Planta (2001) 212: 279–287

Planta
© Springer-Verlag 2001

Functional characterisation of *Nicotiana tabacum* xyloglucan endotransglycosylase (*Nt*XET-1): generation of transgenic tobacco plants and changes in cell wall xyloglucan

Karin Herbers[2,*], Ester P. Lorences[1,], Coral Barrachina[1], Uwe Sonnewald[2]**

[1]U.D. Fisiología Vegetal, Facultad de Ciencias Biológicas, Univ. de Valencia, Dr. Moliner 50, Burjassot, 46100 Valencia, Spain
[2]Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstr. 3, 06466 Gatersleben, Germany

Received: 24 January 2000 / Accepted: 21 July 2000

Abstract. To study the function of xyloglucan endotransglycosylase (XET) in vivo we isolated, a tomato (*Lycopersicon esculentum* Mill.) XET cDNA (GenBank AA824986) from the homologous tobacco (*Nicotiana tabacum* L.) clone named *Nt*XET-1 (Accession no. D86730). The expression pattern revealed highest levels of *Nt*XET-1 mRNA in organs highly enriched in vascular tissue. The levels of *Nt*XET-1 mRNA decreased in midribs with increasing age of leaves. Increasing leaf age was correlated with an increase in the average molecular weight (MW) of xyloglucan (XG) and a decrease in the relative growth rates of leaves. Transgenic tobacco plants with reduced levels of XET activity were created to further study the biochemical consequences of reduced levels of *Nt*XET-1 expression. In two independent lines, total XET activity could be reduced by 56% and 37%, respectively, in midribs of tobacco plants transformed with an antisense construct. The decreased activity led to an increase in the average MW of XG by at least 20%. These two lines of evidence argue for *Nt*XET-1 being involved in the incorporation of small XG molecules into the cell wall by transglycosylation. Reducing the incorporation of small XG molecules will result in a shift towards a higher average MW. The observed reduction in *Nt*XET-1 expression and increase in the MW of XG in older leaves might be associated with strengthening of cell walls by reduced turnover and hydrolysis of XG.

Key words: Cell wall – *Nicotiana* (cell wall) – Transgenic tobacco – Xyloglucan – Xyloglucan endotransglycosylase

**Present address*: SunGene GmbH, Corrensstr. 3, 06466 Gatersleben, Germany
***Present address*: Instituto de Agroquímica y Tecnología de Alimentos, Ap. Correos 73, 46100 Burjassot, Valencia, Spain

Abbreviations: MW = molecular weight; XET = xyloglucan endotransglycosylase: XG = xyloglucan

Correspondence to: E. P. Lorences;
E-mail: ester.lorences@uv.es; Fax: +34-96-3636301

Introduction

Cell walls of plants fulfil protective and structural functions during plant growth and development. This implies that cell walls are dynamic structures able to adapt to diverse internal and external stimuli resulting in modifications of the existing walls during the life cycle of plants. Numerous biochemical analyses of plant cell walls have provided insight into their overall composition (for reviews, see McNeil et al. 1984; Carpita and Gibeaut 1993). Plant cell walls consist of proteins and different carbohydrate polymers: cellulose microfibrils are thought to provide mechanical strength and are embedded in a matrix of polymeric pectins and hemicelluloses. Hemicellulose may be comprised of different proportions of xylans, xyloglucans (XGs) and mixed-linkage glucans depending on the plant species. The XGs, considered to be the main hemicellulosic compounds in dicotyledonous plants, are hydrogen-bonded to cellulose microfibrils resulting in a three-dimensional network (McNeil et al. 1984; Carpita and Gibeaut 1993).

It has long been postulated that the cellulose-xyloglucan cross-links may represent key control points when cells undergo expansion and extension growth as the cellulose microfibrils need to be untethered and reorganised when new material is incorporated into the growing cell wall. Circumstantial evidence for XGs being primary sites of action in this loosening process derive from the finding that auxin-induced elongation of pea epicotyls is associated with XG turnover (Labavitch and Ray 1974). Moreover, auxin-induced growth has been shown to lead to a decrease in the average molecular weight of XGs extracted from azuki bean (Nishitani and Masuda 1983) and pea epicotyls (Talbott and Ray 1992).

Different types of proteins have been identified that may be involved in cell wall loosening. One group of proteins (expansins) originally identified in cucumber was found to induce extension of isolated walls (McQueen-Mason et al. 1992). These proteins belong to multigene families in rice and *Arabidopsis* (Shcherban

et al. 1995) and induce extension by reversibly disrupting non-covalent bonds at the interface of cellulose microfibrils and some matrix components, interestingly other than pectin and xyloglucan (McQueen-Mason and Cosgrove 1995). Another type of protein, which has been termed xyloglucan endotransglycosylase (XET), acts on xyloglucan, mediating transfer of an XG segment from a donor XG polymer to an acceptor, which may be an XG molecule or in a few cases also water (reviewed in Fry et al. 1992; Nishitani 1997). Thus these proteins would comply with a function in the loosening process of cell walls during growth. Data have been compiled showing a positive relationship between plant growth rates and XET activities (Potter and Fry 1993, 1994; Smith et al. 1996; Barrachina and Lorences 1998). Yet the role of XETs in the loosening process has been questioned because XETs do not appear to cause extension of isolated walls in vitro (McQueen-Mason et al. 1993).

After the first clonings of XET cDNAs from nasturtium (*Tropaeolum majus*; de Silva et al. 1993) and *Vigna angularis* (Okazawa et al. 1993) it has been found that XETs and XET-related genes belong to gene families of several members (Xu et al. 1996; Nishitani 1997; Schröder et al. 1998; Akamatsu et al. 1999; Takano et al. 1999). Whereas most XET enzymes catalyze transglycosylation the XET enzyme from nasturtium seeds has been shown to either hydrolyse or transglycosylate XG depending on the substrate concentrations used in in-vitro experiments (Edwards et al. 1986; Farkas et al. 1992; Fanutti et al. 1993). Similarly, a XET isozyme from kiwifruit may hydrolyse or transglycosylate XG depending on the acceptor substrate (Schröder et al. 1998).

It is not only the mode of action that distinguishes the different members of XET families but, in addition, their expression pattern, their responsiveness to different plant hormones, and abiotic stress forms suggesting different functions for XETs under different conditions (Zurek and Clouse 1994; Saab and Sachs 1995; Xu et al. 1995, 1996; Rose et al. 1996; Antosiewicz et al. 1997; Braam et al. 1997; Catalá et al. 1997; Oh et al. 1998; Takano et al. 1999). In order to prove the possible role of XET in modifying cell walls during plant growth or when plants face diverse external stimuli, gain- or loss-of-function experiments need to be performed. In this regard, tobacco XET isoform sense and antisense constructs have been introduced into suspension-cultured tobacco cells (Accession number D86730; Ito and Nishitani 1999). It could be shown that a fluorescein-labelled xyloglucan heptasaccharide was incorporated into XGs and that this incorporation was reduced in cells with decreased levels of the XET protein.

In this study we further report on the functional characterisation of this XET isozyme from tobacco, hereafter named *Nt*XET-1. The tissue-specific expression pattern of *Nt*XET-1 has been analysed. In addition, this is the first report on stably transformed transgenic plants with altered levels of an XET isozyme. We generated transgenic tobacco plants to study how a change in the activity of *Nt*XET-1 would influence XG and cell wall structure at the biochemical level.

Materials and methods

Plant material and tobacco transformation

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN; Vereinigte Saatzuchten, Ebstorf, Germany) were grown in soil in a greenhouse with 16 h supplementary light (200–300 μmol quanta m^{-2} s^{-1}) and 8 h darkness. Relative humidity varied between 60 and 70% and temperatures were adjusted to about 22 and 150 °C during the light and dark phases, respectively. The plants were transformed using *Agrobacterium*-mediated gene transfer using strain C58C1: pGV2260 as described by Rosahl et al. (1987).

Molecular biology techniques

Standard procedures were used for recombinant DNA work (Sambrook et al. 1989). The SDS-PAGE and Western blotting experiments were performed as described by Herbers et al. (1994).

Extraction of RNA and Northern blot analysis

Extraction of RNA and Northern blot analysis experiments were performed as described by Herbers et al. (1994). Northern blots were hybridized with the complete cDNA as shown in Fig. 1A. For hybridization Church buffer was used (Church and Gilbert 1984) at 65 °C for 20 h. Filters were washed three times with 6× SSC at 65 °C for 20 min (1× SSC = 150 mM NaCl, 15 mM Na-citrate).

Screening of a cDNA library

A cDNA library prepared from source leaves of tobacco plants (described in Herbers et al. 1994) was screened using the tomato XET cDNA (GenBank AA824986) as probe. 2×10^5 plaque-forming units were hybridized with radiolabelled XET cDNA.

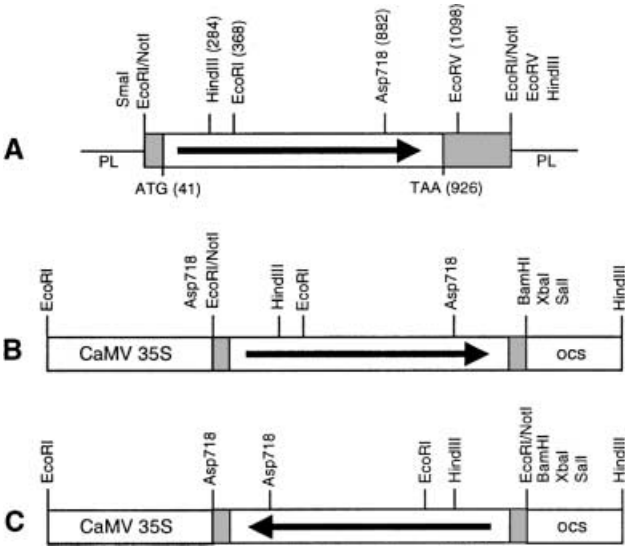

Fig. 1. Structure of *Nt*XET-1 cDNA in pBluescript (**A**) and in the chimeric sense (**B**) and antisense (**C**) constructs. The complete cDNA (1,206 bp) contains an open reading frame from bp 41 to bp 926 (*light grey*). *PL*, polylinker of pBluescript (**A**). The *Sma*I/*Eco*RV fragment was cloned between the cauliflower mosaic virus (CaMV) 35S promoter and the octopine synthase terminator (*ocs*) of pBinAR in sense (**B**) and (**C**) antisense orientation

Purified clones were excised in vivo according to the protocol of Stratagene and characterised by sequencing.

Expression of NtXET-1 protein in Escherichia coli *and immunisation of rabbits*

The *Nt*XET-1 cDNA was amplified from bp 110 to bp 928 with oligonucleotide primers containing a *Bam*HI restriction site. The fragment of about 820 bp was cloned into the *Bam*HI site of pQE9 (Quiagen, Diagen, Düsseldorf, Germany). Induction and purification of the protein was performed according to the manufacturer's instructions (Diagen). Rabbits were immunised and boosted twice subcutaneously with 100 µg protein each time. Antiserum was diluted 1:1,000 for Western blot experiments.

Construction of chimeric NtXET-1 genes for plant transformation

A 1,096-bp fragment including the whole coding region of the *Nt*XET-1 cDNA was isolated as *Sma*I/*Eco*RV fragment from pBluescript (Stratagene) and cloned into the *Sma*I site of *pBin*AR (Höfgen and Willmitzer 1990). Sense and antisense orientation were identified by restriction analysis with *Eco*RI, *Asp*718 and *Hind*III, respectively (Fig. 1).

Assay of XET

Soluble XET activity was assayed as reported by Fry et al. (1992). Purified pine XG (Acebes et al. 1993) or tobacco XG was used as the donor for the endotransglycosylation reaction. [^{3}H]XXXGol, obtained as described by Barrachina and Lorences (1998), was used as the acceptor for the endotransglycosylation reaction. The solution of [^{3}H]XXXGol used for the XET assays had a specific activity of 8.99 TBq mol^{-1}.

Insoluble cell wall-bound XET activity was assayed as described by Barrachina and Lorences (1998).

Extraction and analysis of cell wall polysaccharides

Cell walls were extracted from tobacco midribs followed by sequential extraction of pectic and hemicellulosic polysaccharides as described by Lorences and Zarra (1987).

Xyloglucan was partially purified from the hemicellulosic fraction extracted with 24% KOH as described previously (Acebes et al. 1993). Total sugars of this fraction were determined by the phenol sulfuric acid method (Dubois et al. 1955). The neutral sugar composition of the lyophilisate was determined by GLC (gas-liquid chromatography) to determine the grade of xyloglucan purification and to characterise the response of the iodine staining method (Kooiman 1960) to tobacco midrib xyloglucan.

Neutral sugars were derivatised to alditol acetates as described by Albersheim et al. (1967) and their amount determined by GLC.

Gel permeation chromatography

Gel chromatography of each fraction (4 and 24% KOH) was carried out on a Sepharose CL-4B column (115 cm long, 1.4 cm i.d.) equilibrated in 1 M NaOH. The samples (5 mg lyophilised material) were dissolved in 2 ml 1 M NaOH and eluted with the same solution at a flow rate of 19 ml h^{-1}. Fractions of 2 ml were collected and aliquots of each fraction were assayed for total sugars and xyloglucan content by the methods of Dubois et al. (1955) and Kooiman (1960). The molecular mass of total sugars and xyloglucan was estimated using the formula $MW = \Sigma(M_i \times W_i)/\Sigma\ W_i$, where MW is molecular mass, W_i is total sugar or xyloglucan content of the ith fraction and M_i is molecular mass of the ith fraction estimated from the calibration curves (Nishitani and Masuda 1981).

Results

Isolation of tobacco NtXET-1 cDNA

Using tomato XET as a probe, a tobacco source leaf cDNA library was screened and seven plaque-forming units were purified. Restriction analysis and partial sequencing revealed that the isolated cDNAs were identical. Therefore only the longest cDNA, being 1,206 bp in length, was further analysed (Fig. 1A). The open reading frame encodes 295 amino acids resulting in a protein with a theoretical molecular weight of 33.9 kDa. The XET protein (named *Nt*XET-1) contains a hydrophobic N-terminus, indicating a signal peptide, and two putative glycosylation sites at amino acid positions 14 and 114. Homology searches in databanks revealed near identity to a XET cDNA isolated from tobacco (Nishitani 1996, Accession No. D86730, named EXGT-Nt), except for an arginine residue at amino acid position 202 which is exchanged for threonine in the EXGT-Nt sequence. Ninety percent identity was observed between the *Nicotiana tabacum* XET-1 cDNA and the *L. esculentum* leaf cDNA. The tobacco enzyme belongs to group I of XET-related subfamilies classified according to homology (Nishitani 1997).

Tissue-specific expression of NtXET-1

In order to assign a possible function to this member of the XET-family we performed an extensive expression analysis in reproductive and vegetative tissues (Fig. 2). Under the hybridization conditions described in the

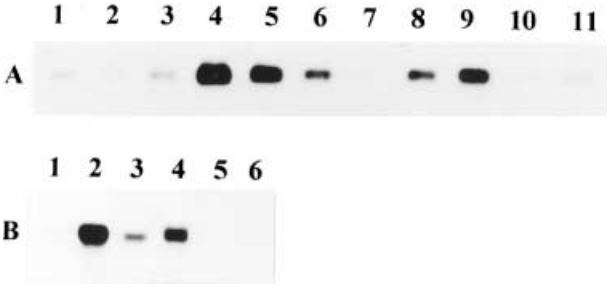

Fig. 2. Tissue-specific expression of *Nt*XET-1 during flower development (**A**) and in vegetative organs (**B**). **A** Levels of *Nt*XET-1 mRNA in green buds (*lane 1*), white buds (*lane 2*), coloured buds (*lane 3*), closed flowers (*lane 4*), open flowers (*lane 5*) and open flowers 24 h after pollination (*lane 6*). Open flowers were used to dissect sepals (*lane 7*), petals (*lane 8*), anthers (*lane 9*), pistils (*lane 10*) and ovary (*lane 11*). **B** Levels of *Nt*XET-1 mRNA in root (*lane 1*), stem (*lane 2*), second-order vein (*lane 3*), midrib (*lane 4*), laminae of source leaves (*lane 5*) and laminae of sink leaves (*lane 6*). An aliquot of 15 µg RNA was loaded per lane

Materials and methods only two bands were hybridizing in a genomic Southern blot using *Nt*XET-1 cDNA as the probe (data not shown). We therefore assume that the Northern blots performed are highly specific for *Nt*XET-1 mRNA and do not hybridize appreciably with other XET-related mRNAs.

Analysis of RNA showed no expression in developing flower buds (Fig. 2A, lanes 1–3). However, flowers showed a stage-specific accumulation of *Nt*XET-1 mRNA when they were fully developed (Fig. 2A, lanes 4 and 5). Levels declined rapidly after pollination (Fig. 2A, lane 6). Dissecting open flowers into their distinct organs revealed the highest expression in petals and anthers (Fig. 2A, lanes 8 and 9) but no expression in sepals, pistil and ovary (lanes 7, 10, 11).

The RNA was also isolated from different vegetative tissues of tobacco plants at the 15-leaf stage (Fig. 2B). Mature source leaves of plants at the 15-leaf stage were dissected into first-order veins (midribs), second-order veins and laminae, and the corresponding RNAs were analysed for *Nt*XET-1 expression. Strong hybridisation signals were visible in second-order veins (lane 3) and midribs (lane 4) whereas hardly any *Nt*XET-1 RNA could be detected in laminae of source (lane 5) and sink leaves (lane 6). *Nt*XET-1 RNA levels were also highly abundant in stem tissue (Fig. 2B, lane 2) and hardly any RNA was detected in roots (lane 1).

To find out whether the tissue specific expression was influenced by leaf age and whether it would correlate with leaf relative growth rates *Nt*XET-1 mRNA levels were analysed in leaves of plants at the 15-leaf stage (Fig. 3A). Steady-state RNA levels hybridizing to *Nt*XET-1 cDNA were highest in upper leaves and decreased with increasing age of the leaves. As smaller leaves contain more veinal tissue in relation to mesophyll tissue as compared to older leaves it was not obvious whether the observed expression pattern suggested developmentally controlled expression of *Nt*XET-1 or expression in tissues associated with veins. To differentiate these possibilities, RNA was isolated from midribs of leaves covering different developmental stages. *Nt*XET-1 was found to be highly expressed in midribs with decreasing levels in source leaves (Fig. 3B).

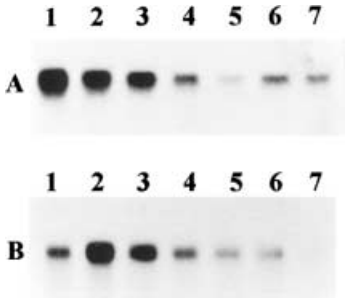

Fig. 3. Expression of *Nt*XET-1 mRNA levels was analysed in leaves of different ages (**A**), and in midribs of leaves of different ages (**B**). *Lanes 1–7* correspond to the 4th, 6th, 8th, 9th, 11th, 12th and 13th leaf (counted from the top) of plants at the 15-leaf stage. An aliquot of 15 μg of total RNA was loaded per lane

Thus *Nt*XET-1 is controlled both in a tissue- and developmental-specific manner in leaves. The Northern blots do not differentiate between *Nt*XET-1 expression in vascular tissue and in parenchyma cells associated with vascular tissue.

Relative growth rates (% per hour) for leaves at different developmental stages were determined as follows (means ± SD, $n = 5$): 0.344 ± 0.148 (leaf 4), 0.398 ± 0.180 (leaf 6), 0.293 ± 0.105 (leaf 8), 0.194 ± .0073 (leaf 9), 0.028 ± 0.009 (leaf 11), 0.025 ± 0.008 (leaf 12) and 0.006 ± 0.001 (leaf 13). The comparison between *Nt*XET-1 mRNA levels (Fig. 3B) and leaf relative growth rates suggests a positive correlation between *Nt*XET-1 expression in midribs and leaf growth.

Activity of XET in tobacco midribs

To analyse whether overall activity would follow the *Nt*XET-1 mRNA pattern, total XET activities were determined in midribs from differently aged leaves of tobacco plants. Midribs were harvested from the 4th, 6th, 9th and 12th leaf (counted from the top) of tobacco plants at the 15-leaf stage and both soluble and insoluble cell-wall-bound XET activities were determined using pine xyloglucan as substrate (Table 1). Total activities (expressed in Bq/kBq per mg fresh weight) consisted of about 80% soluble and 20% insoluble cell-wall-bound XET activity (Table 1). Surprisingly, XET activities varied only slightly between leaves of the different developmental stages. A tendency for lower activity in the 4th leaf and highest activities in the 6th leaf from the top of plants was observed (Table 1). This would follow the RNA expression data to a limited degree (Fig. 3B). However, even with little *Nt*XET-1 transcript detectable in the 12th leaf (Fig. 3B), considerable XET activities were determined. In addition there was no correlation between leaf relative growth rates and total XET activities.

The discrepancy between *Nt*XET-1 mRNA levels and XET activity data suggests either that *Nt*XET-1 is regulated at the post-transcriptional level or that other XET-related enzymes contribute to overall XET activity.

Table 1. Soluble and insoluble XET activity [Bq kBq^{-1} h^{-1} $(mg\ FW)^{-1}$] in midribs of tobacco leaves at different developmental stages. Midribs were harvested from the 4th, 6th, 9th, and 12th leaf of three different plants at the 15-leaf stage. Each treatment was performed in duplicate. Soluble activities were determined using pine xyloglucan as substrate. Values are mean ± SD ($n = 3$)

Leaf	Soluble	Insoluble	Total	Ratio of soluble and insoluble activities
4th	7.2 ± 1.1	2.3 ± 0.3	9.5 ± 1.4	3.1
6th	9.6 ± 1.7	2.7 ± 0.4	12.3 ± 2.1	3.6
9th	8.6 ± 1.2	2.2 ± 1.0	10.8 ± 2.2	3.9
12th	8.1 ± 0.7	2.1 ± 0.9	10.2 ± 1.6	3.8

Construction of chimeric sense and antisense XET genes

Having found a possible correlation between leaf relative growth rate and *Nt*XET-1 m RNA expression levels we decided to alter *Nt*XET-1 activity in transgenic tobacco plants to study the biochemical consequences on cell walls and on leaf growth. To this end the *Nt*XET-1 cDNA was cloned in sense and antisense orientation behind the constitutive cauliflower mosaic virus (CaMV) 35S promoter in pBin19 (for details, see *Materials and methods*, Fig. 1B,C). The constructs were used to transform tobacco plants via *Agrobacterium tumefaciens*.

Screening for transgenic tobacco plants with altered amounts of NtXET-1 mRNA

Plants transformed with the sense (sense*Nt*XET-1) and the antisense (αNtXET-1) construct were screened for *Nt*XET-1 RNA levels in leaves. Of 62 independent sense *Nt*XET-1 lines analysed, 3 showed elevated levels of *Nt*XET-1 RNA (lines 30, 31, 36) in leaf blades (Fig. 4, lanes 1–3). Two *Nt*XET-1 mRNAs of different sizes were detected in the overexpressing lines, the upper band being the one expected (930 bp *Nt*XET-1 plus *ocs* terminator). The smaller mRNA might result from premature transcriptional termination. Of 87 lines transformed with the antisense construct, 4 were found to have reduced levels of *Nt*XET-1 mRNA (α*Nt*XET-1 lines 45, 47, 52, 58) in midribs (Fig. 4B shows plants lines 45 and 47).

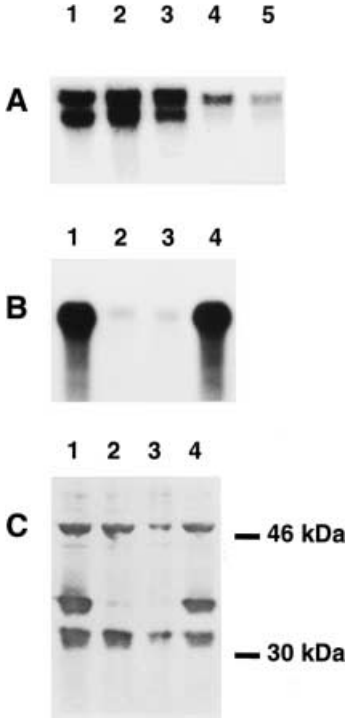

Fig. 4A–C. Altered *Nt*XET-1 mRNA and protein levels in transgenic tobacco plants. **A** Analysis of XET mRNA in plants transformed with the sense construct. The three independent transformants, lines 30 (*lane 1*), 31 (*lane 2*) and 36 (*lane 3*), are compared with two wild-type plants (*lanes 4*, *5*). The RNA was isolated from leaf blades of the 5th leaf of plants at the 8-leaf stage. **B** Analysis of XET mRNA in plants transformed with the antisense construct. The two independent transformants, lines 45 (*lane 2*) and 47 (*lane 3*), are compared with two wild-type plants (*lanes 1* and *4*). The RNA was isolated from midribs of the 5th leaf of plants at the 8-leaf stage. **C** Analysis of XET protein in plants transformed with the antisense construct. The two independent transformants, lines 45 (*lane 2*) and 47 (*lane 3*), are compared with two wild-type plants (*lanes 1*, *4*). Protein was isolated from midribs of the 5th leaf of plants at the 8-leaf stage. Total protein of 15 μg (**A, B**) and 3 μg (**C**) was loaded per lane

To determine whether the altered levels of *Nt*XET-1 mRNA were reflected at the protein level, a rabbit polyclonal antiserum raised against the isolated *Nt*XET-1 protein was used in Western blot experiments. In leaf blades of wild-type and sense *Nt*XET-1 plants, no specific immunoreacting band could be detected (data not shown). Therefore the overexpressing lines were not further investigated. In protein extracts of midribs, three protein bands (of about 31, 36 and 48 kDa) were recognised (Fig. 4C). The protein with an apparent molecular weight of about 36 kDa could be identified as *Nt*XET-1 as this band was nearly completely missing in the antisense lines 45 and 47 (Fig. 4C). The discrepancy between the theoretical and apparent MW might be explained by glycosylation of *Nt*XET-1. It is not likely that the other two immunoreacting proteins represent XET-related proteins because of the substantial size differences.

Analysis of total XET activity in tobacco plants containing reduced amounts of NtXET-1 protein

Soluble and insoluble cell-wall-bound XET activities were determined in leaf midribs of wild-type and α*Nt*XET-1 plants of lines 45 and 47 using tobacco midrib XG as substrate. Plants analysed were at the 17-leaf stage and midribs were derived from the 9th leaf from the top. Activities of both soluble and insoluble cell wall-bound XET were reduced in both lines as compared to the wild type (Table 2), indicating that both activities are likely to be encoded by the same gene. In both lines the insoluble cell-wall-bound XET activity was reduced more than the soluble activity. The residual insoluble cell-wall-bound XET activities of lines 45 and 47 amounted to 23 and 51%, respectively, whereas residual soluble activities were 56 and 71%. Thus, remaining total activities amounted to about 44% and 64% in antisense lines 45 and 47, respectively.

Cell wall composition of midribs isolated from tobacco

We then analysed the composition of cell walls in tobacco midribs, paying particular attention to the alkali-extractable hemicellulose and xyloglucan to investigate the physiological significance of *Nt*XET-1 in this tissue. Cell walls were isolated from midribs derived from the 6th and 9th leaf (counting from the top) of plants at the 15- and 17-leaf stage, respectively. Cell wall components of wild-type plants were differentially extracted and their relative contribution to total cell wall (expressed as percent dry weight) determined. In the

Table 2. Soluble and insoluble XET activity [Bq kBq^{-1} h^{-1}(mg $FW)^{-1}$] in midribs of tobacco leaves with reduced levels of *Nt*XET-1 mRNA and protein. Midribs were derived from the 9th leaf at the 17-leaf stage. Each treatment was performed in duplicate. Soluble activities were determined using tobacco midrib xyloglucan as substrate. Activities are mean values ± SD ($n = 4$)

Genotype	Soluble	Insoluble	Total
Control	4.1 ± 0.98 (100%)	2.52 ± 0.51 (100%)	6.62 ± 1.49 (100%)
αXET line 45	2.3 ± 0.29 (56%)	0.58 ± 0.03 (23%)	2.88 ± 0.32 (43.5%)
αXET line 47	2.9 ± 0.40 (71%)	1.30 ± 0.16 (51%)	4.20 ± 0.56 (63.4%)

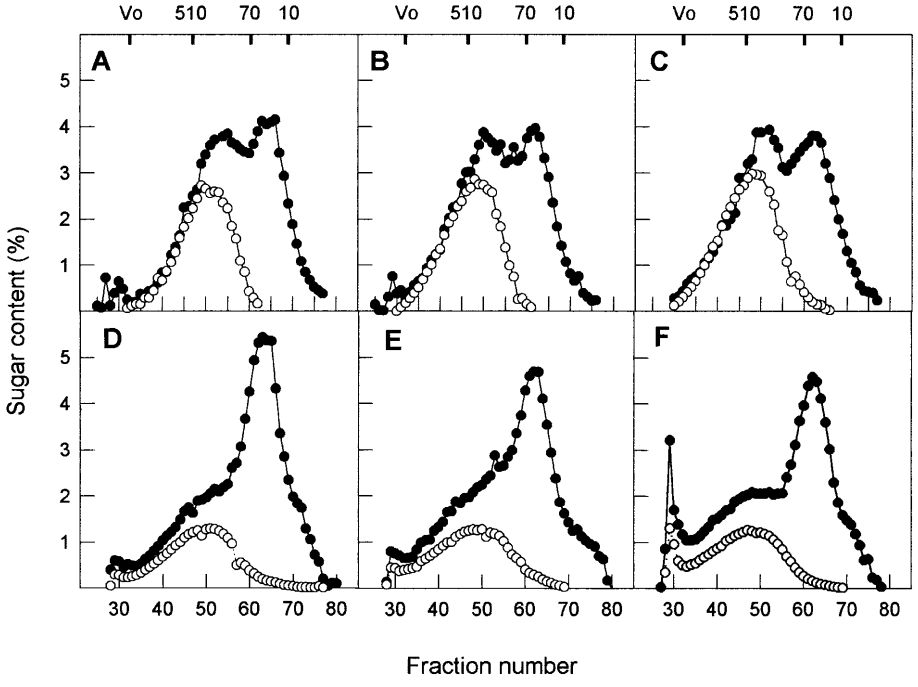

Fig. 5A–F. Distribution of the molecular mass of hemicelluloses of wild-type and antisense plants. Hemicellulose was isolated with 24% KOH and chromatographed on a Sepharose CL-4B column. Fractions were extracted from tobacco midribs at different stages of development and from different lines. **A–C** The 6th leaf from the top of plants at the 15-leaf stage. **D–F** The 9th leaf from the top of plants at the 17-leaf stage. Midribs were harvested from leaves of 10 different plants and the experiment was performed twice with replicas of each treatment. The data represent average values. **A**, **D** Wild type; **B**, **E** αXET line 45; **C**, **F** αXET line 47. All plants were regenerated from tissue culture. *Closed circles*, total sugars; *open circles*, xyloglucan. Calibration scale obtained arising from dextrans is shown at the top. *Vo*, void volume

9th leaf, the ammonium oxalate-extractable pectin was calculated to make up 36% and the hemicelluloses extracted with KOH 57% of the cell wall material. Of the latter, 32% could be isolated by treatment with mild alkali (4% KOH) while 68% was extractable with 24% KOH. In order to determine the sugar composition of the hemicellulose the polysaccharides of the 4% and 24% fractions were analysed. Xyloglucan was only present in the 24% KOH fraction.

We partially purified and analysed tobacco midrib xyloglucan to characterise the response of the iodine staining method. The OD 1 was calculated to correspond to 125 μg ml^{-1}. The amount of xyloglucan represented about 40% (6th leaf at the 15-leaf stage) and 26% (9th leaf at the 17-leaf stage) of the 24% KOH hemicellulose fraction, showing that the proportion of XG with respect to total hemicellulosic sugars differed considerably between leaves at the different developmental stages.

Analysis of XG in midribs of control and NtXET-1 antisense plants

The transgenic plants had no visible phenotype under greenhouse conditions. Yet, to evaluate a putative effect of the approximately 50% reduction in XET activity on XG we decided to analyse whether the molecular mass distribution of XG was altered in the antisense plants. To this end, hemicellulose was extracted with 24% KOH from midribs of the wild type and α*Nt*XET-1 lines 45 and 47, and chromatographed on a Sepharose CL-4B column. Total sugars as well as XGs were determined in each fraction collected (Fig. 5).

The experiment was performed twice using (i) midribs derived from the 6th leaf of plants at the 15-leaf stage (Fig. 5A–C) and (ii) midribs derived from the 9th leaf of plants at the 17-leaf stage (Fig. 5D–F).

Xyloglucan from different leaves of different-aged wild-type plants not only showed quantitative changes in XG with respect to total hemicellulose but also qualitative changes with respect to the MW distribution of XG (Table 3). An increase in the average MW of XG

Table 3. Average molecular weight of xyloglucan in wild-type and antisense XET plants. Average masses were calculated from the values plotted in Fig. 5 (see *Material and methods*). The increase in MW between wild-type plants at different ages (6th leaf and 9th leaf) and between wild-type and antisense XET plants were significantly different (Student's *t*-test)

Leaf	Wild-type	αXET line 45	αXET line 47
6th	683 (100%)	846 (123%)	863 (126%)
9th	838 (100%)	1,026 (122%)	1,357 (162%)

in older leaves of wild-type plants as compared to younger leaves (838 kDa versus 683 kDa) was observed. In addition, the transgenic plants consistently displayed a further increase in the average MW by at least 20%, both in the younger and older leaves (Table 3).

Discussion

The tissue expression of NtXET-1 is under developmental control

The tobacco cDNA (*Nt*XET-1) was isolated from a tobacco leaf cDNA library using a tomato XET cDNA (GenBank AA824986) as the probe. The tobacco cDNA was found to be nearly identical to EXGT-Nt (Nishitani 1996, Accession No. D86730). Expression analysis revealed that *Nt*XET-1 mRNA was confined to veinal tissues in the vegetative part of the plant (Figs. 2B and 3B). This tissue-specific expression was under developmental control: *Nt*XET-1 mRNA levels of midribs were highest in small source leaves (6th leaf from top). Expression levels decreased with increasing leaf age and largely corresponded to leaf relative growth rates. Alterations in the *Nt*XET-1 RNA levels were much more pronounced than total XET activities in leaves of different ages. Several reasons may account for this discrepancy: (i) little turnover of *Nt*XET-1 protein and other posttranscriptional control mechanisms, (ii) other XET activities contribute to total activity in midribs and, (iii) methodological constraints of XET extraction and in-vitro assay for determining in-vivo activities.

In accordance with our data, immunolocalisation studies with antibodies raised against TCH4 by Antosiewicz et al. (1997) revealed XET proteins in differentiating vascular elements and in living elements of mature vascular tissue (among other locations). The authors concluded that XETs might aid in wall modifications required for the formation of xylem and phloem-conducting elements. The tissue-specific expression pattern of *Nt*XET-1 in strongly growing leaves supports the notion that this enzyme is involved in these differentiation and growth processes.

Reduced levels of NtXET-1 in transgenic tobacco led to a decrease in the soluble and insoluble cell-wall-bound XET activities of midribs

To study the consequences of altered *Nt*XET-1 levels on the hemicellulose portion of cell walls, transgenic plants were created. We obtained plants with reduced levels of *Nt*XET-1 mRNA and protein, respectively (Fig. 4B, C). In midribs of the antisense lines, *Nt*XET-1 mRNA and *Nt*XET-1 protein were hardly detectable. Surprisingly, total XET activities were only reduced by about 56% and 37% in lines 45 and 47, respectively (Table 2), suggesting either a regulatory mechanism to activate the remaining protein or the presence of another/other XET enzyme(s) in midribs. We favour the latter possibility because this might equally explain the discrepancy between *Nt*XET-1 mRNA levels and total XET activities in midribs of wild-type plants (Fig. 3B, Table 1). The antisense construct affected both the insoluble as well as the soluble activity, indicating that the same gene encodes a protein that may be both soluble and bound to the cell wall.

Reduced levels of NtXET-1 in tobacco lead to an increase in the average MW of midrib xyloglucan

To study the biochemical consequences of reduced *Nt*XET-1 expression on cell walls the MW distribution of XG was analysed in midribs of wild-type and antisense lines at two developmental stages (Fig. 5, Table 3).

In wild-type plants we found that the XG content strongly decreased with increasing age of the leaves, from 40% in younger leaves to 26% in older leaves in the 24% KOH hemicellulosic fraction. This result shows that cell walls derived from tobacco midribs undergo major changes with respect to XG content during development. In tobacco stalks the hemicelluloses nearly exclusively consist of xylan rather than XG (Eda et al. 1976). In addition, in midribs of wild-type plants, an increase in the average MW of XG was observed in older leaves. This increase in MW was found to be significantly different by the Student's *t*-test.

Thus there appears to be a positive correlation between high *Nt*XET-1 expression, high XG content and lower MW distribution of XG in wild-type plants. It may be hypothesized that alterations in XET activity might be responsible for changes in qualitative and quantitative changes of XG in cell walls of veinal tissues.

This hypothesis is supported by results obtained with the antisense transgenic plants. A decrease in expression and activity of *Nt*XET-1 in the transgenic plants was paralleled by an increase in the average MW of xyloglucan (Fig. 5, Table 3), indicating a significant increase in the degree of polymerisation, verified by the Student's *t*-test.

From this finding we infer that *Nt*XET-1 has a preference for smaller XG molecules as acceptor molecules. A reduction in its action as observed in older leaves may therefore result in a shift towards an XG with higher MW. We have not checked whether *Nt*XET-1 might also function as XG hydrolase. This, however, is less likely, as it belongs to a subfamily of I XETs with transferase activity only (Nishitani 1997), although this classification has recently been questioned by Schröder et al. (1998).

Physiological consequences

We suppose that a reduction in overall *Nt*XET-1 activity with a concomitant increase in the average MW of xyloglucan may result in reinforcement of cell walls: First, cutting and re-joining of XG molecules becomes a less frequent event and secondly, longer XG molecules might be hydrogen-bonded to the cellulose microfibrils over longer extensions.

Thus we assume that *Nt*XET-1 has a role during differentiation and growth of the vascular tissue by incorporating new XG molecules. In addition, during leaf development *Nt*XET-1 levels decrease, which might result in reduced grafting of XG molecules and, on average, longer XG molecules, leading to more stable cell walls in older leaves. This might have consequences for plant-pathogen and plant-virus interactions.

We still have to investigate whether an increase in the average MW of XG leads to higher stability of the cell wall of midribs and whether extensibility of cell walls as well as plant-pathogen interactions are altered in the transgenic plants.

We thank Martin Ganal (IPK, Gatersleben, Germany) for the tomato XET cDNA. We also thank Christiane Prüßner and Anita Winger for technical assistance. We are grateful to Andrea Knospe and Sybille Freist for transformation of tobacco plants and tissue culture work. Part of the work was supported by the Deutscher Akademischer Austauschdienst and the Spanish Goverment within the frame of the programme "Projektbezogener Personenaustausch mit Spanien", "Acciones Integradas Hispano-Alemanas".

References

Acebes JL, Lorences EP, Zarra I (1993) Pine xyloglucan. Occurrence, localization and interaction with cellulose. Physiol Plant 89: 417–422

Albersheim P, Nevins DJ, English PD (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr Res 5: 340–345

Antosiewicz DM, Purugganan MM, Polisensky DH, Braam J (1997) Cellular localization of *Arabidopsis* xyloglucan endotransglycosylase-related proteins during development and after wind stimulation. Plant Physiol 115: 1319–1328

Akamatsu T, Hanzawa Y, Ohtake, Takahashi T, Nishitani K, Komeda Y (1999) Expression of endoxyloglucan transferase genes in *aucalis* mutants of *Arabidopsis*. Plant Physiol 121: 715–721

Barrachina C, Lorences EP (1998) Xyloglucan endotransglycosylase activity in pine hypocotyls. Intracellular localization and relationship with endogenous growth. Physiol Plant 102: 55–60

Braam J, Sistrunk ML, Polisensky DH, Xu W, Purugganan MM, Antosiewicz DM, Campbell P, Johnson KA (1997) Plant responses to environmental stress: regulation and functions of the *Arabidopsis* TCH genes. Planta 203: S35–S41

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1–30

Catalá C, Rose JKC, Bennett AB (1997) Auxin regulation and spatial localization of an endo-1,4-β-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. Plant J 12: 417–426

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995

de Silva J, Jarman CD, Arrowsmith DA, Stronach MS, Chengappa S, Sidebottom C, Reid JSG (1993) Molecular characterization of a xyloglucan-specific endo-(1–4)-β-D-glucanase (xyloglucan endotransglycosylase) from nasturium seeds. Plant J 3: 701–711

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1955) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356

Eda S, Ohnishi A, Kato K (1976) Xylan isolated from the stalk of *Nicotiana tabacum*. Agric Biol Chem 40: 359–364

Edwards M, Dea ICM, Bulpin PV, Reid JSG (1986) Purification and properties of a novel, xyloglucan-specific endo(1–4)-β-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus*). J Biol Chem 261: 9489–9494

Fanutti C, Gidley MJ, Reid JSG (1993) Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1–4)-β-D-glucanase) from the cotyledons of germinated nasturtium seeds. Plant J 3: 691–700

Farkas V, Sulova Z, Stratilova E, Hanna R, Maclachlan G (1992) Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. Arch Biochem Biophys 298: 365–370

Fry SC, Smith RC, Hetherington PR, Potter I (1992) Endotransglycosylation of xyloglucan: a role in cell wall yielding? Curr Top Plant Biochem Physiol 11: 42–62

Herbers K, Prat S, Willmitzer L (1994) Functional analysis of a leucine aminopeptidase from *Solanum tubersoum* L. Planta 194: 230–240

Höfgen R, Willmitzer L (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). Plant Sci 66: 221–230

Ito H, Nishitani K (1999) Visualization of EXGT-mediated molecular grafting activity by means of a fluorescent-labeled xyloglucan oligomer. Plant Cell Physiol 40: 1172–1176

Kooiman P (1960) A method for the determination of amyloid in plant seeds. Rec Trav Chim Pays-Bas 79: 675–678

Labavitch JM, Ray PM (1974) Relationship between promotion of xyloglucan metabolism and induction of elongation by indoleacetic acid. Plant Physiol 54: 499–502

Lorences EP, Zarra I (1987) Auxin-induced growth in hypocotyl segments of *Pinus pinaster* Aiton. Changes in molecular weight distribution of hemicellulosic polysaccharides. J Exp Bot 38: 960–967

McNeil M, Darvill AG, Fry SC, Albersheim P (1994) Structure and function of the primary cell walls of plants. Annu Rev Biochem 53: 625–663

McQueen-Mason SJ, Cosgrove DJ (1995) Expansin mode of action on cell walls. Plant Physiol 107: 87–100

McQueen-Mason SJ, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4: 1425–1433

McQueen-Mason SJ, Fry SC, Durachko DM, Cosgrove DJ (1993) The relationship between xyloglucan endotransglycosylase and in-vitro cell wall extension in cucumber hypocotyls. Planta 190: 327–331

Nishitani K (1996) GenBank Accession No. D86730

Nishitani K (1997) The role of endoxyloglucan transferase in the organization of plant cell walls. Int Rev Cytol 173: 157–206

Nishitani K, Masuda Y (1981) Auxin-induced changes in the cell wall structure. Changes in the sugar compositions, intrinsic viscosity and molecular weight distributions of matrix polysaccharides of the epicotyl cell wall of *Vigna angularis*. Physiol Plant 52: 482–494

Nishitani K, Masuda Y (1983) Auxin-induced changes in the cell wall xyloglucans: Effects of auxin on two different subfractions of xyloglucans in the epicotyl cell wall of *Vigna angularis*. Plant Cell Physiol 24: 345–355

Oh M-H, Romanov WG, Smith RC, Zamsky E, Sasse J, Clouse SD (1998) Soybean *BRU*1 encodes a functional xyloglucan endotransglycosylase that is highly expressed in inner epicotyl tissues during brassinosteroid-promoted elongation. Plant Cell Physiol 39: 124–130

Okazawa K, Sato Y, Nakagawa T, Asada K, Kato I, Tomita E, Hishitani K (1993) Molecular cloning and cDNA sequencing of endoxyloglucan transferase, a novel class of glycosyltransferase that mediates molecular grafting between matrix polysaccharides in plant cell wall. J Biol Chem 268: 25364–256368

Potter I, Fry SC (1993) Xyloglucan endotransglycosylase activity in pea internodes. Effects of applied gibberellic acid. Plant Physiol 103: 235–241

Potter I, Fry SC (1994) Changes in xyloglucan endotransglycosylase (XET) activity during hormone-induced growth in lettuce and cucumber hypocotyls and spinach cell suspension cultures. J Exp Bot 45: 1703–1710

Rosahl S, Schmidt R, Schell J, Willmitzer J (1987) Expression of a tuber-specific storage protein in transgenic tobacco plants: demonstration of an esterase activity. EMBO J 6: 1155–1159

Rose JKC, Brummell DA, Bennett AB (1996) Two divergent xyloglucan endotransglycosylases exhibit mutually exclusive patterns of expression in *Nasturtium*. Plant Physiol 110: 493–499

Saab IN, Sachs MM (1995) Complete cDNA and genomic sequence encoding a flooding-responsive gene from maize (*Zea mays* L.) homologous to xyloglucan endotransglycosylase. Plant Physiol 108: 439–440

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) Molecular cloning and sequence analysis of expansins – a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc Natl Acad Sci USA 92: 9245–9249

Schröder R, Atkinson RG, Langenkämper G, Redgwell RJ (1998) Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. Planta 204: 242–251

Smith RC, Matthews PR, Schünmann PHD, Chandler PM (1996) The regulation of leaf elongation and xyloglucan endotransglycosylase by gibberellin in "Himalaya" barley (*Hordeum vulgare* L.). J Exp Bot 47(302): 1395–1404

Takano M, Fujii N, Higashitani A, Nishitani K, Hirasawa T, Takahashi H (1999) Endoxyloglucan transferase cDNA isolated from pea roots and its fluctuating expression in hydroponically responding roots. Plant Cell Physiol 40: 135–142

Talbott LD, Ray PM (1992) Changes in molecular size of previously deposited and newly synthesized pea cell wall matrix polysaccharides. Plant Physiol 98: 369–379

Xu W, Purugganan MM, Polisensky DH, Antosiewicz DM, Fry SC, Braam J (1995) *Arabidopsis* TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. Plant Cell 7: 1555–1567

Xu W, Campbell P, Vargheese AK, Braam J (1996) The *Arabidopsis* XET-related gene family: environmental and hormonal regulation of expression. Plant J 9: 879–889

Zurek DM, Clouse SD (1994) Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. Plant Physiol 104: 161–170