Two tomato \(\alpha\)-expansins show distinct spatial and temporal expression patterns during development of nematode-induced syncytia

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Cyst nematodes induce specific syncytial feeding structures within the root which develop from an initial cell by successive incorporation of neighbouring cells through local cell wall dissolutions followed by hypertrophy of included cells. Expansins are known to induce cell wall relaxation and extension in acidic pH, and they are involved in many processes requiring wall modification from cell expansion to cell wall disassembly. We studied the expression pattern of tomato (\textit{Lycopersicon esculentum} L., cv. Money Maker) expansins during development of syncytia induced by the potato cyst nematode (\textit{Globodera rostochiensis} Woll.). Based on semi-quantitative reverse transcription–polymerase chain reaction, two expansin genes, \textit{LeEXPA4} and \textit{LeEXPA5}, were selected for detailed examinations because their expression was either elevated in infected roots (\textit{LeEXPA4}) or specifically induced in the root upon nematode infection (\textit{LeEXPA5}). Both genes have distinct spatial and temporal expression patterns that may reflect their different roles in syncytium development. \textit{LeEXPA4} transcripts were localized predominantly in parenchymatous vascular cylinder cells surrounding syncytia. This finding suggests that \textit{LeEXPA4} might be involved in cell wall disassembly or relaxation, mediating syncytium expansion and/or development of conductive tissues. By contrast, \textit{LeEXPA5} transcripts were localized in enlarging syncytial elements. Similarly, in immunogold localization experiments, polyclonal antibodies localized the \textit{LeEXPA5} protein in cell walls of syncytial elements. This expression pattern suggests that \textit{LeEXPA5} gene is specifically involved in enlargement of cells incorporated into syncytium.

Introduction

Cyst nematodes, such as \textit{Globodera rostochiensis}, have the unique ability to induce morphological and physiological changes in the host root, leading to the formation of a syncytial feeding site. The syncytium serves as the only source of nutrients for the parasite during its entire life and has structural features of transfer cells (Jones 1981). Second-stage juveniles (J2) induce syncytia preferentially in the root elongation zone by releasing gland secretions into single selected plant cell. The

\textbf{Abbreviations} – BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; dNTP, deoxyribonucleotide triphosphate; FITC, fluorescein isothiocyanate; \textit{LeEXPA}, tomato \(\alpha\)-expansin gene; \textit{LeEXPB}, tomato \(\beta\)-expansin gene; RT-PCR, reverse transcription–polymerase chain reaction.
syncytium expands along the vascular cylinder by formation of openings in the walls between neighbouring cells (Grundler et al. 1998). As a result, protoplasts fuse and cells become syncytial elements (=members of a multinucleate nematode feeding site). Developing syncytium shows typical features such as proliferation of cytoplasm, enlargement of nuclei, proliferation of plastids and mitochondria, reorganization of the vacuolar system and general hypertrophy (Golinowski et al. 1997). The syncytium is a consequence of a sophisticated process of mutual exchange of molecular signals and responses between parasite and host. It is generally accepted that nematode secretions induce changes in plant gene expression, although the triggering effectors are still unknown. A number of nematode parasitism genes have been cloned (Gao et al. 2003, Vanholme et al. 2004) and many differentially expressed plant genes have been identified in parasitized root (Gheysen and Fenoll 2002), but the key molecular signalling events during this interaction are still not understood.

Cell wall rearrangement is one of the most interesting events taking place during syncytium development. These modifications comprise different, sometimes opposite processes often occurring at the same time. Just after syncytium induction, two basic processes occur. On one side, cell wall extension and thickening take place in the hypertrophied, enlarged syncytial elements; on the other side, cell wall openings are formed via local cell wall dissolution between cells incorporated into the syncytium. These processes also occur during syncytium expansion in the subsequently incorporated cells at distal parts of syncytium. At early stages of syncytium development, cell wall openings are formed by widening of plasmodesmata, while in older stages, their formation is independent from plasmodesmata (Grundler et al. 1998). Concomitantly with the local cell wall loosening and lyses, the outer syncytial wall becomes thickened to resist the osmotic pressure that increases in syncytia up to 9000–10 000 hPa (Böckenhoff and Grundler 1994). An extensive system of cell wall ingrowths is formed in mature syncytia at places bordering xylem tracheary elements (Golinowski et al. 1996, Jones 1981).

It is evident that a set of diverse enzymes and other proteins is required to lead to the observed rearrangements during syncytium differentiation. The complexity and synchronization of processes involved in cell wall modifications suggest that cyst nematodes recruit host cell wall-modifying enzymes and proteins to transform plant cells into elaborate feeding sites. Indeed, plant genes encoding polygalacturonases (Mahalingam et al. 1999) and endo-β-1,4-glucanases (Goellner et al. 2001) have been found to be upregulated in syncytia. Expansins were also shown to be involved in syncytium induction and maintenance (Wieczorek et al. 2006). They have the unique property of cell wall loosening in an acidic pH (<5) and thus enable in vivo and in vitro cell wall extension (McQueen-Mason et al. 1992). Interestingly, they do not show any enzymatic activity, and their mechanism of action is still not finally resolved. It is suggested that they disrupt non-covalent bonds between cellulose microfibrils and matrix polysaccharides in the cell wall (McQueen-Mason and Cosgrove 1994, Samper-dro and Cosgrove 2005, Yennawar et al. 2006). As a result of their activity, the cell wall is relaxed and stretched and thus facilitates turgor-mediated growth and the action of cell wall-degrading or -modifying enzymes, like transglycosylases or hydrolases (Rose et al. 1997). Expansins play an important role in physiological processes requiring cell wall modifications such as cell growth, xylem vessel differentiation, fruit ripening and organ abscission (Cho and Cosgrove 2000, Fleming et al. 1999, Im et al. 2000, Rose et al. 2000). Based on phylogenetic sequence analysis, they form a multigene superfamily consisting of four families, namely α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Kende et al. 2004). In tomato, 13 EXPA have been cloned and published till now (http://www.bio.psu.edu/expansins/genes.htm). Their expression patterns have been described in different organs and under different conditions. For example, LeEXPA1 is expressed during fruit ripening (Rose et al. 1997), LeEXPA2 is expressed in expanding tissues and in auxin-treated hypocotyls (Caderas et al. 2000, Reinhardt et al. 1998) and LeEXPA18 mediates cell expansion that accompanies cell division (Reinhardt et al. 1998, Vogler et al. 2003). In germinating tomato seeds, mRNAs of LeEXPA4, -8 and -10 were found (Chen and Bradford 2000, Chen et al. 2001). The specific functions for all tomato expansin genes are not clearly determined, as on one hand, a single gene is expressed in different organs and tissues, while on the other hand, several genes are expressed at the same time and location (Brummell et al. 1999).

Recently, microarray experiments have demonstrated the induction of LeEXPA5 in tomato roots infected with root knot nematode *Meloidogyne javanica* (Bar-Or et al. 2005). Gal et al. (2006) using in situ RT-PCR localized its transcripts in cells surrounding giant cells, which suggests that this gene could be involved in the formation of gall tissue. Moreover, root knot nematode multiplication was reduced in transgenic tomato roots carrying LeEXPA5-silencing construct delivered by *Agrobacterium rhizogenes* transformation. Although both root knot nematodes and cyst nematodes exhibit similar parasitic strategy (sedentary endoparasitism), the multinucleate feeding structures they induce, giant cells and syncytia, respectively, develop by different mechanisms and differ.
structurally (Jones 1981, Jones and Northcote 1972a, 1972b). Therefore, it is not surprising that different genes are induced during these two distinct parasitic interactions (Gheysen and Fenoll, 2002; Jammes et al., 2005; Puthoff et al., 2003). These differences are clearly evident in expression profiles of expansin genes. In the model interactions Arabidopsis–Heterodera schachtii (Wieczorek et al., 2006) and Arabidopsis–M. incognita (Jammes et al., 2005), significant differences were found in the expression pattern of expansin genes between syncytia and giant cells. For example, AtEXPA7 is upregulated in galls, while it is downregulated in syncytia. A global and detailed analysis of expansin genes expression at transcript level in Arabidopsis roots infected with H. schachtii, Wieczorek et al. (2006) showed upregulation of at least 10 and silencing of 2 expansin genes upon nematode infection. They also found that the expression of expansin genes is temporarily and spatially coordinated. However, the results of this study were not sufficient to presume the specific role of each expansin gene during syncytium development.

In the present work, the expression of 10 expansin genes: LeEXPA1, -2, -3, -4, -5, -8, -9, -10, -11 and -18 was investigated during the interaction between G. rostochiensis and tomato. Two expansin isofoms (LeEXPA4 and -5) were selected for detailed spatial and temporal analyses of their transcript distribution. In addition, LeEXPA5 protein was immunolocalized in nematode-infected roots.

Materials and methods

Plant and nematode culture

Tomato (Lycopersicon esculentum L., cv. Money Maker) seeds were surface sterilized in 70% (v/v) ethanol for 3 min and in 30% (v/v) commercial bleach for 10 min. After three baths in sterile water, seeds were germinated on petri dishes containing 1.2% (w/v) agar (Sigma, St. Louis, MO) at 20°C in dark. Five-day-old seedlings were transferred into Gamborg’s B_5 medium (Sigma; pH 6.2) supplemented with 2% (w/v) sucrose and 1.5% (w/v) agar. Petri dishes were sealed and kept in a growth chamber (Sanyo MLR350) at 18°C with 16-h light (80 μmol m⁻² s⁻¹) and 8-h dark photoperiod. After 10 days of growth, aerial parts of plants were cut off and each root system was inoculated with about 500 freshly hatched second-stage juveniles of G. rostochiensis Woll. (pathotype Ro1). The juveniles were obtained from dry cysts and surface sterilized as described by Goverse et al. (2000). The G. rostochiensis cysts were kindly provided by Dr. Hans Helder (Laboratory of Nematology, Wageningen University, Netherlands).

Semi-quantitative RT-PCR

Total RNA was isolated from uninfected segments of root elongation zone and dissected root segments containing syncytia collected 7 days after inoculation. The samples were frozen in liquid N₂, and total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Three independent tissue samples were collected. The quantity and the quality of the isolated RNA were tested with Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA). Specific oligonucleotides flanking the protein-coding sequences of tomato expansins were designed to amplify mRNA templates (Table 1). It was impossible to design specific primer sequences for LeEXPA6 and -7; thus, they were not included in this study. Internal control reactions were performed using primers designed for the sequence of tomato 18S ribosomal cDNA (Table 1). The reverse transcription reaction mixture (20 μl) consisted of 1 × reverse transcription (RT) buffer, 0.5 mM of each dNTP, 20 μM of random primers (oligo(dN)6), 10 U of RNasin.

### Table 1. Sequences of primer pairs used in semi-quantitative RT-PCR analysis of LeEXPA genes and constitutively expressed 18S rRNA gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Expected product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeEXPA1</td>
<td>U82123</td>
<td>5’-GAATTGTTACCAAATTTTTCATCC-3’</td>
<td>5’-ATGAGGTTAGAAGATCGATG-3’</td>
<td>724</td>
<td>55</td>
</tr>
<tr>
<td>LeEXPA2</td>
<td>AF096776</td>
<td>5’-GCTCACACCATTCATTTT -3’</td>
<td>5’-CTCCCAAACTCCTACCC -3’</td>
<td>825</td>
<td>55</td>
</tr>
<tr>
<td>LeEXPA3</td>
<td>AF094879</td>
<td>5’-AAGAGGACACCGCTCTCT -3’</td>
<td>5’-AGGTCCAGAACGCTACGA -3’</td>
<td>750</td>
<td>57</td>
</tr>
<tr>
<td>LeEXPA4</td>
<td>AF094888</td>
<td>5’-GCTGCAATATGATGCCTAC -3’</td>
<td>5’-AGGTTCCACGTTGACATC -3’</td>
<td>723</td>
<td>55</td>
</tr>
<tr>
<td>LeEXPA5</td>
<td>AF094898</td>
<td>5’-GCTTCTATGATCTTATTTAATG-3’</td>
<td>5’-GCTTTAACAGGAATTGTC -3’</td>
<td>722</td>
<td>57</td>
</tr>
<tr>
<td>LeEXPA6</td>
<td>AF184232</td>
<td>5’-TTAACTGCTACATTGCTTAC -3’</td>
<td>5’-TGATTGAAATTCACACG -3’</td>
<td>573</td>
<td>52</td>
</tr>
<tr>
<td>LeEXPA9</td>
<td>AF123340</td>
<td>5’-AGAGGATAGAAGGTTAGAATTCC -3’</td>
<td>5’-ATGCCTTCATTCCTATTTAAG -3’</td>
<td>745</td>
<td>54</td>
</tr>
<tr>
<td>LeEXPA10</td>
<td>AF184233</td>
<td>5’-AGTGTCCATGCAATATGACG -3’</td>
<td>5’-AGTAAACTGTCGCCTTTG -3’</td>
<td>699</td>
<td>55</td>
</tr>
<tr>
<td>LeEXPA11</td>
<td>AJ218775</td>
<td>5’-AATGCATTATCATACATG -3’</td>
<td>5’-CTCATAAAACTTACCCCG -3’</td>
<td>815</td>
<td>52</td>
</tr>
<tr>
<td>LeEXPA18</td>
<td>AJ004997</td>
<td>5’-TTCAGAGAATGAGAGTACCT -3’</td>
<td>5’-TCATGAAATGAGGGATCC -3’</td>
<td>713</td>
<td>55</td>
</tr>
</tbody>
</table>

18S rRNA | X51576 | 5’-GGGTTGACGGCCGAGAAT -3’ | 5’-CGGGACAGGGAGCCGAGA -3’ | 353 | 59 |
containing syncytia (S) 7 days after inoculation. The cDNA was diluted to a concentration of 10 ng μL⁻¹ and used for several polymerase chain reaction (PCR) reactions with specific primers (Table 1). Reactions with 18S rRNA were performed with 1:10 000 diluted cDNA mixture. The PCR mixture (30 μL) contained 1 μL (LeEXPA4, -9, -18), 2 μL (LeEXPA1, -2, -3, -10, -11) or 3 μL (LeEXPA5, -8) of RT mixture, 1× PCR buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.5 μM of forward and reverse gene-specific primers and 2.5 U BioTherm Taq DNA polymerase. The following thermal cycling conditions were introduced: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, annealing at temperature indicated in Table 1 for 30 s, 72°C for 1 min and 1 cycle at 72°C for 3 min. All reactions were performed using MJ Research (Waltham, MA) PTC-200 Gradient Cycler. Each sample was tested at least twice, and representative results of PCRs are shown in Fig. 1. PCR products were checked by subjecting 18 μL of each sample to electrophoresis in 1.5% (w/v) agarose/TAE gels containing 0.5 μg ml⁻¹ ethidium bromide using 10 ng of 18S rRNA as a control on template of total RNA isolated from segments of elongation zone of uninfected roots (R) and root segments containing syncytia at different developmental stages (1, 3, 5, 7, 10 and 14 days after syncytium induction) were fixed in 4% (w/v) paraformaldehyde in microtubule stabilizing buffer (MSB) buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO₄ · 7H₂O, pH 6.9) for 2 h at room temperature. Then, samples were washed four times with MSB buffer for 30 min and subsequently dehydrated in a graded ethanol with 10 mM DTT (Sigma) series: 10, 30 and 50% at room temperature and 70, 96, 100, 100 and 100% at 4°C for 15 min each.

Samples were embedded in butyl-methyl-methacrylate (BMM) resin (Fluka, Buchs, Switzerland) according to Baskin et al. (1992) or LR White resin (Sigma) as described by Dykstra and Reuss (2003). BMM-embedded material was cut on 3.0-μm-thick sections on a Leica RM2165 microtome. The sections were adhered to MJ Research Superfrost microscope slides for 2 h at 50°C and used for in situ RT-PCR followed by hybridization, in situ hybridization alone or for immunolocalization. Samples embedded in LR White were cut into the 150-nm-thick sections on a Leica UCT ultramicrotome, collected on formvar-coated 150-mesh nickel grids and used for immunogold labelling.

**Synthesis of sscDNA digoxigenin-labelled probes**

Two subsequent PCRs were performed to synthesize digoxigenin (DIG)-labelled sscDNA probes for LeEXPA4 or -5. The following primer pairs were used: for LeEXPA4 forward primer: 5′-CCGATTAAGCGGATGATGACG-3′ and reverse primer: 5′-CCATTGCCATCGTTATCA-CAC-3′ to amplify a 317-bp product and for LeEXPA5 forward primer: 5′-GCTTTTATGCTATCTTTTATG-3′ and reverse primer: 5′-CCGGATCACACGTATAGC-3′ to amplify a 250-bp product. In the first PCR, both primers (forward and reverse) were added to the reaction mixture and dsDNA fragments were amplified using Expand High Fidelity PCR System (Roche, Mannheim, Germany) on templates of LeEXPA4 or -5 RT-PCR products purified using QIAquick Gel Extraction Kit (Qiagen). In the second PCR, two separate parallel labelling reactions with forward or reverse primer for both expansin genes were performed on template of the first PCR products to synthesize sense or antisense sscDNA DIG-labelled probes. This PCR was carried out using PCR DIG Probe Synthesis Kit (Roche). The thermal cycling conditions used in both PCRs were as follows: 94°C for 2 min; 35 cycles: 95°C for 15 s, 64°C for LeEXPA4 or 60°C for LeEXPA5 primers for 30 s, 72°C for 45 s; and final elongation at 72°C for 7 min. Unincorporated nucleotides were removed using Mini Quick Spin DNA columns (Roche). Results of purification and probe concentrations were determined on the 1.5% (w/v) agarose/TAE gels.

**Collection of infected plant material for in situ transcripts and protein localization**

Inoculated roots were examined in vivo every 12 h under stereomicroscope to determine precisely the time of syncytium induction. Root pieces containing syncytia were subjected to in situ RT-PCR followed by hybridization, in situ hybridization alone or for immunolocalization. Two subsequent PCRs were performed to synthesize digoxigenin (DIG)-labelled sscDNA probes for LeEXPA4 or -5. The following primer pairs were used: for LeEXPA4 forward primer: 5′-GGAGTAAGCGGAGTGATGACG-3′ and reverse primer: 5′-CCATTGCCATCGTTATCAC-3′ to amplify a 317-bp product and for LeEXPA5 forward primer: 5′-GGTCTTTATGCTATCTTTTATG-3′ and reverse primer: 5′-CCGGATCACACGTATAGC-3′ to amplify a 250-bp product. In the first PCR, both primers (forward and reverse) were added to the reaction mixture and dsDNA fragments were amplified using Expand High Fidelity PCR System (Roche, Mannheim, Germany) on templates of LeEXPA4 or -5 RT-PCR products purified using QIAquick Gel Extraction Kit (Qiagen). In the second PCR, two separate parallel labelling reactions with forward or reverse primer for both expansin genes were performed on template of the first PCR products to synthesize sense or antisense sscDNA DIG-labelled probes. This PCR was carried out using PCR DIG Probe Synthesis Kit (Roche). The thermal cycling conditions used in both PCRs were as follows: 94°C for 2 min; 35 cycles: 95°C for 15 s, 64°C for LeEXPA4 or 60°C for LeEXPA5 primers for 30 s, 72°C for 45 s; and final elongation at 72°C for 7 min. Unincorporated nucleotides were removed using Mini Quick Spin DNA columns (Roche). Results of purification and probe concentrations were determined on the 1.5% (w/v) agarose/TAE gels.

**Fig. 1.** RT-PCR products obtained after reactions with specific primer pairs designed for expansin genes: LeEXPA1, -2, -3, -4, -5, -8, -9, -10, -11, -18 and 18S rRNA as a control on template of total RNA isolated from segments of elongation zone of uninfected roots (R) and root segments containing syncytia (S) 7 days after inoculation.
In situ hybridization

In situ hybridization was performed on sections of uninfected roots and roots with syncytia collected 1, 3, 5, 7, 10 and 14 days after syncytium induction. BMM resin was removed in 100% acetone, and sections were rehydrated in millipore water for 5 min. Sections were subjected to proteinase K digestion [1 μg ml⁻¹ proteinase K (Sigma)] in 50 mM Tris–HCl, pH 7.5] at 37°C for 15 min and rinsed twice in millipore water for 10 min. After short baths in a graded ethanol series (10, 30, 50, 70, 80, 90 and 100%), for 10 s each, slides were air-dried for 45 min at room temperature. Hybridization mixture composed of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 250 ng ml⁻¹ tRNA (Sigma), 100 μg ml⁻¹ Poly(A) (Sigma), 300 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1× Denhard’s solution (Amersham, Uppsala, Sweden), 0.01 mM DTT and 1 μg ml⁻¹ DIG-labelled ssDNA antisense or sense probe was distributed on slides, and slides were incubated overnight at 45°C in MJ Research PTC-100 Thermal Cycler. Then, they were washed in 2× sodium chloride sodium citrate (SSC) (300 mM NaCl, 30 mM sodium citrate, pH 7.2) at room temperature for 25 min and in three baths of 0.2× SSC at 51°C for 25 min each. After three brief washes with sterile water (for 1 min each), slides were subjected to immunodetection.

In situ RT-PCR followed by in situ hybridization

Sections of uninfected roots and root segments containing syncytia corresponding to all tested developmental stages were subjected to in situ RT-PCR followed by in situ hybridization. The BMM resin was removed, and sections were treated with proteinase K as described above. DNA was digested with DNasel [100 mM sodium acetate, 5 mM MgSO₄, pH 5.5, 1 kU ml⁻¹ DNasel RNase-free (Roche), 1 kU ml⁻¹ RNase Inhibitor (Roche)] for 8 h at 37°C. The in situ RT-PCR mixture consisted of 1× RT-PCR buffer (Titan One Tube RT-PCR Kit, Roche), 1 U per slide RNase Inhibitor (Roche), 0.25 mM each dNTP, 2 μM of each forward and reverse primers (the same as used for ssDNA probe synthesis), 3 μl per slide Titan enzyme mix (Roche) and 1× Self Seal Reagent (MJ Research). The following RT-PCR program was introduced: 50°C for 30 min; 10 cycles of: 94°C for 30 s, 64°C for LeEXPA4 primers or 60°C for LeEXPA5 primers for 30 s, 68°C for 2 min, and then, 40 cycles of: 94°C for 30 s, 64°C for LeEXPA4 primers/60°C for LeEXPA5 primers for 30 s, 68°C for 2 min + additional 5 s for each successive cycle; 68°C for 10 min and 92°C for 1 min. After thermal cycling, the slides were soaked for 5 min in 2× SSC at room temperature and the amplified products were hybridized with dscDNA DIG-labelled probes produced as described for ssDNA probes, but to the second PCR, both primers (1 μM of each) were added. The composition of the hybridization mixture was the same as described above, but instead of ssDNA probe, 500 ng ml⁻¹ of DIG dscDNA probe was added. Amplified cDNA and dscDNA probes were denatured at 95°C for 10 min and hybridized as described above.

Immunodetection of DIG

After in situ hybridization and in situ RT-PCR followed by hybridization, two different detection methods of DIG were implemented. For chromogenic detection, slides were incubated in buffer (100 mM Tris, 150 mM NaCl, pH 7.5) at room temperature for 5 min. Non-specific binding sites were blocked with 1% (w/v) BSA (Sigma) in the same buffer for 30 min. Then, the slides were incubated with 1:500 diluted anti-DIG alkaline phosphatase-conjugated antibody (Roche) for 1 h at 37°C. After two washes for 15 min in the buffer, slides were incubated in the same buffer with the pH set at 9.5 for 10 min. BCIP/NBT mixture (Sigma) was deposited on the slides, and colour development reaction was allowed to run for 4–6 h at room temperature in the dark. The reaction was stopped by submerging slides into stopping buffer (10 mM Tris–HCl, 1 mM Na₂-EDTA, pH 8.0) for 10 min. Sections were photographed on Kodak Gold colour negative films (200 ASA) using an Olympus (Tokyo, Japan) AX70 PROVIS microscope equipped with an Olympus SC35 photographic device.

The fluorescent detection was performed using Fluorescent Antibody Enhancer Set for DIG Detection (Roche). Signal was detected using an Olympus AX70 PROVIS microscope equipped with U-M61002 fluorescence filter set and photographed as described above.

Western blot analysis

Polyclonal rabbit anti-LeEXPA5 antibody (Sigma Genosys, Cambridge, UK) raised against 14-amino acid oligopeptide from C-terminal part of deduced amino acid sequence of LeEXPA5 protein (accession number AAD13633, AA: 204–218) was used for Western bloting and immunolocalization.

Cell wall proteins were extracted from uninfected as well as infected (7 days after syncytium induction) root segments according to McQueen-Mason and Cosgrove (1995) and concentrated at Microcon Centrifugal Devices YM-10 (Millipore, Billerica, MA). Extracted proteins were separated on SDS-PAGE (4.5% stacking and 12% running) polyacrylamide gels at 25 mA for 2.5 h and blotted to the Polyvinylidene difluoride (PVDF) membrane (HybondP; Amersham) under 35 V for 2.5 h. Membranes were
blocked in 3% (w/v) BSA (Sigma) in Tris-buffered saline (TBS: 135 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 7.5) for 1 h at room temperature. They were incubated with polyclonal anti-LeEXPA5 antibody diluted 1:500 with 3% (w/v) BSA (Sigma) in TBS for 1 h at room temperature and then washed twice for 10 min in TBS containing 0.2%
In situ analysis of expansin genes and RT-PCR was performed with primer pairs designed for 10 expansin genes. Results revealed upregulation of specific genes with known full cDNA sequence apparently up-regulated in infected roots.

Immunolocalization

From semi-thin BMM sections, resin was removed, and unspecific binding sites were blocked with 3% (w/v) BSA in TBS for 1 h at room temperature. Slides were incubated at room temperature with anti-LeEXPA5 antibody diluted 1:100 in TBS containing 3% (w/v) BSA for 1 h. After four 10-min washes in TBS, slides were incubated with goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) diluted 1:1000 in 1% (w/v) dried non-fat milk in TBS for 1 h. Slides were washed four times in TBS supplemented with 0.05% (v/v) Tween 20 for 10 min and twice in water for 5 min. Signal was photographed as described above.

Ultra-thin LR White sections were blocked in 2% (w/v) BSA in TBS for 1 h. They were incubated at room temperature in anti-LeEXPA5 antibody diluted 1:100 in TBS for 1 h. After three washes in pure TBS, the grids were incubated for 1 h in 1:50 diluted goat anti-rabbit antibody conjugated to 10-nm colloidal gold particles (Sigma). Grids were washed three times in TBS for 10 min and twice in distilled water for 15 min. Sections were counterstained with 2% (w/v) uranyl acetate for 15 min and washed in distilled water. As a control, pre-immune serum was used. Immunogold-labelled sections were examined under a JEOL (Tokyo, Japan) JEM 1220 transmission electron microscope operating at 80 kV.

Results

RT-PCR reveals upregulation of specific expansin genes

RT-PCR was performed with primer pairs designed for 10 expansin genes and 18S rRNA as a control (Table 1) and with equal amounts of RNA extracted from segments of elongation zone of uninfected roots or segments of infected roots containing 7-day-old syncytia (Fig. 1). All primers gave products of the predicted sizes (Table 1). Bands of the same intensity were obtained after control reactions with 18S rRNA primers. Except for LeEXPA5, the tested expansins were also detected in the elongation zone of uninfected roots. LeEXPA1, -2, -4, -5, -11 and -18 were found to be expressed at higher levels in the parasitized roots than in elongation zone of uninfected roots. LeEXPA5 was of special interest because unlike other expansin genes, it was not expressed in uninfected control roots. Therefore, it was chosen for a detailed temporal and spatial expression analysis in roots containing syncytia. Additionally, LeEXPA4 was selected for in situ localization studies as a representative of expansin genes with known full cDNA sequence apparently up-regulated in infected roots.

In situ localization of LeEXPA4 and -5 mRNA

Longitudinal sections of uninfected roots and roots containing syncytia corresponding to all studied developmental stages were subjected to in situ RT-PCR followed by hybridization or in situ hybridization alone. The hybridization signal was detected using chromogenic or fluorescent detection method. For each examined timepoint, the reactions were carried out in parallel with both in situ techniques as well as with both detection methods to have a comparison of signal localization to avoid artefacts. Due to the fact that all implemented methods gave comparable and reproducible transcript distribution patterns on the sections of corresponding developmental stages (e.g., see Figs. 2D–G and 3D–G), only selected data are presented.

In situ experiments revealed that LeEXPA4 mRNA was present in some parenchymatous vascular cylinder cells of uninfected roots (Fig. 2A). In roots containing 3- (Fig. 2B) or 5- (Fig. 2C) day-old syncytia, transcripts of LeEXPA4 were found mostly in the peripheral parts of syncytia and in some parenchymatous vascular cylinder cells located next to the syncytia. On sections of 7-day-old syncytia (Fig. 2D, E), LeEXPA4 transcripts were not detected in the cytoplasm of syncytial elements; however,

Fig. 3. In situ analysis of LeEXPA5 mRNA distribution in uninfected tomato root (A, B) and cyst nematode-infected roots containing syncytia (C–L) carried out using in situ hybridization with ssDNA antisense probe (D, H, I) and sense probe (K) or in situ RT-PCR followed by hybridization (A–C, E–G, J, L). Hybrids were detected using chromogenic detection of DIG signal is visible as purple staining; C, E, F, L) or fluorescent detection of DIG using FITC-conjugated antibody (signal is visible as green fluorescence; A, B, D, G–K). (L) Control reaction for chromogenic detection of DIG performed without anti-DIG alkaline phosphatase-conjugated antibody. Bars, 10 μm. (A, B) Sections of uninfected roots, (C) section of 1-day-old syncytium, (D, E) sections of 3-day-old syncytia, (F, G) sections of 5-day-old syncytia, (H) section of 7-day-old syncytium, (I) section of 10-day-old syncytium, (J) section of 14-day-old syncytium, (K) section of 3-day-old syncytium and (L) section of 5-day-old syncytium. LR, lateral root primordium; N, nematode; S, syncytium; X, xylem.
the parenchymatous vascular cylinder cells next to the syncytium still exhibited relatively high level of LeEXPA4 expression. The same distribution pattern of LeEXPA4 mRNA was also observed in 10- (Fig. 2F, G) and 14- (Fig. 2H) day-old syncytia. The control hybridization with LeEXPA4 sscDNA sense probe did not give any detectable signal (Fig. 2I).

In situ localization of LeEXPA5 transcripts indicated their presence only in the outer cell layer of emerging lateral root primordium in the uninfected root (Fig. 3A, B). In roots infected with nematodes, LeEXPA5 was upregulated just after syncytium induction, as its mRNA was detected in the initial syncytial cell (Fig. 3C). On sections of 3- (Fig. 3D, E) and 5- (Fig. 3F, G) day-old syncytia, the signal indicating LeEXPA5 expression was observed only in the syncytial elements but not in the cortical or vascular cylinder cells surrounding syncytia. The level of LeEXPA5 mRNA tended to decrease in the central part of syncytia located next to nematode head in 7- (Fig. 3H) and 10- (Fig. 3I) day-old syncytia. By contrast, the distal, newly incorporated syncytial elements and some cells adjacent to them exhibited the presence of LeEXPA5 mRNA. The cells expressing LeEXPA5 were mainly located between conductive elements and syncytia. In samples of 14-day-old syncytia, expression of LeEXPA5 could be found neither in syncytial elements nor in other root cells (Fig. 3J).

The level of LeEXPA4 mRNA changed in roots containing 7- (Fig. 4D) and 10- (data not shown) day-old syncytia where LeEXPA5 was present mainly in the cell walls of recently incorporated syncytial elements and less abundantly in the cell walls of older parts of syncytia located next to nematode head. In addition, the LeEXPA5 protein was also localized in the walls of cells neighbouring such syncytia, especially in some parenchymatous cells of the vascular cylinder. The specificity of the reaction was tested by replacing anti-expansin antibody with pre-immune rabbit serum that did not provide any signal (Fig. 4E).

To study the distribution of the LeEXPA5 protein at the ultrastructural level, ultra-thin sections of roots containing 3-day-old syncytia were subjected to immunogold labelling and examined under transmission electron microscope. The LeEXPA5 protein was most abundant in the walls of enlarging syncytial elements. Gold particles were dispersed throughout the cell wall (Fig. 4F). The labelling indicated that LeEXPA5 protein was also present in Golgi apparatus and in Golgi-derived vesicles, located next to the syncytial wall (Fig. 4G). Interestingly, gold labelling was missing in syncytial walls around cell wall openings (Fig. 4H). However, single gold particles were detected in vesicles or in syncytial cytoplasm next to locally digested syncytial wall. Weak gold labelling was observed throughout the whole syncytial cytoplasm.

**Immunolocalization of LeEXPA5**

LeEXPA5 protein was localized using a polyclonal antibody directed against a part of its C-terminal amino acid sequence. The reactivity of the antibody was tested in Western blot analysis (Fig. 4A). The antibody recognized only one band in the line containing cell wall proteins extracted from root segments containing syncytia. The approximate molecular weight of 27 kDa resembled the expected molecular weight of LeEXPA5. No band was visible in the cell wall protein fraction isolated from uninfected root segments.

The results of immunolocalization experiments performed on sections of uninfected and infected roots confirmed the results obtained using in situ methods. In uninfected roots, the LeEXPA5 protein was localized exclusively in the cell walls of lateral root primordia when they emerge from the main root (Fig. 4B). It was detected in 3- (data not shown) and 5- (Fig. 4C) day-old syncytia where the fluorescent signal appeared in all syncytial elements. The distribution pattern of the LeEXPA5 protein changed in roots containing 7- (Fig. 4D) and 10- (data not shown) day-old syncytia where LeEXPA5 was present mainly in the cell walls of recently incorporated syncytial elements and less abundantly in the cell walls of older parts of syncytia located next to nematode head. In addition, the LeEXPA5 protein was also localized in the walls of cells neighbouring such syncytia, especially in some parenchymatous cells of the vascular cylinder. The specificity of the reaction was tested by replacing anti-expansin antibody with pre-immune rabbit serum that did not provide any signal (Fig. 4E).

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interaction with cyst nematode was characterized only in model involved in this parasitic process (Wieczorek et al. 2006). Added to the list of plant cell wall-modifying proteins galacturonases (Mahalingam et al. 1999) are induced endo-1,4-glucanases (Goellner et al. 2001) and polygalacturonases (Mahalingam et al. 1999) are induced during syncytium formation. Recently, expansins were added to the list of plant cell wall-modifying proteins involved in this parasitic process (Wieczorek et al. 2006). However, the expression of expansins in roots infected with cyst nematode was characterized only in model interaction Arabidopsis-H. schachtii and focused only on transcript analysis (Wieczorek et al. 2006). This analysis revealed that expression of AtEXPA3, -6, -8, -10 and -16 is specifically induced in roots containing syncytia, and they are not expressed in coeval uninfected roots. AtEXPA1, -4, -15 and -20 and AtEXPB3 are expressed in roots without syncytia, and they are upregulated in roots containing syncytia. In addition, AtEXPA7 and -18 were found to be silenced upon infection. In this study, we found that α-expansins are involved in the development of syncytia induced by potato cyst nematode in tomato roots. Six α-expansins (LeEXPA1, -2, -4, -5, -11 and -18) were shown to be expressed at higher levels at or around nematode feeding sites. Expression of only one expansin, LeEXPA5, was specifically triggered in the infected roots (Fig. 1). Considering the number of expansin genes upregulated in syncytia induced in Arabidopsis roots and that in most plant organs and tissues, more than one or two expansins are expressed (Brummell et al. 1999, Wu et al. 2001), it is very likely that other, not yet identified, tomato expansin genes play a specific role during syncytium development in tomato roots.

Multiple techniques were implemented to localize expansin expression in cyst nematode-infected Arabidopsis roots (promotor::GUS lines, in situ RT-PCR performed on sections of 7-day-old syncytia; Wieczorek et al. 2006) to give basic information about functions of studied genes. Introduction of in situ analysis performed on sections representing different timepoints of syncytium development allowed us to propose the possible roles of LeEXPA4 and -5 during syncytium development.

Based on Northern blot analysis, LeEXPA4 was not detected in tomato roots (Brummell et al. 1999). However, using in vitro RT-PCR, we were able to detect the LeEXPA4 transcripts in roots, and thereafter, we localized them specifically in the parenchymatous cells of the vascular cylinder of uninfected roots (Fig. 2A). In roots containing syncytia, LeEXPA4 was still expressed mainly in parenchymatous vascular cylinder cells surrounding syncytia and in distal peripheral parts of syncytia (Fig. 2B–H). Due to its expression pattern (Fig. 2A–H), LeEXPA4 can be classified, according to Wieczorek et al. (2006), to category I that groups AtEXPA1, -4, -15 and -20 in Arabidopsis.

On the basis of our findings and the results of other authors, we suggest two possible functions for LeEXPA4 in infected root. First, LeEXPA4 protein could be involved in differentiation of conductive elements. Syncytia are induced to accomplish nematode nutritional demands and require efficient influx of water and nutrients. In fact, syncytium development triggers differentiation of additional vascular elements (Golinowski et al. 1996) in which LeEXPA4 might be involved. This hypothesis could explain why LeEXPA4 was found in many organs like stem, flower, leaf and maturing fruit (Brummell et al. 1999). In addition, LeEXPA4 belongs to the phylogenetically conserved subgroup A of α-expansin genes (Link and Cosgrove 1998), and it was noted that most members of this subgroup play significant roles in development of primary and secondary vascular tissues (Gray-Mitsumune et al. 2004). Recently, it was shown by Muller et al. (2007) that expression of ZmEXPA4, another member of subgroup A of α-expansin genes, is correlated with leaf tissue widening and is localized in differentiating xylem vessels and around them. The authors concluded that ZmEXPA4 may be involved in differentiation of xylem elements.

Milioni et al. (2001) proposed that expansins mediate xylem differentiation by disassembly of the primary cell wall. Accordingly, LeEXPA4 may be involved in wall degradation processes, and thus, it seems to be not only restricted to the specific developmental process. Second, LeEXPA4 could mediate tissue weakening and softening in the infected roots as it was shown during seed germination (Chen and Bradford 2000). The weakening of tissues surrounding syncytia could facilitate intensive intrusive syncytium growth, as syncytial elements undergo extensive hypertrophy and mature syncytium occupies a large volume of the vascular cylinder.

Contrary to LeEXPA4, expression of LeEXPA5 in uninfected roots was detected only using in situ RT-PCR followed by hybridization in emerging lateral root primordia (Fig. 3A, B). The lateral root emerges primarily by cell expansion rather than by mitotic activity of the apical meristem (Malamy and Benfey 1997). In this study, in vitro RT-PCR analysis did not reveal presence of LeEXPA5 transcripts, as used RNA template was isolated from root elongation zone, where lateral roots are not yet induced. According to its expression pattern, LeEXPA5 can be classified to category II, which contains genes
expressed in syncytia and other parts of the roots (e.g., root meristems) but not in the healthy tissues surrounding syncytia (i.e., tissues not involved in syncytium proliferation). In *Arabidopsis*, *AtEXPA6*, -8 and -10 belong to this category (Wieczorek et al. 2006).

In nematode-infected roots, expression of *LeEXPA5* strongly correlates with syncytium expansion. At the beginning of the syncytium development (1–5 days after syncytium induction), all syncytial elements exhibit *LeEXPA5* expression (Fig. 3C–G) as they undergo hypertrophy. In roots containing 7- to 10-day-old syncytia, the expansion process as well as *LeEXPA5* transcript distribution is restricted to the distal parts of syncytium, located along conductive tissues, and to cells adjacent to syncytia being prepared to fuse with syncytia (Fig. 3H, I). These results indicate that, although a syncytium is a single cytoplasmic entity, different syncytial elements show different gene expression patterns as they undergo distinct processes. The targeting of mRNA of *LeEXPA5* to different parts of syncytium might be facilitated by cytoskeleton, which undergoes extensive reorganization during syncytium development (de Almeida Engler et al. 2004). The lack of *LeEXPA5* expression at detectable levels at 14 dpi (Fig. 3) may indicate that syncytia have reached their final size and their expansion is less intense or that at this stage, other expansins are activated.

The process of incorporation of new cells into syncytium besides of cell wall extension involves the formation of cell wall openings. Thus, immunolocalization with rabbit polyclonal anti-*LeEXPA5* antibody was performed to find in which type of cell wall modification (wall extension or degradation), *LeEXPA5* is involved. The protein was localized almost exclusively in the walls of expanding syncytial elements (Fig. 4C, D, F), and it was absent in vicinity of forming cell wall openings (Fig. 4H). This indicates that the possible role of *LeEXPA5* during syncytium formation is to promote cell expansion by increasing cell wall extensibility rather than to enable the formation of wall openings by increasing accessibility of cell wall components to hydrolytic enzymes. The last event may not demand expansin activity, or other expansins are involved in this process. The involvement in cell expansion has been proposed for *LeEXPA5* also during fruit development, as its transcripts were most abundant in tomato fruits at immature green and early mature green stages (Brummell et al. 1999), when rapid cell expansion takes place (Gillaspy et al. 1993). In addition, *LeEXPA5* was the only expansin in which expression was found to be activated in tomato roots infected with root knot nematode *M. javanica* (Bar-Or et al. 2005), and it was suggested that *LeEXPA5* could play a role in cell expansion during the development of gall tissue (Gal et al. 2006).

The closest known homologue of tomato, *LeEXPA5* (96% of deduced amino acid identity) is a putative expansin (*upa6*) isolated from *Capsicum annuum*. *upa6* was also shown to be involved in cell expansion (Marois et al. 2002) and to be activated by type III effector AvrBs3 protein from *Xanthomonas campestris* in the leaf cells that undergo hypertrophy. It seems that *LeEXPA5* mediates cell expansion during developmental processes occurring in healthy plants as well as during pathogenic processes and that its function is to enable cell expansion. The pathogenic bacterium, cyst and root knot nematodes might control plant gene expression at the same checkpoint.

In immunogold labelling experiments, it was observed that *LeEXPA5* protein is equally distributed over syncytial wall, and similar distribution pattern was observed for another expansin by Hoffmann-Benning et al. (1994). Localization of expansin in dictyosomes (Fig. 4G) was also noticed by other authors and confirms proposed, on the basis of the presence of signal peptide, secretory pathway for expansins (Cosgrove et al. 2002, Hoffmann-Benning et al. 1994).

In our study, we investigated in detail the temporal and spatial expression patterns of *LeEXPA4* and -5 in cyst nematode-infected tomato roots. The results demonstrated that *LeEXPA4* and -5 are specifically upregulated upon nematode infection. In addition, both genes have different patterns of transcript distribution, reflecting their possibly different roles during syncytium development. *LeEXPA4* might be involved in cell wall weakening and disassembly during tissue differentiation and/or syncytium expansion, whereas *LeEXPA5* is supposed to take part in cell wall extension during hypertrophy of syncytial elements. The process of syncytium enlargement among root cells may involve three activities: mechanical turgor-driven pressure of syncytial elements onto syncytial wall (Böckenhoff and Grundler 1994), *LeEXPA5* activity in the syncytial wall and also local weakening of surrounding cell walls mediated by *LeEXPA4*.

Although challenging, due to the interference of functional redundancy (Brummell and Harpster 2001, Cosgrove et al. 2002), it would be interesting to generate transgenic plants with silenced *LeEXPA* members to provide further evidences of expansin gene functions in syncytium development and their impact on nematode development.

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