabidopsis* leaves were used as controls. In an additional analysis, GUS activity in the leaves of old (2–3 weeks) and young (4–7 days) cucumber plants was compared. The results shown are representative for most of transformation events for each construct.

The assay for GUS activity in *Arabidopsis* seedlings was performed under conditions that eliminated any touch induction. The seeds of *Arabidopsis* T$_2$ transgenic plants were sown on solid MS medium (2% agar; Difco, Detroit Mich.) in vertically placed Petri dishes, and plants were grown at 22 °C for 7 days under light conditions (intensity: 150 µmol m^{-2} s^{-1}). GUS activity was assayed following tissue fixation in 2% (w/v) paraformaldehyde by the same method as adopted for the *XTH22* gene (Iliev et al. 2002).

Promoter activity was assayed in the cucumber somatic embryos by replacing the culture medium with the X-Gluc working solution (the same concentration as used above) followed by a 6-h incubation. The reaction was stopped with 70% ethanol, and the embryos and cell aggregates were observed under a stereomicroscope (Olympus SZ, Olympus Optical, Japan) or inverted microscope (Olympus BX60, Olympus Optical). For the whole-mount microscopic observation, embryos were cleared with Hoyer's solution (see following).

XET in vivo co-localisation assay

The *in vivo* assay for XET 'action' (=co-localisation of XET activity and the accessible, endogenous donor substrate molecules) was carried out according to Vissenberg et al. (2000) with minor modifications. The xyloglucan–oligosaccharide–sulphorhodamine conjugates (XGO-SRs) were synthesised as described by Fry (1997), with the oligosaccharides consisting of a mixture of XET substrates, mainly the nona-, octa- and hepta-saccharides: XLLG, XXLG and XXXG. These XGO-SRs were dissolved at a concentration of 6.5 µM in 25 mM Na-succinate buffer, pH 5.5; the same concentration of cellobiose-SR was also dissolved in Na-succinate buffer and used as a control.

Cucumber somatic embryos at various stages were collected from the medium and placed for 1 h in 250 µl of cellobiose-SR or XGOs-SR working solutions, in the dark, following the protocol of Vissenberg et al. (2000). For an improved substrate penetration, 1 µl of TRITON-X100 (Sigma, St. Louis, Mo.) was added to 250 µl of working solution. The somatic embryos were then incubated in an ethanol:acetic acid (6:1) solution for 1 h, briefly washed with 70% ethanol and incubated at room temperature for 6 h in Hoyer's solution (chloral hydrate:glycerol:water, 8:1:2). For subsequent observations, the samples were kept in 10% glycerol (Sigma) solution. Fluorescence was examined by confocal laser scanning microscopy under an Olympus FV-500 system (Olympus) equipped with a 568-nm argon–krypton laser. The signal was detected between 540 and 520 nm.

Results

Bioinformatic analysis of the *Cs-XTH1* and *Cs-XTH3* 5′ regulatory regions

Previously, we reported that two cucumber *XTH* genes, *CsXTH1* and *CsXTH3*, were similarly engaged in somatic embryogenesis (Linkiewicz et al. 2004; Malinowski et al. 2004; Wiśniewska et al. 2012). These genes are also the closest paralogues within 31-member XTH gene family in cucumber and belong to group I/II (Supplementary Figure S1, Supplementary Table S1; Wóycicki et al. 2011). As expected, the nucleotide sequence conservation reaching 75% identity within coding sequence does not extend to the 5′ regulatory regions of *CsXTH1* and *CsXTH3* (no significant similarity found). Focusing on the occurrence and distribution of potential regulatory motifs, it is still difficult to find clear similarities in studied regions. Most of the presumptive *cis* elements detected were previously characterised as being stress-related (Fig. 1; Chen et al. 2002). Many motifs have been found in the promoters of ethylene- (GCC-box

and ERE; Ohme-Tagaki et al. 2000), salicylic acid- (W-box, Eulgem et al. 2000, S-box; Kirsch et al. 2000) and ABA-inducible genes (W-box, Rushton et al. 1996; ABRE; Busk and Pages 1998; E-box and G-box; Li and Hall 1999). In addition, numerous MYB motifs are usually present in stress-inducible promoters (Chen et al. 2002; Reichmann 2002). E-box elements, which are also present in *Cs-XTH* genes promoters, were found to be the binding site for the BIM1 transcription factor during brassinosteroid signalling (Yin et al. 2005). BR sensing is also an important element of the plant's response to external factors.

Cs-XTH1 and *Cs-XTH3* promoter activity in roots

To determine which promoter region is the most important for the activity of each *Cs-XTH* gene, we used transgenic *Arabidopsis* plants with promoter deletion fragments fused to a GUS coding sequence. Schematic representation of the constructs used is shown in Fig. 1.

The GUS assay performed on the 7-day-old *Arabidopsis* seedlings under conditions excluding the involvement of external mechanical stimuli showed that both the *Cs-XTH1* and *Cs-XTH3* promoters were active in the roots. This observation is in agreement with previous data from Northern blot analysis where the strongest transcription of both these genes was shown to occur in roots (Malinowski et al. 2004). High promoter activity was observed only in those parts of the root where growth by means of elongation was taking place or had taken place, and no activity was observed in the root meristematic region (Fig. 2). Promoter activity in other parts of the seedlings was low or even undetectable.

Although promoter activity was observed in all of the transformants (Figs. 2, 3a), the shortest promoter fragments (−275pCs-XTH1 and −244pCs-XTH3) gave only residual GUS staining, whereas longer versions of promoter fragments (i.e. −467 and −928 for *Cs-XTH1*, −430 and −610 for *Cs-XTH3)* gave comparable staining, mainly in roots of transgenic *Arabidopsis* seedlings. This suggests that regions from −275 to −467 for *Cs-XTH1* and from −244 to −430 for *Cs-XTH3* are responsible for the strong expression level.

Influence of mechanical factors

In a heterologous system (*Arabidopsis*), both promoters were activated following leaf wounding (Fig. 3a). The shortest promoter fragments (constructs −275pCs-XTH1 and −244pCs-XTH3) gave the weakest GUS staining. No differences in promoter fragment activity during the 60-min period between the first and third incision were observed (Fig. 3a, e) in either the homologous (cucumber) or heterologous (*Arabidopsis*) system. GUS staining of *Arabidopsis* mature plants showed high promoter activity in tension-bearing regions such as the dehiscence (Fig. 3b, c) and abscission (Fig. 3b) zones of the siliques and flowers (data not shown). Touch-inducible activity of both promoters was also observed (Fig. 3d). Cucumber plants with the −928pCs-XTH1 reporter constructs that were regenerated from somatic embryos also reflected the wound-inducible

Fig. 2 The promoter activity of *Cs-XTH1* (**a**) and *Cs-XTH3* (**b**) genes in *Arabidopsis* during seedling growth. The images show 7-day-old seedlings grown vertically on the medium surface under vegetative conditions. The highest activity was observed in roots, except for the root tip region. The shortest deletion fragments resulted in only residual GUS activity. The length of the promoter fragments is shown below the images. GUS activity was checked after fixation in 2% (w/v) paraformaldehyde
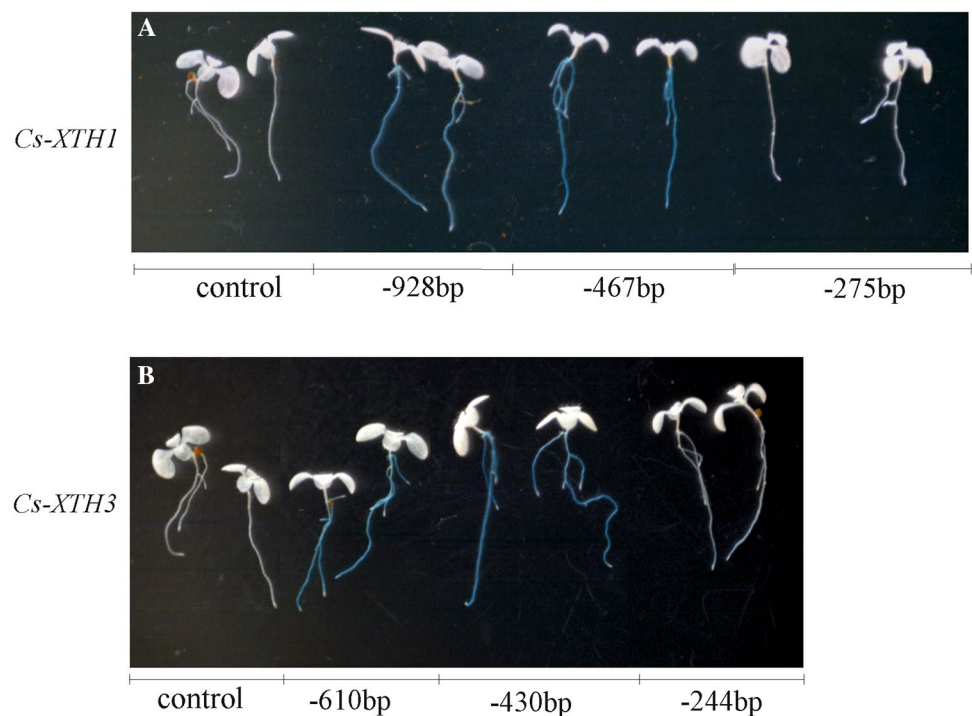
Fig. 3 Mechanical stimuli can induce the *Cs-XTH1* and *Cs-XTH3* promoter activity. **a** Expression patterns in response to wounding by incision. **b, c** GUS activity revealed by −928p*Cs-XTH1* and −610p*Cs-XTH3* promoter fragments in the tension-bearing regions of the *Arabidopsis* silique. This pattern was observed for all but the shortest constructs. **d** Touch response. For mechanical treatment, *Arabidopsis* inflorescence stalks were gently touched by the finger tip in sites indicated by arrows. The example shown here is the −467pCs-XTH1::GUS transgenic *Arabidopsis* plant. This response was observed for all but the shortest promoter::GUS fusions. **e** Wound-inducible activity observed for the −928-bp fragment of the *Cs-XTH1* promoter in cucumber transgenic plants. The scale bars represent 1 cm
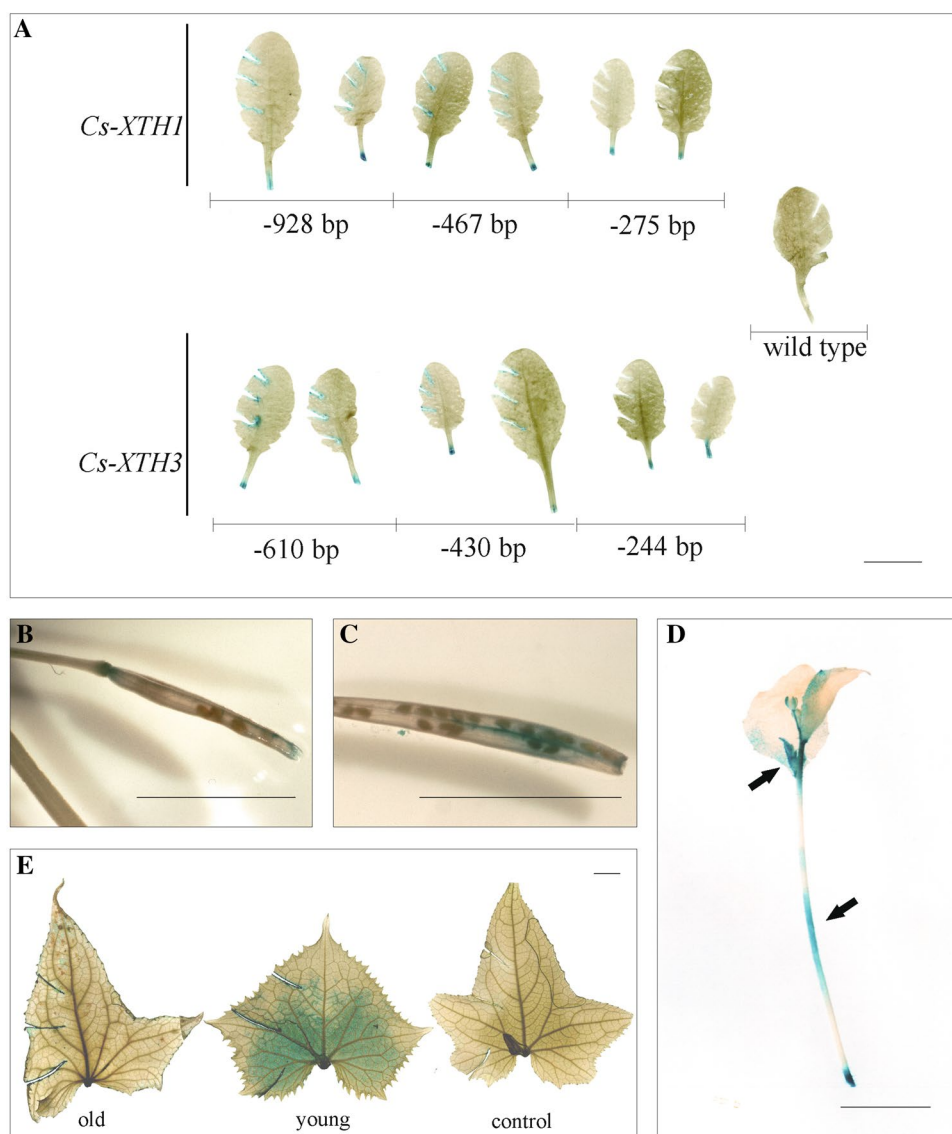
promoter activity; however, additional staining was observed in the central part of younger leaves (Fig. 3e).

The activity of *Cs-XTH1* and *Cs-XTH3* in cucumber somatic embryos

Transgenic somatic embryos (T_0 generation) were obtained following the transformation of cucumber embryogenic suspension cultures with the −928Cs-XTH1::GUS and −610Cs-XTH3::GUS constructs. The promoter activity observed in transgenic somatic embryos resembled the *in situ* mRNA localization pattern previously reported for these two genes (Malinowski et al. 2004). The activity of both promoter fragments was strongest in the cotyledonary part of the embryos (Fig. 4c–e, g for *Cs-XTH1*; Fig. 4h–j for *Cs-XTH3*). In proliferating or degrading somatic embryos, equal

GUS staining of the external cell layers of embryos was observed (Fig. 4f, k) for both promoters. In addition, staining of the differentiated root hairs was observed in germinated somatic embryos (Fig. 4f, k). The strong GUS staining of transgenic cell lines was also observed in small aggregates having the ability for further differentiation. No GUS activity was observed in the control wild-type cucumber somatic embryos (Fig. 4a, b).

In vivo localisation of XET action in cucumber somatic embryos

Monitoring the gene transcriptional activity (e.g. by promoter::reporter gene fusions) may give a hint in explaining its role. Posttranscriptional, translational, and posttranslational regulations and protein relocation can modify the site of gene action. Moreover in the case of proteins having

Fig. 4 The activity of the *Cs-XTH1* and *Cs-XTH3* promoters in cucumber somatic embryos: **a, b** Control, wild-type somatic embryos; **c–g** − 928pCs-XTH1::GUS transgenic somatic embryos; **h–k** − 610pCs-XTH3::GUS transgenic somatic embryos. Both promoters were most active in the peripheral region of the cotyledons. High promoter activity was also observed in proliferating embryos (**f, k**) where the blue staining was present in developed root hairs. The scale bars represent 100 µm
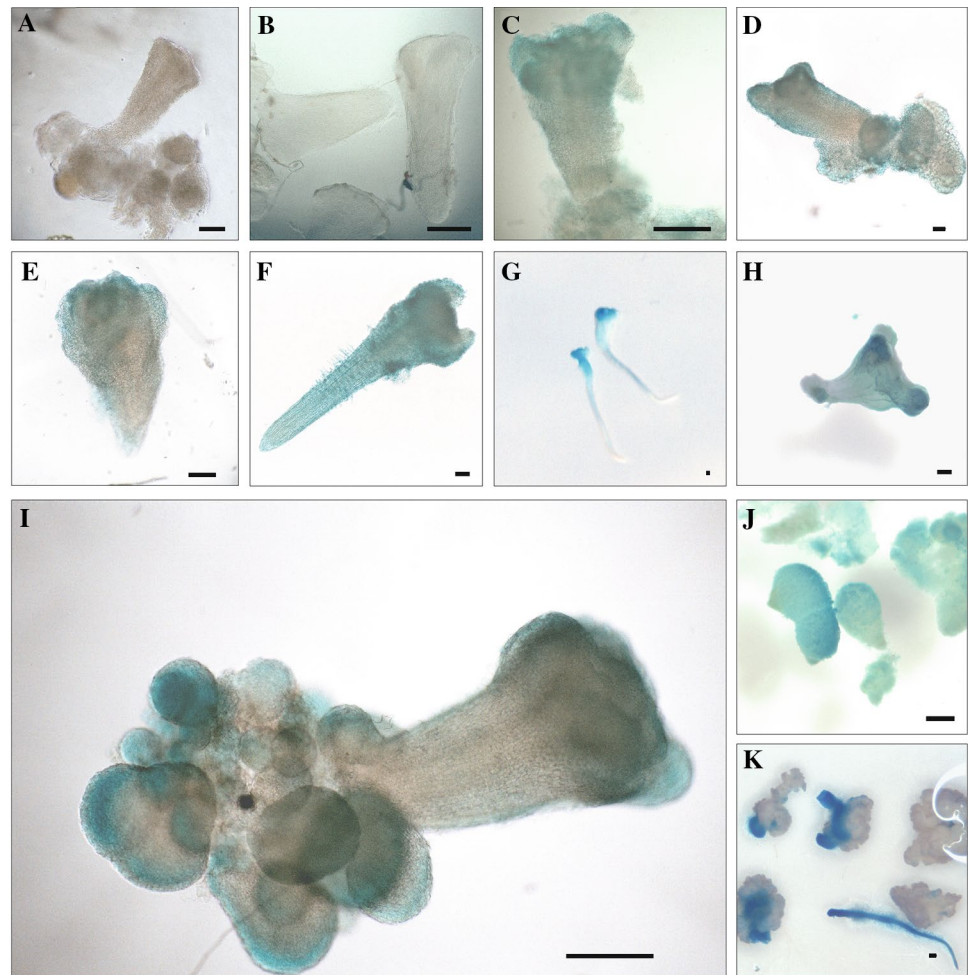
an enzymatic activity, the substrates availability matters. Using the fluorescently labelled acceptor substrate, we may see more precisely where do studied genes act (or other co-localised family members). The localisation of XET action in somatic embryos revealed two different fluorescence patterns corresponding to embryo development modifications observed in embryogenic cucumber cultures. These modifications can be described as shifting the embryo development towards faster growth of apical or basal part. In somatic embryos with arrested or slightly changed development of the cotyledonary parts, the highest XET action was observed in the middle part of the embryo, in an intermediate region between the future root and hypocotyl. This region is known as the presumptive site of peg formation during cucumber seed germination (Takahashi 1997). Observed XET action was specifically localised, and no (or only residual) action was present in the aggregates bearing somatic embryos (Fig. 5a, b). In some in vitro-germinated somatic embryos, strong elongation of this region of high XET action was observed (Fig. 5f). In somatic embryos having well-developed cotyledons, a lower XET action was observed (Fig. 5g, h). However, in this case, the whole embryo body showed

fluorescence with the highest XET action being localised in the cotyledons. An extremely strong fluorescence was also observed around the incision of torpedo stage embryo (Fig. 5c). The cell aggregates that accompanied somatic embryos showed strong fluorescence too (Fig. 5i). Only low-level fluorescence was observed in control embryos incubated with cellobiose-SR, which is not an effective XET acceptor substrate (this background was also observed for non-incubated embryos): the fluorescence measurements taken after the embryos had been incubated with cellobiose—SR were set as a threshold level for the confocal microscope calibration (Fig. 5j). It should be pointed out the higher-than-background (thus distinguishable) XET action was observed only in the most external layers, except for the root–hypocotyl intermediate region where fluorescence was observed as a ring encompassing several cell layers (Fig. 5e). These data, however, do not exclude the presence of much lower XET activity inside the embryo which was impossible to detect on cellular level in whole-mount experiments because of the relatively big size of the experimental object and, therefore, low magnification used for the confocal microscopy observations.

Fig. 5 XET action in cucumber somatic embryos. After a 1-h incubation in the cellobiose-SR (control—J) or XGOs-SR working solutions, somatic embryos and cell aggregates were observed under the confocal microscope. The pictures in Nomarski optics are shown on the left of each object studied except for (F). The scale bars represent 100 μm. **a** XET action in a late heart stage somatic embryo. **b** XET action in an early torpedo stage somatic embryos. **c** Torpedo stage wounded. **d, e1, e2, f** Somatic embryos with arrested cotyledons and strongly elongating root. **g** Somatic embryo with well-developed cotyledons and elongated root. **h** Somatic embryo showing a balanced development, with a proper plumular part. **i** An aggregate composed of small cells. **j** An aggregate with adjacent somatic embryos; control incubation in which the XGO-SR working solution has been replaced by its cellobiose-SR counterpart
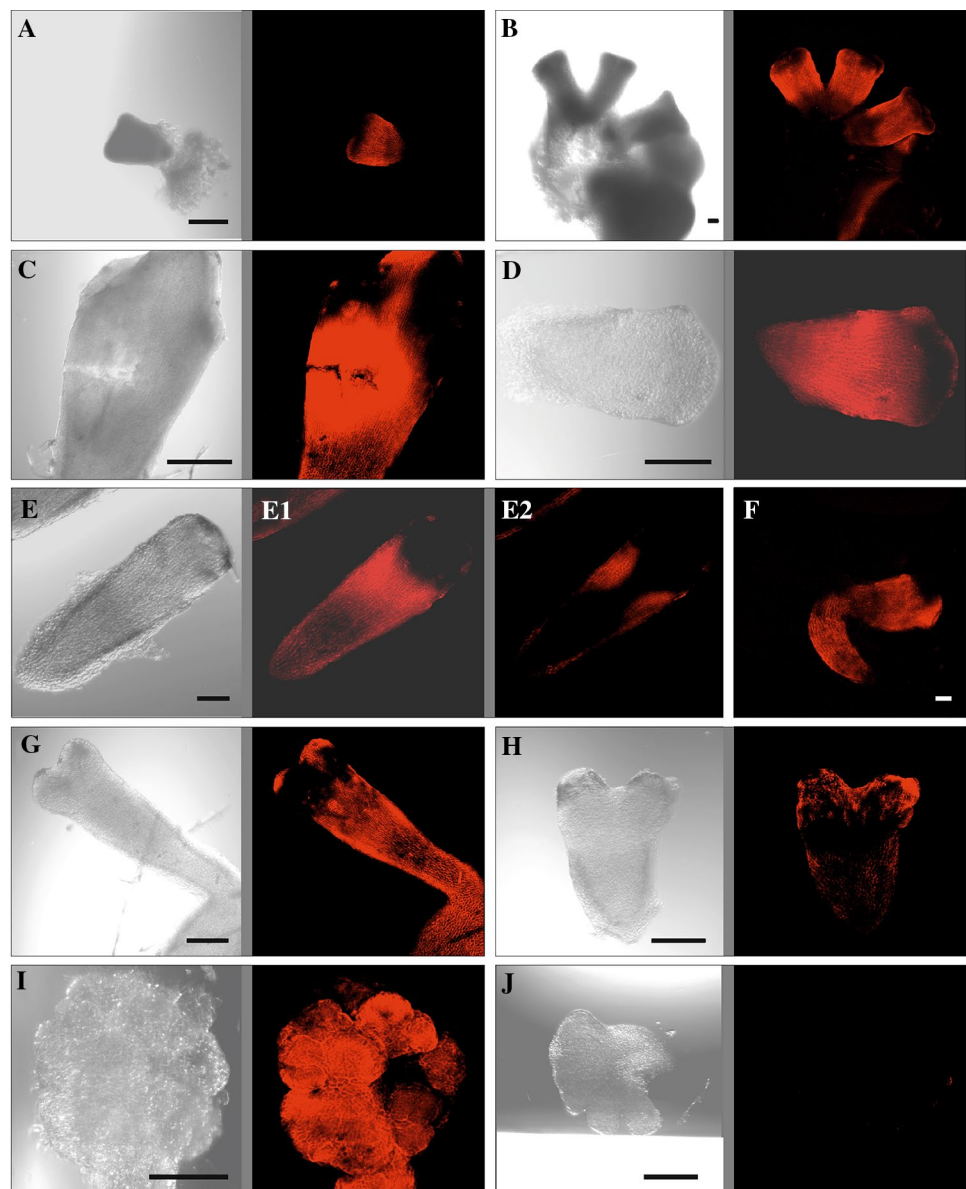
Discussion

Cucumber embryogenic suspension cultures provide an efficient and useful means of studying gene expression during embryogenesis (Malinowski et al. 2004; Grabowska et al. 2009; Wiśniewska et al. 2012, 2013), mainly because there is no need for plant regeneration and de novo induction of somatic embryogenesis. Previously, we described two cucumber *XTH* genes, *CsXTH1* and *CsXTH3*, which were involved in somatic embryogenesis (Linkiewicz et al. 2004; Malinowski et al. 2004; Wiśniewska et al. 2012). Here, we present the promoter::GUS fusions analyses of these genes as well as localisation of XET action in cucumber somatic embryos.

The *Cs-XTH1 and Cs-XTH3* genes showed developmental regulation. We suggest that this developmental regulation may be correlated to mechanical stress generated internally during tissue growth and differentiation, a proposal which is supported by the activity of both promoters in the silique dehiscence and abscission zones, where several tensions occur (Fig. 3b, c). The strong promoter activity in elongating parts of roots (Fig. 2) and growing cotyledons of cucumber somatic embryos (Fig. 5g, h) can also be related to internal local changes in turgor pressure. The widespread roles of mechanical stimuli in plant development were discussed by Braam (2005).

The observed XET action in regions of the somatic embryos highly responsive to gravity changes (predicted sites of peg formation; Fig. 5a, b, d–g) or in cotyledons

capable of further growth (Fig. 5g, h) can also be correlated to mechanical changes at the surface of the embryo. When cucumber seeds germinate, they can develop the peg in response to the direction of applied gravity changes (Takahashi et al. 1997). Saito et al. (2004) suggested that this gravistimulation response is related to auxin sensing. During suspension culture growth, the gravitational disturbances are obvious and, consequently, many genes are mechanically activated (Kieran et al. 2000). Soga et al. (2001) reported that xyloglucan metabolism can be modified in response to gravity changes. Such responses could explain the pattern of XET action observed in the cucumber somatic embryos. However, the question of why the plant needs such specific developmental regulation of *XTH* genes is beyond the scope of the present investigation.

Besides postulated mechanical stress engagement both in plant development and studied genes regulation, there could be other stress factors involved. The diversity of potential development- and stress-related *cis* elements that we have detected in the regulatory regions of *Cs-XTH1* and *Cs-XTH3* (supplementary Table 1) reflects the possible complexity of their regulation. The analysis of GUS activity driven by 5′-truncated promoters of *Cs-XTH1* and *Cs-XTH3* allowed us to identify the promoter regions which are necessary for their efficient transcription. However, further investigations are required to discover the factors which bind to them.

There is a number of stress factors which are often linked to tissue culture and somatic embryogenesis. This in turn can significantly modify embryo development and the activity of particular genes. In contrast to zygotic embryos, which are to some extent isolated from the external environment (seed coat, endosperm, ovule sac), somatic embryos are exposed to external factors that may be able to influence their development. The lack of an endosperm disturbs nutrition, influences the mechanical properties of the region surrounding the embryo and even modifies the size of the embryo (Bonello et al. 2002; Berger 2003; Garcia et al. 2005). A somatic embryo developing in suspension culture may be exposed to hypoxia stress (Merkle et al. 1995). Chapman et al. (2000) reported that one of the most important features during the differentiation and subsequent development of somatic embryos is its ability to arrest or moderate the growth of some cell layers in highly hypotonic conditions (Chapman et al. 2000). Several reports describe the crucial role of cell wall-modifying enzymes, such as glucanases (Helleboid et al. 1998) or peroxidases (Cordewener et al. 1991), in this process.

The promoter activities of *Cs-XTH1* and *Cs-XTH3* observed in the heterologous system (*Arabidopsis thaliana*) used in the present investigation reveal that external mechanical factors are able to play an important role in the regulation of the expression of these genes. Both genes were rapidly induced by wounding (Fig. 3a). *Cs-XTH1* promoter

activity in the cucumber plants was also induced by wounding (Fig. 3e). As with the *XTH22* gene (Braam and Davis 1990), touch induction of both *XTH* genes was also observed (Fig. 3d).

Based on the results presented here, it would appear that there are two equivalent regulatory mechanisms driving the expression of *Cs-XTH1* and *Cs-XTH3*—one developmental and the second adaptive. In many of the processes related to embryogenesis, there is a possible interplay between these mechanisms. The expression of many genes active during somatic embryogenesis can be also induced by stress factors (reviewed in Fehér et al. 2003). Moreover, somatic embryogenesis itself can be induced by stress factors (Gaj 2002; Ikeda-Iwai et al. 2003).

We hypothesise that the expression of the two cucumber *XTH* genes investigated here can be correlated to the need for local cell wall strengthening by the integration of newly secreted xyloglucan molecules during somatic embryo development in a highly stressful environment (Thompson et al. 1997; Sasidharan et al. 2011). However, the involvement of these genes in cell wall loosening by increasing later molecular rearrangement (Thompson and Fry 2001) cannot be also excluded. Moreover, the specific function of the genes analysed should be under additional influence of the activity of redundant *XTH* family members and molecular structure of substrates available (Eklöf and Brumer 2010; Nishikubo et al. 2011).

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