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A gibberellin-regulated xyloglucan endotransglycosylase gene is expressed in the endosperm cap during tomato seed germination

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Abstract

Xyloglucan endotransglycosylases (XETs) modify xyloglucans, major components of primary cell walls in dicots. A cDNA encoding an XET (*LeXET4*) was isolated from a germinating tomato (*Lycopersicon esculentum* Mill.) seed cDNA library. DNA gel blot analysis showed that *LeXET4* is a single-copy gene in the tomato genome. *LeXET4* mRNA was strongly expressed in germinating seeds, was much less abundant in stems, and was not detected in roots, leaves or flower tissues. During germination, *LeXET4* mRNA was detected in seeds within 12 h of imbibition with maximum mRNA abundance at 24 h. Tissue prints showed that *LeXET4* mRNA was localized exclusively to the endosperm cap region. Expression of *LeXET4* was dependent on exogenous gibberellin (GA) in GA-deficient (*gib-1* mutant) tomato seeds, while abscisic acid, a seed germination inhibitor, had no effect on *LeXET4* mRNA expression in wild-type seeds. *LeXET4* mRNA disappeared after radicle emergence, even though degradation of the lateral endosperm cell walls continued. The temporal, spatial and hormonal regulation pattern of *LeXET4* gene expression suggests that XET has a role in endosperm cap weakening, a key process regulating tomato seed germination.

Key words: Cell wall, germination, *Lycopersicon esculentum*, xyloglucan endotransglycosylase, XET.

Introduction

In seeds in which the embryo is embedded in rigid endosperm tissue, two opposing forces govern germination. One is the growth potential provided by embryo expansion, while the other is the physical restraint of the endosperm. Radicles of gibberellin (GA)-deficient mutant (*gib-1*) tomato (*Lycopersicon esculentum* Mill.) seeds cannot emerge from the seed in the absence of exogenous GA, but can do so when the endosperm tissue opposite the radicle tip (endosperm cap) is removed, leading to the conclusion that weakening of the endosperm cap is a prerequisite for radicle emergence (Groot and Karssen, 1987). Cell wall modification is considered to be a major factor controlling the weakening process (Groot *et al.*, 1988), resulting in extensive physical changes in endosperm cap cell walls during germination (Sánchez *et al.*, 1990; Nonogaki *et al.*, 1998; Toorop *et al.*, 2000).

Models of the primary cell wall describe it as a dynamic network of cellulose and hemicellulose embedded in a matrix of pectic polysaccharides plus structural proteins (Carpita and Gibeaut, 1993). Physical changes in plant cell walls could result from several types of modification, including cleavage of the backbone of the major matrix polymers, weakening of the non-covalent bonds between polysaccharides, and breakage of cross links between matrix polymers (Cosgrove, 1999). Since cell wall hydrolysis is associated with endosperm cap weakening (Watkins *et al.*, 1985; Sánchez *et al.*, 1990), wall hydrolases are regarded as good candidates to control the weakening process. Many cell wall hydrolases or their

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expressed mRNAs have been identified from tomato endosperm caps, including endo-β-mannanase (Groot *et al*., 1988; Nonogaki *et al*., 2000), polygalacturonase (Sitrit *et al*., 1999), β-1,3-glucanase and chitinase (Wu *et al*., 2001), β-1,4-glucanase, arabinosidase and others (reviewed in Bradford *et al*., 2000), but the precise biochemical mechanisms of action of these enzymes in endosperm weakening remain unclear (Bewley, 1997).

Expansins, novel wall proteins proposed to be involved in cell wall expansion (McQueen-Mason *et al*., 1992), are also expressed in germinating seeds (Chen and Bradford, 2000; Chen *et al*., 2001). Lacking significant hydrolytic activity, expansins are proposed to function by disrupting hydrogen bonding between cellulose and hemicellulose polymers (Cosgrove, 1999). In dicots, the principal hemicellulosic component in the primary cell walls is xyloglucan, which is thought to form a tightly bound, non-covalent association with cellulose (McCann *et al*., 1990). Since this cellulose/xyloglucan network is believed to represent a major constraint to turgor-driven cell expansion (Whitney *et al*., 1999), breakage of these critical loading-bearing linkages or associations may be an essential feature of wall loosening (Catala *et al*., 2000). In addition, expansins are expressed in association with fruit ripening (Rose *et al*., 1997; Brummell *et al*., 1999) and abscission (Cho and Cosgrove, 2000), indicating that they are involved in wall disassembly processes where expansion does not occur. This is also the case in seed germination, where expression of an expansin gene (*LeEXP4*) specifically in the tomato endosperm cap was correlated with weakening of this tissue during germination (Chen and Bradford, 2000).

Xyloglucan endotransglycosylases (XETs) may also be involved in the modification of the cellulose/xyloglucan network. XETs catalyse both the endo-type splitting of a xyloglucan molecule and the linking of the newly generated reducing end to a non-reducing end of another xyloglucan molecule or oligosaccharide (Fry *et al*., 1992; Nishitani and Tominaga, 1992). This lengthening and rearrangement of xyloglucans may release tension and accommodate wall expansion. Depending on the relative size of acceptor molecules, transglycosylation by XET can increase or reduce the length of polysaccharides, which could result in either cell wall expansion or wall disassembly (Campbell and Braam, 1999). Like expansins, XETs are expressed both in growing tissues and in ripening fruits (Rose and Bennett, 1999). XETs are also expressed in other developmental processes where cell wall modification or disassembly occurs, such as aerenchyma formation (Antosiewicz *et al*., 1997; Saab and Sachs, 1996) and reserve mobilization following germination in xyloglucan-storing seeds (de Silva *et al*., 1993; Fanutti *et al*., 1993; Rose *et al*., 1996; Tine *et al*., 2000).

It is likely that expansins, XETs and other cell wall hydrolases co-operate in cell wall modification to achieve specific developmental results (Rose and Bennett, 1999; Catala *et al*., 2000). This hypothesis is supported by an expansin action assay using cellulose/hemicellulose composites, which indicated that the target of expansins in the cell walls might be the cellulose/xyloglucan matrix (Whitney *et al*., 2000). By co-ordinate action on the same substrate, expansins and XETs might co-operatively loosen cell walls to accommodate expansion or facilitate disassembly. Since tissue-specific expression of an expansin (Chen and Bradford, 2000) and of several cell wall hydrolases (Sitrit *et al*., 1999; Bradford *et al*., 2000; Nonogaki *et al*., 2000) has been correlated with endosperm cap weakening during tomato seed germination, it was tested whether XET genes are also expressed in this tissue. The identification and characterization of an XET gene (*LeXET4*) that is expressed specifically in the endosperm cap of germinating tomato seeds prior to radicle emergence is reported here.

Materials and methods

Plant materials

Tomato seeds of either wild type (cv. Moneymaker; MM) or the homozygous GA-deficient (*gib-1*) mutant were used in the study. The *gib-1* mutant and its isogenic parent line were originally obtained from Dr Cees Karssen, Wageningen Agricultural University, The Netherlands. Field-grown *gib-1* plants were sprayed three times per week with 100 μM GA_{4+7} to revert the dwarf habit and allow more vigorous growth and fertility. Seeds were extracted immediately after fruits were harvested, treated with 0.25 M HCl, dried to 6% moisture content (fresh weight basis) and stored at 0 °C until used (Ni and Bradford, 1993). For germination, seeds were incubated at 25 °C in the dark in 9 cm diameter Petri dishes on top of two layers of filter paper moistened with 12 ml of either distilled water, 100 μM GA_{4+7}, or 100 μM abscisic acid (ABA).

RNA isolation, PCR amplification and cDNA library screening

Total RNA was extracted from imbibed tomato seeds. Whole seeds (100) were pulverized in liquid N_2 and the frozen material transferred to 2 ml of extraction buffer (10 mM Tris-HCl pH 8.2, 100 mM LiCl, 1 mM ethylenediaminetetraacetate [EDTA], 1% [w/v] sodium dodecyl sulphate [SDS], 25 mM dithiothreitol [DTT]) in a ground glass homogenizer on ice. The slurry was further homogenized and centrifuged at 15 000 *g* for 1 min at 4 °C. Extraction was carried out following a modification of the phenol/SDS method (Ausubel *et al*., 1987). Degenerate PCR primers were designed based on two conserved amino acid domains according to the alignment of deduced amino acid sequences from known XET genes (Okazawa *et al*., 1993; Aubert and Herzog, 1996) as follows: (5′)GA(G/A)CA(C/T)GA(C/T)GA(G/A)AT(A/C/T)GA(C/T)TT(C/T)G and (3′)TC(A/C/G/T)GT(G/A)CA(G/A)TA(G/A)-TT(G/A)TA(A/G/T)AT(A/C/G/T)G. Total RNA extracted from germinating seeds was used as the template for reverse transcription-polymerase chain reactions (RT-PCR). After amplification for 35 cycles (94 °C for 1 min, 50 °C for 1.5 min, and 72 °C for 1.5 min), the resulting ~500 base pair fragment was cloned into pCR2.1 vector (Invitrogen, Carlsbad,

CA). DNA sequences were determined with universal primers T3 and M13-forward, using an Applied Biosystems model 377 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). After the sequence was confirmed to be homologous to known XETs, the PCR fragment was used as probe to screen a cDNA library prepared from *gib-1* seeds imbibed in 100 μM GA_{4+7} in order to obtain a full-length cDNA. The cDNA was labelled with enhanced chemiluminescence (ECL) nucleic acid labelling reagents (ECL kit, Amersham Life Science, Arlington Heights, IL) at 37 °C for 10 min, then was added to prehybridization solution at a final concentration of 10 ng ml^{-1}. Hybridization was for 2 h at 42 °C, followed by washing twice at 42 °C with 6 M urea, 0.5% saline sodium citrate (SSC; low stringency) or 0.2% SSC (high stringency) for 20 min, and twice at room temperature with 2×SSC for 5 min. Independent inserts in the library vector pBK-CMV were sequenced. The longest cDNA named *LeXET4* (Genbank Accession No. AF186777) was selected for further characterization.

Genomic DNA extraction and gel blot analysis

For Southern blotting, genomic DNA was isolated from young tomato leaves as described previously (Murray and Thompson, 1980) and modified (Bernatzky and Tanksley, 1986). Aliquots (10 μg) were digested with restriction enzymes, fractionated on a 0.8% (w/v) agarose gel, and transferred to Hybond-N^+ membranes (Amersham). Probes were prepared by PCR using primers from the 3′-untranslated region of the gene. Probe labelling, prehybridization, hybridization, washing, and visualization of the membrane were performed as described for cDNA library screening using ECL, except that hybridization was for 16 h.

Northern blot analysis

Total RNA was extracted from seeds or from root, leaf, flower, and stem tissues of tomato plants as described above. Total RNA (10 μg) from each sample was subjected to electrophoresis on 1.2% (w/v) agarose/10% (v/v) formaldehyde denaturing gels, transferred to Hybond N^+ membrane and UV-crosslinked. The 3′ terminal region of *LeXET4* was used as a gene-specific probe for all Northern blots and tissue printing. Digoxigenin (DIG)-labelled RNA probe was prepared by transcription using T7 or T3 RNA polymerase. Hybridization and washing followed the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN) with slight modification. The membrane was hybridized overnight with DIG-labelled probe at 65 °C, washed once at room temperature with 2×SSC and 0.1% SDS and twice at 72 °C with 0.2×SSC and 0.1% SDS. The membrane was rinsed with washing buffer for 5 min and transferred to blocking buffer (5% milk powder in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]). One hour later, antibody was added and incubated for another 30 min. The membrane was washed twice with washing buffer (maleic acid buffer with 0.05% Tween 20) and incubated in detection buffer for 4 min before substrate (Lumi-Phos^{R}530; Lumigen, Southfield, MI) was added for 5 min. Exposure times were from 20 min to 2 h depending on the signal strength.

Tissue printing

After 24 h of imbibition as described above, tomato seeds were bisected using a razor blade. The cut surfaces were pressed onto a Hybond-N^+ membrane for 10–15 s before the tissue was removed. The membrane was cross-linked using UV light and hybridized with RNA probes (both sense and antisense). Hybridization and washing conditions were as for Northern blots. Signals were detected by incubating the membranes in 0.18 M Tris-HCl buffer (pH 8.8) containing 0.025 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate, 0.1 mg ml^{-1} nitroblue tetrazolium and 2 mM $MgCl_2$ (Nonogaki *et al.*, 2000). Reaction times varied from 1 h to overnight depending on the signal strength.

Results

Cloning and phylogenetic and genomic analyses of LeXET4 cDNA

A 500 bp band was amplified from RNA of germinating tomato seeds by RT-PCR using degenerate primers corresponding to two conserved regions of known XET genes. Sequence analysis indicated the existence of one XET homologue. A cDNA library from germinating tomato seeds was screened with this PCR product and a full-length cDNA was isolated. When compared with XET sequences in the database, this cDNA was clearly an XET homologue, but was distinct from known tomato XETs and was therefore named *LeXET4* (Genbank Accession No. AF186777). *LeXET4* encoded a predicted peptide of 295 amino acids with an *N*-terminal signal peptide sequence of 22 amino acids when analysed using signal peptide prediction software (http://www.cbs.dtu.dk/services/SignalP/). An ATG codon initiated an open reading frame at nucleotide 24 and a TAA consensus stop codon was present at nucleotide 909. When the deduced amino acid sequence of *LeXET4* was aligned with other tomato XET genes and with two other XET genes expressed in seeds of *Arabidopsis thaliana* (L.) Heynh. and nasturtium (*Tropaeolum majus* L.), respectively (Fig. 1), *LeXET4* showed the highest homology (81%) with tomato *LeXET1*, which is expressed in tomato leaves and young fruits (Catala *et al.*, 1997, 2000). The deduced amino acid sequence of *LeXET4* contained the features conserved in known XET genes, including a hydrophobic signal peptide region, a central DEIDFEFLG sequence shared with β-glucanases (amino acids 105–113 in LeXET4), an *N*-linked glycosylation consensus site (N-X-S/T) adjacent to the conserved DEIDFEFLG motif (amino acids 114–116 in LeXET4), and two pairs of cysteines in the more variable carboxyl-terminal region (Fig. 1; Campbell and Braam, 1999). The amino acid sequences of representative full-length cDNAs of XET genes were used to generate a phylogenetic tree (Fig. 2). LeXET4 was most closely related to Group 1 XETs, one of the two major groups of XET sequences (Campbell and Braam, 1999). Even though *LeXET4*, *AtXTR8* (Aubert and Herzog, 1996), *TmNXG1* (de Silva *et al.*, 1993), and *TmXET1* (Rose *et al.*, 1996) are all expressed in seeds, *AtXTR8* and *TmNXG1* belong

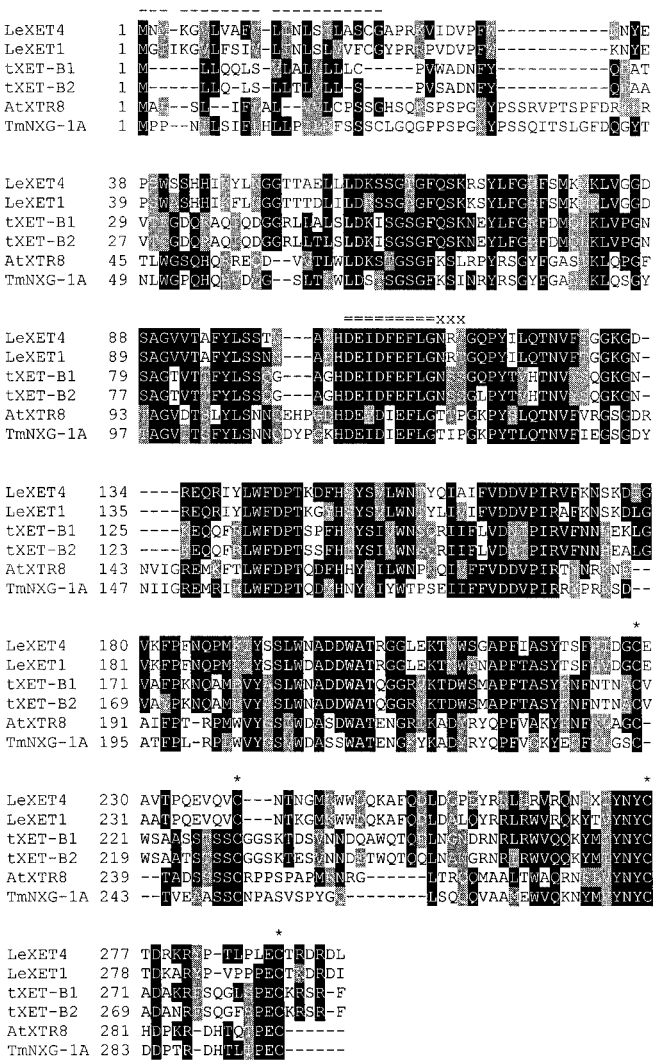

Fig. 1. Multiple alignment of the deduced amino acid sequences of full-length XET cDNAs from different species. LeXET4 (AF186777), LeXET1 (D49539), tXET-B1 (X82685), tXET-B2 (X82684), AtXTR8 (X92975), and TmNXG-1A (X68254) were aligned by the MEGALIGN program (DNASTAR Inc., Madison, WI) using the Clustal algorithm. Identical amino acids are in reverse colour and conservative substitutions are shaded using the online Boxshade program (http://www.ch.embnet.org/software/BOX_form.html). A signal sequence is indicated by a single dashed line over the LeXET4 sequence; a double dashed line denotes a region conserved between XETs and β-glucanases (amino acids 105–113 in LeXET4); xxx denotes a consensus *N*-linked glycosylation site (amino acids 114–116); asterisks mark two pairs of conserved cysteines near the carboxyl terminal region.

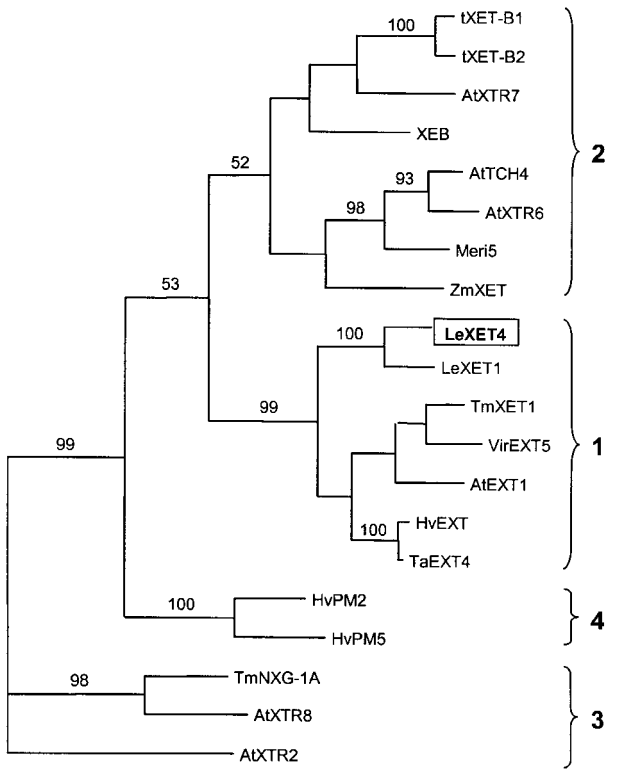

Fig. 2. Phylogenetic tree generated with the deduced amino acid sequences of full-length XET-related genes. *Arabidopsis* XTR2 was used as outgroup. Alignments were made using the default parameters of the MEGALIGN program based on the Clustal algorithm, and PAUP*4.0 was used to generate the phylogenetic tree. The XET-related genes include: TmNXG-1A (X68254) and TmXET1 (L43094) from nasturtium; AtEXT1 (D16454), AtXTR2 (U43487), AtTCH4 (U27609), AtXTR6 (U43488), AtXTR7 (U43489), AtXTR8 (X92975), Meri5 (D63508) from *Arabidopsis*; LeXET1 (D16456), LeXET4 (AF186777), tXET-B1 (X82685), tXET-B2 (X82684) from tomato; VirEXT5 (D16458) from azuki bean (*Vigna angularis* (Willd.)); HvEXT (X91659), HvPM2 (X91660), HvPM5 (X93173), and XEB (X93175) from barley; TaEXT4 (D16457) from wheat (*Triticum aestivum* L.), and ZmXET (U15781) from maize (*Zea mays* L.). The major groups indicated along the right side are consistent with those in previous publications (Campbell and Braam, 1999).

to Group 3, while *LeXET4* and *TmXET1* belong to Group 1 (Fig. 2).

The 3′-terminal untranslated region of *LeXET4* was used as the probe for genomic DNA gel-blot analysis. The probe strongly hybridized with single genomic DNA fragments following *Hin*dIII and *Bam*HI digestion (Fig. 3), indicating that *LeXET4* is a single-copy gene and that the probe is gene-specific. An RNA probe transcribed from the same region was used subsequently to hybridize with the RNA gel blots and tissue prints.

Spatial and temporal expression patterns of LeXET4

To examine the tissue specificity of *LeXET4* expression, total RNA from tomato roots, stems, leaves, flowers, and germinating seeds was hybridized with the *LeXET4*-specific RNA probe. A strong signal was detected from germinating seeds and a weak signal from stems, but no expression was detected from other tissues (Fig. 4). Thus, *LeXET4* mRNA is preferentially expressed in imbibed seeds.

The temporal expression pattern of *LeXET4* mRNA was determined in tomato seeds imbibed for 6, 12, 18, 24, 30, 36, and 42 h. *LeXET4* mRNA could be detected after 12 h and accumulated maximally at 24 h of imbibition, then decreased somewhat but remained present at 36 and 42 h (Fig. 5). Radicle emergence under these

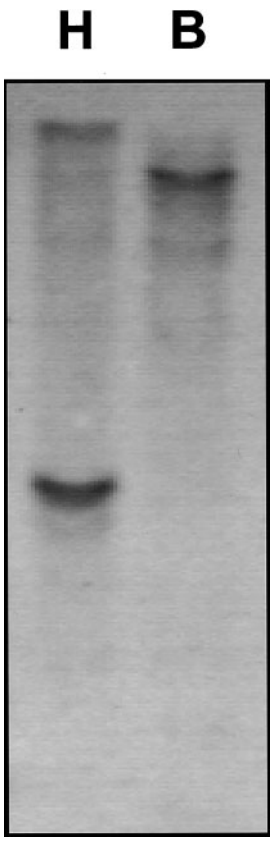

Fig. 3. Genomic DNA gel blot analysis of the tomato XET gene *LeXET4*. Tomato genomic DNA (10 μg) was digested by *Hin*dIII (H) and *Bam*HI (B), respectively, and subjected to gel blot hybridization using a gene-specific cDNA probe amplified by PCR using primers corresponding to the 3′-untranslated region of *LeXET4*. Strong hybridization was detected only to single bands of ~4 kb (H) and ~20 kb (B).

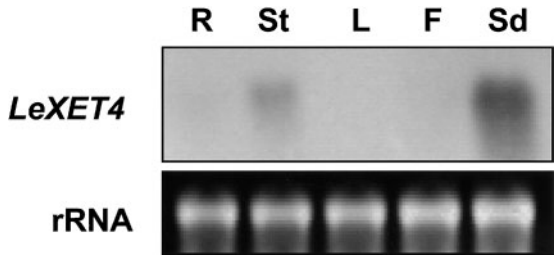

Fig. 4. RNA gel blot analysis of *LeXET4* mRNA abundance in different tissues. Total RNA was extracted from root (R), stem (St), leaf (L), and flower (F) tissues of tomato plants or from seeds imbibed for 24 h (Sd). Total RNA (10 μg) from each sample was separated by electrophoresis and hybridized with a *LeXET4*-specific cDNA probe. The lower panel shows ethidium bromide-stained rRNA to indicate the relative RNA loading of each lane.

conditions would begin to occur in a small percentage of seeds at 48 h.

Tissue printing was used to localize *LeXET4* mRNA in imbibed and germinated seeds. Wild-type MM seeds were imbibed for 24 h, bisected, and the cut surfaces were printed onto a membrane. The membranes were incubated with either antisense or sense RNA probes for

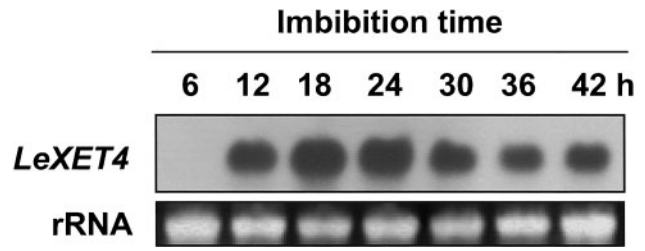

Fig. 5. RNA gel blot analysis of the timing of *LeXET4* expression in germinating seeds. Total RNA was extracted from germinating seeds after different times of imbibition. Total RNA (10 μg) from each sample was separated by electrophoresis and hybridized with a *LeXET4* gene-specific probe. The lower panel shows ethidium bromide-stained rRNA to indicate the relative RNA loading of each lane.

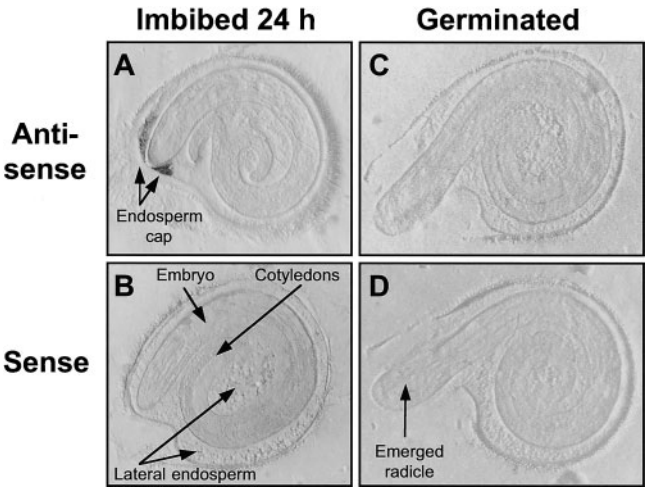

Fig. 6. Tissue printing to localize *LeXET4* expression. Tomato seeds imbibed for 24 h (A, B) or 60 h (C, D) were bisected and the cut faces were printed onto two membranes. One set of membranes was hybridized with antisense probe (A, C) and the other set was hybridized with sense probe (B, D). *LeXET4* mRNA was localized specifically to the endosperm cap prior to radicle emergence (A), but was not detected in germinated seeds (C). No hybridization was detected in any tissue using the sense probe (B, D).

LeXET4 and hybridization was detected by a colour reaction. Expression of *LeXET4* mRNA was detected only in the micropylar endosperm cap tissue prior to radicle emergence (Fig. 6A). Tissue prints were also made of seeds after radicle emergence (60 h after imbibition), but no signal was detected in any seed tissues (Fig. 6C). No hybridization was detected using sense probes (Fig. 6B, D), indicating that the hybridization in Fig. 6A is specific for *LeXET4* mRNA.

Accumulation of LeXET4 mRNA is induced by exogenous GA in gib-1 seeds but is not affected by ABA in wild-type seeds

Tissue printing was also performed to determine the expression pattern of *LeXET4* in response to hormones. For each treatment, ten seeds were bisected and printed on membranes. *LeXET4* mRNA accumulated exclusively

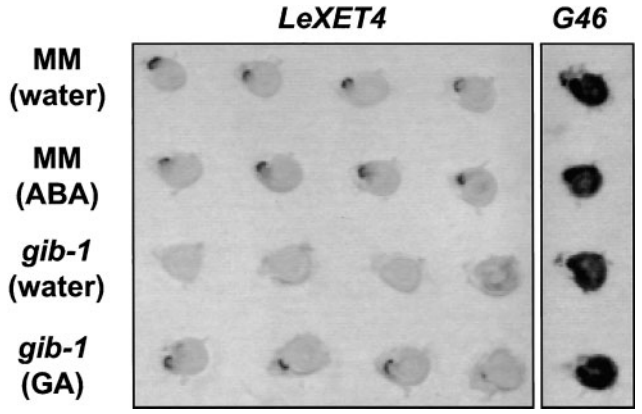

Fig. 7. Tissue printing for detection of *LeXET4* in wild-type and *gib-1* tomato seeds under different conditions. After 24 h of imbibition of MM seeds in water or 100 μM ABA, and of *gib-1* seeds in water or 100 μM GA_{4+7}, seeds were bisected and the cut surfaces were printed onto two membranes. One membrane was hybridized with antisense *LeXET4* RNA and the other one was hybridized with RNA of *G46*, which codes for a constitutive ribosomal protein (Cooley *et al*., 1999). Endosperm cap-specific expression was consistently detected in endosperm caps of MM seeds in water or ABA and in *gib-1* seeds in GA (four seeds shown of ten tested for each condition). The *G46* panel of representative prints shows that RNA was transferred uniformly to the membrane by tissue printing.

in the endosperm caps of MM seeds imbibed in either water or 100 μM ABA (Fig. 7). No expression was observed in *gib-1* seeds in water, but imbibition in 100 μM GA_{4+7} resulted in *LeXET4* mRNA accumulation specifically in the endosperm caps (Fig. 7).

Discussion

XETs are associated with diverse developmental processes, including growth (de Silva *et al*., 1994; Smith *et al*., 1996), fruit ripening (Maclachlan and Brady, 1994; Redgwell and Fry, 1993; Schroeder *et al*., 1998), aerenchyma formation in roots (Saab and Sachs, 1996) and leaves (Antosiewicz *et al*., 1997), and reserve mobilization in seeds during and after germination (de Silva *et al*., 1993; Tine *et al*., 2000). In germinated nasturtium seeds, XETs were proposed to be responsible for the mobilization of cell wall xyloglucan reserves (Fanutti *et al*., 1993). Two different nasturtium XET genes, *TmXET1* and *TmNXG1*, exhibited mutually exclusive expression patterns following germination, with the first being expressed in all vegetative tissues except cotyledons, whereas transcripts of the second were detected solely in cotyledons, suggesting distinct physiological roles for these two XETs (Rose *et al*., 1996).

Similarly, the specific tissue localization and timing of *LeXET4* expression (Figs 5, 6A, 7) suggests a physiological role for this XET in tomato seed germination. In wild-type MM seeds, *LeXET4* mRNA was detected from 12 h after imbibition (Fig. 5), when endosperm cap weakening can first be detected (Groot and Karssen, 1987; Chen and Bradford, 2000; Toorop *et al*., 2000; Wu *et al*., 2001). *LeXET4* mRNA was exclusively localized to the micropylar endosperm cap tissue prior to radicle emergence (Fig. 6A), and was not detected in either the endosperm cap or the lateral endosperm of germinated seeds (Fig. 6C). The lateral endosperm cell walls are degraded following radicle emergence to provide nutrients for seedling growth, but if XET activity were required for this, a different isoform of XET would apparently need to be expressed, as in the case of endo-β-mannanase. Mannans are major hemicellulosic constituents of tomato endosperm cell walls, and mannanases are proposed to be involved in both endosperm cap weakening and lateral endosperm degradation (Bewley, 1997). Distinct endo-β-mannanase genes are expressed in the endosperm cap prior to radicle emergence (*LeMAN2*) and in the lateral endosperm after radicle emergence (*LeMAN1*) (Nonogaki *et al*., 2000). This implies that weakening of the endosperm cap and degradation of cell wall carbohydrate reserves in the lateral endosperm are distinct physiological processes that utilize specific enzyme isoforms. As the library screened to obtain the *LeXET4* cDNA was prepared from seeds imbibed for only 24 h (prior to radicle emergence), it would not be expected to contain any XET cDNAs that might be expressed only after germination. Additional XET genes expressed in the lateral endosperm or in the embryo following germination may remain to be discovered in tomato.

The expression pattern of *LeXET4* in *gib-1* seeds is also consistent with a role for XET in endosperm cap weakening. When imbibed in water, no expression of *LeXET4* was detected in *gib-1* seeds (Fig. 7), and no weakening of the endosperm cap occurred (Groot and Karssen, 1987; Chen and Bradford, 2000). However, when weakening was induced by exposure to GA (Groot and Karssen, 1987; Chen and Bradford, 2000), *LeXET4* expression occurred specifically in the endosperm cap region (Fig. 7). This localized pattern of induction by GA was also observed for other endosperm cap-specific genes, such as *LeEXP4* (Chen and Bradford, 2000) and *LeMAN2* (Nonogaki *et al*., 2000). Even though the entire seed was in contact with GA solution, the induction of *LeXET4*, *LeEXP4*, and *LeMAN2* mRNA accumulation occurred only in the endosperm cap, indicating that both cell type and GA are involved in regulating gene expression. GA can induce expression of other genes in the *gib-1* tomato embryo within 12 h of imbibition (Cooley *et al*., 1999; Chen *et al*., 2001), indicating that the restriction of *LeXET4* expression to the endosperm cap was not due to an inability of GA to access other tissues. In *Arabidopsis* seeds, expression of the XET gene *AtXTR8* increased just prior to radicle emergence and continued to be present during early seedling growth (Aubert and

Herzog, 1996). As in tomato, GA induced expression of this gene in seeds of the GA-deficient *ga1-3* mutant of *Arabidopsis*. GA also enhanced expression of XET genes in growing leaves of both wild-type and GA-responsive dwarf mutants of barley (*Hordeum vulgare* L.) (Smith *et al*., 1996) and rice (*Oryza sativa* L.) (Uozu *et al*., 2000), indicating that regulation of expression of XET genes by GA is not limited to seeds. Other plant hormones, including auxin and brassinosteroids, have also been shown to regulate expression of XET genes in a tissue-specific manner (Xu *et al*., 1995, 1996; Catala *et al*., 2000; Uozu *et al*., 2000).

ABA did not inhibit *LeXET4* mRNA accumulation (Fig. 7), which is consistent with the lack of effect of ABA on *LeEXP4*, *LeMAN2* and most other endosperm cap-specific genes isolated from germinating tomato seeds (Bradford *et al*., 2000; Chen and Bradford, 2000; Nonogaki *et al*., 2000; *GluB* is an exception, Wu *et al*., 2001). ABA was required for an increase in XET activity associated with growth maintenance in maize (*Zea mays* L.) roots under water stress (Wu *et al*., 1994). However, while ABA inhibits radicle emergence of tomato seeds, it does not block the major initial phase of endosperm cap weakening (Toorop *et al*., 1996, 2000; Chen and Bradford, 2000; Wu *et al*., 2001). Thus, the correlation between endosperm cap weakening and expression of *LeXET4* and other cell wall-modifying proteins in this tissue is maintained.

The substrate of XET is xyloglucan, which is a major hemicellulosic component in dicot cell walls. However, unlike nasturtium cotyledons, xyloglucan is not a major storage component in the cell walls of tomato endosperm caps, based on their low xylose content (only ~2% of total wall sugars) compared to that in, for example, the tomato embryo (~13% of total wall sugars) (Dahal *et al*., 1997). Instead, mannan is the major hemicellulose in the endosperm, with mannose representing ~60% of total wall sugars, compared to only 30% mannose in embryo cell walls (Groot *et al*., 1988; Dahal *et al*., 1997). With the limited amount of xylose (and thus, xyloglucan) in the endosperm walls, it is reasonable to ask whether XET can play an important role in wall disassembly. However, these values are calculated as a fraction of total cell wall sugars. If the additional storage mannans in the endosperm are disregarded, the percentage of xylose in the remaining cell wall fraction is higher. In addition, when the sugars released from endosperm caps into the incubation medium were analysed, xylose represented approximately 10% of the released sugars (after hydrolysis) (Dahal *et al*., 1997). Thus, xylose-containing components were released preferentially relative to their percentage composition in the cell walls. However, the testa has a comparatively high xylose content (20% of total wall sugars; Dahal *et al*., 1997), and some of the xylose released into the medium could have originated from the testa. As an extracellular enzyme, XET could act on the testa, which presents an additional impediment to radicle emergence external to the endosperm cap (Hilhorst and Downie, 1996). Cellular and biochemical studies of the localization of LeXET4 protein and of its enzymatic action on cell walls of tomato seed tissues will be required to determine what substrates are present and whether they are modified or hydrolysed by the enzyme.

Even though xyloglucan apparently is not a major wall component in endosperm cap tissue, the cellulose/xyloglucan network might still comprise critical load-bearing linkages that would need to be modified to allow wall disassembly with the co-operation of expansin and other enzymes, as has been proposed (Rose and Bennett, 1999). In a study to probe expansin action, Whitney *et al*. found that expansin protein was active on polymer composites containing xyloglucan but not on those made with mannan polymers (Whitney *et al*., 2000). This and related studies concluded that the cellulose/xyloglucan matrix is indeed the site of expansin action, and inferred that the cellulose/xyloglucan network may be the primary determinant of mechanical properties in growing walls (Whitney *et al*., 1999, 2000). Expansin (*LeEXP2*) and XET (*LeEXT1*) genes were co-ordinately expressed early in tomato fruit development and in auxin-treated tomato hypocotyls (Catala *et al*., 1997, 2000), and the present study's results similarly show co-ordinate expression of specific expansin (*LeEXP4*; Chen and Bradford, 2000) and XET (*LeXET4*; Fig. 6A) genes in the endosperm cap, potentially allowing co-operation between these two cell wall-modifying factors. By acting on the same substrate, XET and expansin might co-operatively modify the cellulose/xyloglucan network to accommodate various physiological processes, in this case leading to endosperm cap weakening. On the other hand, expansin and XET might not be sufficient to result in complete endosperm cap weakening. The presence of multiple cell wall hydrolases such as mannanase, polygalacturonase and others in the same tissue (Sitrit *et al*., 1999; Bradford *et al*., 2000; Nonogaki *et al*., 2000) supports the concept of concerted and sequential action of cell wall-modifying proteins in tomato endosperm cap weakening.

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