Increased extensin levels in Arabidopsis affect inflorescence stem thickening and height

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Abstract

Extensins are hydroxyproline-rich glycoproteins which are abundant in the cell walls of higher plants and whose precise function has been the subject of much speculation. In order to investigate this, transgenic A. thaliana plants were generated containing the Arabidopsis Atext1 extensin coding sequence under the transcriptional control of the strong constitutive CaMV 35S promoter. Northern analysis and RT-PCR identified transgenics with high constitutive levels of Atext1 mRNA. Hydroxyproline assays confirmed that transgenic plants which over-expressed Atext1 contained high levels of hydroxyproline-rich protein. Phenotypic analysis of the transgenics showed that there were no significant phenotypic effects on the timing of different developmental stages or on the general form of the plant. However, transgenics with high extensin levels showed an enhanced increase in stem thickness, with an indirect effect on stem height. This effect, is, however, rather small, with a reduction in height of between 5–8% depending on the transgenic line being analysed.

Key words: Arabidopsis, Atext1, cell wall, extensin, hydroxyproline, phenotype, stem thickness.

Introduction

The primary cell wall of plants is an extremely complex and dynamic structure that fulfils many different functions. The strength of the wall determines cell mechanical properties, and therefore, cell size and shape (Veytsman and Cosgrove, 1998; McNeil et al., 1984), collectively contributing to plant morphology (Varner and Lin, 1989; Taiz, 1984). The cell wall also acts as a protective shield against herbivory, pathogen attack, and various environmental stresses (Schindler, 1998) and is also involved in the storage and release of signalling molecules, carbohydrates, metal ions, and other materials (Cosgrove, 1999). Of the proteins which are present in the plant cell wall, hydroxyproline-rich glycoproteins (HRGPs) of which extensins are the most well studied, have been implicated in nearly all aspects of plant growth and development including pollen recognition and fertilization (Wu et al., 2001), cell division and differentiation (Ito et al., 1998; Keller and Lamb, 1989), the cessation of cell elongation (Cleland and Karlens, 1967; Ito et al., 1998; Sadava and Chrispeels, 1973), abscission and senescence (Merkouropoulos and Shirsat, 2003), and responses to biotic and abiotic stress (Showalter, 1993; Mazau and Esquerre-Tugayé, 1986). Extensins are characterized by a repetitive Ser-Hyp motif and have been shown to be developmentally regulated, tissue- and cell-specific, and induced by a variety of environmental stresses (Showalter, 1993). High levels of expression in regions of the plant subject to tensile stress (Tiré et al., 1994; Shirsat et al., 1996) and their induction by the weight-loading of whole plant organs (Shirsat et al., 1996), have led many authors to suggest that these proteins play a mechanical role in cell walls, in addition to their proposed roles in pathogen defence, the assembly of new cell walls and the deposition of lignin. While these studies have provided good evidence for the involvement of extensins in these responses, almost all these experiments can be classed as correlative, where induction of extensin mRNA or extensin driven reporter genes has been correlated with the inducing signal or stress. Very few studies have provided direct and unequivocal evidence for the function of extensins.

In early attempts to provide direct evidence for the proposed functions of extensin, researchers used various
methods to alter the amount of extensin in the cell wall. In some of the earliest function-related studies, the formation of protein-bound hydroxyproline (Hyp) in *Avena sativa* L. coleoptiles was inhibited in the presence of auxin by the addition of free Hyp to the growth medium, leading to increased extensibility (Cleland, 1967). Conversely, etiolated *Pisum sativum* L. seedlings that were exposed to ethylene showed an increase in protein-bound Hyp (Ridge and Osborne, 1970). Both of these methods were used (Esquerre-Tugayé et al., 1979) to modify HRP levels in *Cucumis melo* L. seedling cell walls before examining their subsequent response to pathogen infection. These methods are, however, far from ideal. The use of ethylene to up-regulate HRGPs is complicated by its role in a diverse array of plant growth and developmental processes including germination, senescence, abscission, flowering, and fruit ripening (reviewed in Abeles, 1973; Roberts and Tucker, 1984). The application of free Hyp to *A. sativa* coleoptiles inhibits auxin-induced cell elongation by an unknown mechanism (Cleland, 1967) and it is therefore likely that other systems in the cell are affected in addition to changes in HRP content.

The transgenic approach to altering extensin levels in plants and examining the effect on phenotype was first employed by Memelink who transformed *Nicotiana tabacum* L. plants with sense and antisense extensin (*pCNT1*) constructs under the control of the strong constitutive CaMV 35S promoter (Memelink et al., 1993). Transgenic plants displayed a range of Hyp levels and soluble extensin content, showing that expression of the CaMV 35S::pCNT1 gene construct in *N. tabacum* resulted in changes in total extensin protein levels. A similar approach (Jamet et al., 2000) used the Ext1.2A gene in transgenic *N. tabacum*. Both these studies demonstrated that a sense/antisense approach was effective in altering extensin levels, however, neither study was able to demonstrate a definite phenotypic effect as a result of altering extensin levels.

It was speculated that, as these studies were looking for gross morphological changes in the transgensics (which were not seen), a more precise approach to the analysis of the timing of plant developmental stages and the use of a homologous system might yield more significant data. Due to its general morphology and growth habit, *A. thaliana* is more amenable to growth analysis studies than *N. tabacum*. *A. thaliana* is an annual plant with an 8-week life cycle, well-defined vegetative and floral organs (e.g. rosette and inflorescence) and, unlike the perennial *N. tabacum*, has many growth parameters that are able to reach maximum levels in a short period of time. These include overall inflorescence height and maximum rosette diameter. Therefore, a more comprehensive analysis of growth and development is possible in this species.

Accordingly, it was decided to over-express the well-characterized *Arabidopsis thaliana* L. *Aext1* gene (Merkouropoulos et al., 1999) which is highly expressed in root tissue, but with low levels of expression in the rosette and floral organs. Analysis of transgenic *A. thaliana* plants containing an *Aext1* promoter, β-glucuronidase (GUS) reporter gene system showed that the *Aext1* promoter was activated in a developmental and stress-induced manner (Merkouropoulos and Shirsat, 2003). The *Aext1::GUS* fusion gene has also been shown to be expressed in response to mechanical wounding, pathogen infection, and in response to treatment with signalling intermediates (salicylic acid and methyl jasmonate) which are known to be involved in plant defence. This paper describes the construction of the CaMV 35S::Aext1 fusion gene and the biochemical and phenotypic effect of its over-expression in transgenic *A. thaliana* plants.

**Materials and methods**

**Plant material and growth analysis**

*A. thaliana* (L.) Heynh. seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Ecotypes Landsberg *erecta* (Ler-0, NASC line NW20) and Wassilewskija (Ws-0, NASC line N1602) were used in this work. *A. thaliana* seeds were sown in seed trays containing a 5:3 mixture of autoclaved B&Q Multi-purpose Compost (B&Q plc., Chandlers Ford, Hampshire, UK) and J Arthur Bower’s Vermiculite (William Sinclair Horticulture Limited, Lincoln, Lincolnshire, UK). Germination times were stratified by incubation at 4 °C in the dark for 3 d. Trays were then transferred to a 20–25 °C growth room with a 16/8 h light/dark cycle. The soil was kept moist by watering with dH₂O, and plants were fed every 2 weeks with full-strength Hoaglands Solution (Hoagland and Arnon, 1950). Where seeds were sown on selective plates, seeds were surface-sterilized and then placed on 0.8% agar, half-strength Murashige and Skoog basal media plates containing 20 mM sucrose and 35 μg ml⁻¹ kanamycin sulphae. Plates were wrapped in foil and stored at 4 °C for 3 d to stratify germination times and then transferred to a growth cabinet under a 16/8 h light/dark cycle at 20 °C. When seedlings had reached the 4-leaf stage, they were transferred to soil. Soil-based growth analysis was conducted on plants sown straight to soil and continued until the first seed pod shattered.

**CaMV35::Aext1 plasmid construction and A. thaliana transformation**

The *Aext1* coding sequence was excised from the plasmid pGM2 (Merkouropoulos et al., 1999) by digesting with *BshH1* and *XhoI*. The resultant 1.256 kb fragment was gel-purified, blunt-ended with DNA Polymerase I Large (Klenow) Fragment (Promega Corporation), and ligated into the *Smal* site of the pUC18 plasmid (Clontech Limited). The *Aext1* coding sequence was then excised from pUC18 with *BamH1* and *SacI* and ligated into the same restriction sites within the binary vector pBIN m-gfp5-ER (donated by Jim Haseloff, Cambridge University) and sequenced to verify that the plasmid border sequences remained intact. This binary vector construct (pKBS) was further transformed into *Agrobacterium tumefaciens* LBA4404 via tri-parental mating according to Elliott and Shirsat (1998). *A. thaliana* plants were transformed by the simple floral dipping method (Clough and Bent, 1998), modified from (Bechtold et al., 1993). Successfully transformed seed lines were selected on the basis of kanamycin resistance. All subsequent analyses were performed on T₂ plants and their progeny.
Genomic DNA isolation and Southern blot analysis

Plant genomic DNA was extracted from 0.25–0.75 g A. thaliana leaf and inflorescence tissue using the CTAB cetyl trimethyl ammonium bromide extraction buffer method (Doyle and Doyle, 1987) and stored at −20 °C. Genomic DNA was digested with HindIII and SacI, separated on a 1.0% agarose TAE gel and transferred by capillary blotting to Hybond-N nylon membranes (Amersham Pharmacia Biotech, Amersham, UK). The 871 bp CaMV 35S promoter sequence was labelled with $[{}^{32}P]$-dCTP by random primer labelling and used as a probe for Southern hybridizations as described (Elliott and Shirsat, 1998). Transgenic plants showing full-length, single copy insertions of the CaMV 35S::Atext1 transgene with no evidence of recombination were used for further study.

Total RNA isolation, RT-PCR and northern blot analysis

Total RNA was isolated from A. thaliana root or leaf and inflorescence tissue according to the method described by Hooks et al. (2004) and stored at −80 °C. Isolated total RNA was treated with deoxyribonuclease I (Sigma-Aldrich) to remove genomic DNA contamination completely according to the manufacturer’s instructions. Reverse transcription was carried out on 50 ng DNase-treated RNA using the Qiagen SensiscriptTM RT Kit (Qiagen Limited, Crawley, West Sussex, UK) and an oligo-dT primer (Promega Corporation) according to the manufacturer’s instructions. Amplification of RT products (cDNA) was conducted using the Qiagen HotStarTag™ DNA Polymerase Kit (Qiagen Limited, Crawley, West Sussex, UK) and reaction products were visualized on a 1.0% agarose TAE gel. Endogenous Atext1 mRNA was amplified using the primer pairs Atext1-inF (5′-CCACACAAACCTACCTTTAC-3′) and Atext1-inR (5′-CAGGATCTGGTCAGTTC-3′) designed from the 3′ transcribed but untranslated region of this gene, spanning a putative intron. Atext1 mRNA transcribed from the CaMV 35S promoter in the transgenics was amplified using the primer pairs Atext1-inF and pKB5-inR (5′-GCGAATTTTGCAAGACTTC-3′) designed from the 3′ transcribed but untranslated region of this gene, spanning a putative intron and incorporating sequences specific to the transgene construct. For northern analysis, the RNA was separated on a 1.3% agarose gel containing formaldehyde and transferred by capillary blotting to a Hybond-N nylon membrane (Amersham Pharmacia Biotech). Northern analysis was conducted using the Qiagen HotStarTag™ DNA Polymerase Kit (Qiagen Limited, Crawley, West Sussex, UK) and an oligo-dT primer (Promega Corporation) according to the manufacturer’s instructions. Amplification of RT products (cDNA) was conducted using the Qiagen HotStarTag™ DNA Polymerase Kit (Qiagen Limited, Crawley, West Sussex, UK) and reaction products were visualized on a 1.0% agarose TAE gel. Endogenous Atext1 mRNA was amplified using the primer pairs Atext1-inF (5′-CCACACAAACCTACCTTTAC-3′) and Atext1-inR (5′-CAGGATCTGGTCAGTTC-3′) designed from the 3′ transcribed but untranslated region of this gene, spanning a putative intron. Atext1 mRNA transcribed from the CaMV 35S promoter in the transgenics was amplified using the primer pairs Atext1-inF and pKB5-inR (5′-GCGAATTTTGCAAGACTTC-3′) designed from the 3′ transcribed but untranslated region of this gene, spanning a putative intron and incorporating sequences specific to the transgene construct. For northern analysis, the RNA was separated on a 1.3% agarose gel containing formaldehyde and transferred by capillary blotting to a Hybond-N nylon membrane (Sambrook et al., 1989). The Atext1 coding sequence was radio-labelled with $[{}^{32}P]$-dCTP by random primer labelling and used as a probe in northern hybridizations as previously described (Elliott and Shirsat, 1998).

Hydroxyproline assays

The total Hyp content of A. thaliana leaf and inflorescence tissue was measured using the assay method first described (Kivirikko and Liesmaa, 1959) and later modified by Fry (1988).

Selection of traits for comparing wild-type and transgenic plants

The phenotypic traits measured during this work, and used to compare wild-type and transgenic plants, were based on an initial study into the variability of individual traits within an untreated wild-type population. Wild-type Wassilewskija and Landsberg erecta plants were grown under standard environmental conditions using a 16 h day-length and various parameters were measured throughout their life cycle. These results were then used to calculate the coefficient of variation (CV) for each parameter. Each calculation was based on at least 20 plants. The CV associated with each value expresses the standard deviation (SD) of the data set as a percentage of the average (CV=[SD/average]×100). In this work, this measurement describes the variability of a particular characteristic within a single plant line (e.g. maximum plant height). A CV of less than 15% for a particular characteristic indicates a highly reproducible result that can be used in comparisons with other transgenic or wild-type lines (Boyes et al., 2001). Only traits that gave CV values below 15% for both ecotypes were used in the larger study to compare wild-type plants with those expressing altered Atext1 protein levels.

Parameters that were found to have low natural variation were maximum plant height and the timing of plant development. Development was assessed by the time taken for each plant to reach predetermined growth stages, as defined by Boys et al. (2001), adapted from the BASF, Bayer, Ciba-Geigy, Hoechst (BBCH) scale (Lancashire et al., 1991). Characteristics that gave CV values >15%, and were therefore not used in the subsequent analysis, included seedling root development, maximum rosette size, rosette leaf area, and the number of secondary bolts or lateral branches.

Results

Construction of the CaMV 35S::Atext1 fusion gene and transformation of A. thaliana plants

The cloning strategy was designed to insert the Atext1 coding sequence in the sense orientation downstream of the constitutive CaMV 35S promoter in the binary vector pBIN mgfp5-ER. The Atext1 fragment was successfully subcloned into the pUC18 vector in the sense orientation to give the recombinant plasmid pKB3. The Atext1 insert from pKB3 was then used to create the binary plant expression vector pKB5 (Fig. 1a). The sequence across the fusion junction is shown in Fig. 1b.

Transgenic A. thaliana plants were generated by transformation with A. tumefaciens containing the pKB5 binary plasmid. As the hypothesis was that transgenic A. thaliana plants expressing high Atext1 protein levels would either show differences in growth patterns or changes in development, it was decided to transform two A. thaliana ecotypes (Landsberg erecta and Wassilewskija) which have

Fig. 1. (a) Plasmid map of plant transformation vector pKB5, showing major restriction sites and features. Arrows indicate the 5′ to 3′ orientation. Chimaeric kanamycin resistance gene: nopaline synthase promoter (NOS prom): neomycin phosphotransferase (NPT II): nopaline synthase terminator (NOS ter). LB and RB refer to the left and right border sequences of the binary vector. (b) Border sequences of the inserted Atext1 coding sequence in the binary vector pKB5, showing restriction sites and the position of the Atext1 coding sequence in relation to the CaMV 35S promoter and NOS terminator. The ATG Atext1 start codon is underlined.

different morphologies. Landsberg erecta (Ler) has blunt siliques, short petioles (reaching only 16–25 cm in overall height), usually upright stems, and a compact inflorescence while Wassilewskija (Ws) is characterized by a large rosette, long narrow leaves, and an overall height of up to 40 cm.

Successful transformants from these two A. thaliana ecotypes were selected on the basis of kanamycin resistance and further selected by Southern analysis. Genomic DNA was extracted from 16 transgenic kanamycin-resistant T2 lines from both ecotypes and probed with the radiolabelled 871 bp CaMV 35S promoter sequence. Plant lines showing a single hybridizing band of the correct size were used for further analysis: lines showing evidence of recombination in the transgene were discarded (data not shown).

**Northern and RT-PCR analysis of transgenic A. thaliana plants carrying the CaMV 35S::Atext1 fusion gene**

Northern blot analysis of the transgenic Ler and Ws plant lines was performed using the 1.256 kb Atext1 coding sequence as a radiolabelled probe (Fig. 2a, b). This analysis showed that the transgenic A. thaliana plants expressed high levels of Atext1 mRNA in their leaf and inflorescence tissue. Transgenic Ler lines 5.2, 5.4, and 5.5 (Fig. 2a, lanes 4–6) show high levels of Atext1 expression, whereas expression was not detected in line 5.1 (lane 3). Expression can be seen in root tissue from the wild-type plant (lane 1), but not in leaf and inflorescence tissue (lane 2).

Atext1 mRNA in the transgenic Ler lines accumulated to levels far higher than the level seen in the roots of wild-type plants (compare lanes 4, 5, and 6 with lane 1 in Fig. 2a). Root tissue has previously been shown to have the greatest Atext1 transcript accumulation compared with other tissues in A. thaliana (Merkouropoulos et al., 1999; Merkouropoulos and Shirsat, 2003). Expression levels in the transgenic Ws lines were similar: all transgenic lines (Fig. 2b, lanes 3–11) showed high levels of Atext1 expression in leaf tissue. At this level of exposure, no expression was seen in root or leaf and inflorescence tissue in the wild-type plant (lanes 1, 2).

Comparisons of Atext1 transcript accumulation between wild-type and transgenic A. thaliana plants were repeated using RT-PCR analysis on the same total RNA samples as those used for northern hybridization. This was done in order to distinguish between expression from the endogenous Atext1 gene and expression from the introduced CaMV 35S::Atext1 transgene. Figure 3a and b show that expression of Atext1 driven from the CaMV 35S promoter is far higher than expression from the endogenous gene in all transgenic lines. In addition, the primers chosen for amplification of the endogenous Atext1 gene were designed across the putative 95 bp intron contained in the 3' transcribed but untranslated region (Merkouropoulos et al., 1999; Merkouropoulos and Shirsat, 2003). Expression levels far higher than the level seen in the roots of wild-type plants (compare lanes 4, 5, and 6 with lane 1 in Fig. 2a). Root tissue has previously been shown to have the greatest Atext1 transcript accumulation compared with other tissues in A. thaliana (Merkouropoulos et al., 1999; Merkouropoulos and Shirsat, 2003). Expression levels in the transgenic Ws lines were similar: all transgenic lines (Fig. 2b, lanes 3–11) showed high levels of Atext1 expression in leaf tissue. At this level of exposure, no expression was seen in root or leaf and inflorescence tissue in the wild-type plant (lanes 1, 2).

**Fig. 2.** (a) Northern blot of total RNA extracted from wild-type and transgenic Landsberg erecta root and leaf tissue and probed with the radiolabelled Atext1 coding sequence. Lane 1, Landsberg erecta root tissue; 2, L. erecta leaf tissue; 3, 4, 5, and 6, leaf tissue from the CaMV 35S::Atext1 transgenic T2 lines 5.1, 5.2, 5.4, and 5.5. The panel above the autoradiograph shows ethidium bromide-stained 28S RNA from the same tracks as used for northern analysis to demonstrate loading equivalents. (b) Northern blot of total RNA extracted from wild-type and transgenic Wassilewskija root and leaf tissue and probed with the radiolabelled Atext1 coding sequence. Lane 1, wild-type Wassilewskija root tissue; 2, wild-type Wassilewskija leaf tissue; 3–11, transgenic leaf tissue from the CaMV 35S::Atext1 T2 lines 5.6, 5.7, 5.8, 5.9, 5.10, 5.11, 5.12, 5.13, and 5.14. The panel above the autoradiograph shows ethidium bromide-stained 28S RNA from the same tracks as used for northern analysis to demonstrate loading equivalents.

**Fig. 3.** (a) RT-PCR products from wild-type and transgenic Landsberg erecta lines. Lane a, PCR using actin-2 primers; lane b, PCR using primers designed to detect endogenous Atext1 expression; and lane c, PCR using primers designed to detect transgenic CaMV-driven Atext1 expression. 1, negative control with no cDNA; 2, wild-type root; 3, wild-type leaf; 4–7 transgenic Ler lines 5.1, 5.2, 5.4, and 5.5. (b) RT-PCR products from wild-type and transgenic Wassilewskija lines. Lane a, PCR using actin-2 primers, lane b, PCR using primers designed to detect endogenous Atext1 expression, and lane c, PCR using primers designed to detect transgenic CaMV-driven Atext1 expression. 1, negative control with no cDNA; 2, wild-type root; 3, wild-type leaf; 4–10 transgenic Wassilewskija lines 5.6, 5.8, 5.9, 5.10, 5.11, 5.13, and 5.14.
Effects of altered extensin levels in Arabidopsis

Phenotypic analysis of transgenic A. thaliana plants carrying the CaMV 35S::AtExt1 transgene

The transgenic A. thaliana lines used for phenotypic analyses were those shown to have significantly different levels of total Hyp in their leaf and inflorescence tissue. These plant lines are for Landsberg erecta: wild type, 5.4, and 5.5 (in order of increasing Hyp content), and for Wassilewskija: wild type, 5.10, 5.6, 5.8, and 5.13. In addition to these lines, growth analyses were also performed on transgenic Ler and Ws lines which showed no increase in Hyp content (lines 5.1 and 5.14, respectively) in order to provide vector controls for the comparisons (i.e. to determine whether changes in morphology were due to changes in Hyp level or due to the presence of a transgene).

Changes in overall plant morphology (e.g. growth habit, leaf shape etc.) were not observed between wild-type and transgenic lines for either ecotype. In order to examine any developmental differences between A. thaliana plants transformed with the CaMV 35S::AtExt1 transgene and the wild type, phenotypic analyses of wild-type and transgenic plants were conducted using the growth stages defined by Boyes et al. (2001), adapted from the BASF, Bayer, Ciba-Geigy, Hoechst (BBCH) scale (Lancashire et al., 1991). Initial plate-based analyses measured differences in the time taken for both wild-type and transgenic seeds to reach defined Boyes developmental stages: the time required for seed germination, radicle emergence, hypocotyl and cotyledon emergence between wild type and transgenic plants were recorded and for leaf development, the time taken for cotyledons to open fully and for the first two rosette leaves to reach 1 mm in length was measured. No significant differences were found between the time taken for wild-type plants and transgenic plants with high hydroxyproline levels to reach these defined developmental stages (data not shown).

The period between Boyes stages 1.04 and 5.00 encompasses the transition from vegetative seedling development to mature floral development, and the period between stages 6.00 and 8.00 encompasses the time taken for flower pollination and silique maturation. Analyses conducted on soil-grown plants showed that, although the time to reach certain developmental stages differed significantly in some cases between the transgenics and the wild type, the patterns observed did not correlate with the measured differences in total Hyp content between the plant lines (data not shown).

Analysis of inflorescence growth in wild-type A. thaliana and transformed lines carrying the CaMV 35S::AtExt1 transgene

Figure 5a and b show the maximum height of inflorescence stems in the transgenic Ler and Ws CaMV 35S::AtExt1 lines compared with the wild type. In each ecotype the two transgenic lines with the highest Hyp content showed a reduction in overall height compared with the wild type.

Figure 4. Comparison of total Hyp content in transgenic Ler and Ws A. thaliana CaMV 35S::AtExt1 lines with wild-type levels (expressed as percentages of either Ws or Ler Hyp levels). Measurements were taken in triplicate. SD bars are shown. The dotted line indicates the 100% baseline, i.e. wild-type levels for either Ws or Ler. Statistically significant results (using a 2-tailed Student’s T-test) are highlighted with asterisks, significant at the 90% level (*) and 95% level (**).
type. The difference was significant in lines 5.4, 5.8, and 5.13 and highly significant in line 5.5. In a separate experiment (Fig. 6), the total inflorescence length (including side branches) and fresh mass was measured at growth stage 6.00 (first flower open) in the Landsberg erecta wild-type line and transgenic lines 5.1, 5.4, and 5.5 (this experiment was not conducted with the Ws ecotype). Individually, these two measurements showed no significant differences between wild-type and transgenic plants, however, a pattern was evident in the calculated values of fresh mass mm\(^{-1}\) stem. Figure 6 shows that the two transgenic lines 5.4 and 5.5 both have increased fresh mass mm\(^{-1}\) of stem compared with the wild-type control. The difference was significant for line 5.4 (95%) and highly significant for line 5.5 (99.9%). However, no difference was observed between the wild type and the vector-control line 5.1, suggesting that this difference could indeed be due to the increased Hyp levels in lines 5.4 and 5.5. Increased fresh mass mm\(^{-1}\) in the transgenic inflorescence stems also indicates that the stems are thicker (or denser) than those in Ler wild-type plants. While significant, it must be emphasized that these differences are surprisingly small compared with the wild type, in general, the difference is between 5–8% depending on the transgenic line. It was also examined whether the differences in overall height of the inflorescence stems were due to alterations in growth rate (data not shown). The growth curves of inflorescence stems from transgenics expressing high levels of hydroxyproline and the wild-type were identical for the first 13 d of growth, but from days 13–26, divergence between the transgenic and wild-type growth curves in both Ws and Ler stems was seen. During this period, the rate of stem growth started to decline in each of the wild-type lines. However, this decline occurred at a faster rate in the transgenics, reaching a plateau two days earlier than the wild-type in each case and accounting for the differences observed in maximum height.

Discussion

**Atext1 mRNA levels in A. thaliana plants carrying the CaMV 35S::Atext1 transgene**

Reverse transcriptase PCR (RT-PCR) was initially used to identify transgenic plants expressing the CaMV 35S::Atext1 transgene. Figure 3a and b show low levels of endogenous Atext1 gene expression in leaf and inflorescence tissue, higher levels in root tissue, and the presence of transgenic Atext1 transcripts in each of the transgenic lines. Northern hybridization analysis on the transgenic lines showed very high levels of Atext1 mRNA in leaf tissue from each of the transgenic lines, excluding Ler line 5.1 (Fig. 2a, b), these levels are higher than those seen in the root. In many plant species, it has been noted that expression levels of extensin in the root are far higher than those seen in the leaf (Evans et al., 1990), in the current work, it is obvious that the CaMV 35S promoter is able to drive constitutive expression in the leaf and inflorescence tissues to levels far higher than those normally seen in the wild-type root.
The analysis of \textit{Atext1} expression levels by both RT-PCR and northern hybridization provided complementary results. Compared with northern analysis, the RT-PCR method appeared to be more sensitive in detecting low levels of \textit{Atext1} transcripts. For example, a low level of \textit{CaMV 35S::Atext1} mRNA in Ler line 5.1 was seen using RT-PCR (Fig. 3a, lane 3), but not in the northern analysis. In addition, the RT-PCR results also suggest that steady-state \textit{Atext1} mRNA levels in wild-type plants vary between the Ws and Ler ecotypes, being higher in the latter. However, the PCR method was less able to quantify differences between samples containing high transcript levels. Differences in \textit{CaMV 35S::Atext1} gene expression between Ws transgenic lines were more obvious in the results from the northern analysis (Fig. 2b).

\textbf{Changes in hydroxyproline content in transgenic leaf tissue}

Figure 4 shows that total Hyp levels in leaf and inflorescence tissues are altered in transgenic \textit{A. thaliana} lines carrying the \textit{CaMV 35S::Atext1} transgene, compared with wild-type controls. The greatest difference is shown by Ws line 5.13, which shows a 3.5-fold increase in total Hyp content compared with wild-type Ws, showing that expression of the \textit{CaMV 35S::Atext1} transgene is able drastically to affect total Hyp levels in \textit{A. thaliana} leaves and inflorescences. In previous work (Memelink \textit{et al.}, 1993) transgenic \textit{N. tabacum} plants with the extensin p\textit{CNT1} gene under the control of the \textit{CaMV 35S} promoter were generated and transgenics that displayed up to a 4-fold increase in leaf and inflorescence Hyp content compared with wild-type controls were isolated. Wild-type levels of Hyp in leaf and inflorescence tissues were $\approx 0.3 \mu g$ mg$^{-1}$ dry mass, compared with $\approx 1.3 \mu g$ mg$^{-1}$ dry mass in the highest \textit{CaMV 35S::pCNT1} transgenic. These values compare well with results obtained in the current study: transgenic Ws and Ler lines had leaf and inflorescence Hyp levels of 0.34 $\mu g$ mg$^{-1}$ and 0.60 $\mu g$ mg$^{-1}$, respectively, and the highest expressing line (5.13) showed leaf Hyp levels 3.5-fold higher than the Ws control.

The total Hyp content measured in \textit{A. thaliana} tissues includes hydroxyproline from proteins encoded by other \textit{A. thaliana} extensin and HRGP genes, for example, the extensin genes \textit{AtExt2-5} (Yoshiba \textit{et al.}, 2001) and \textit{RSH} (Hall and Cannon, 2002). As \textit{Atext1} transcript levels are normally low in mature leaf tissue (Merkouropoulos \textit{et al.}, 1999), Hyp levels in wild-type Ws and Ler plants are most likely to be wholly derived from these other sources. The difference between steady-state Hyp content in Ws and Ler ecotypes is interesting and could be explored further, especially as the \textit{RSH} ecotype (with nearly double the leaf Hyp content than Ws) shows a dwarf phenotype. Whether this dwarf phenotype is actually due to higher hydroxyproline levels is unknown.

\textbf{Transgenics over-expressing extensin have stems of increased thickness}

All previous studies examining the effect of altered extensin levels on plant morphology were conducted on \textit{Nicotiana tabacum} L. plants. A preliminary report in 1991 suggested that the over-expression of a \textit{Daucus carota} L. extensin gene in \textit{N. tabacum} affected plant size (Cooper \textit{et al.}, 1991), although no further work on this system was published. When the phenotypic effect of changing extensin levels in the cell walls of \textit{N. tabacum} plants by altering expression of the tobacco p\textit{CNT1} gene was examined (Memelink \textit{et al.}, 1993), the authors detected no obvious change in morphology in the F1 populations and no significant differences in plant height during 9 weeks of growth. They concluded that \textit{N. tabacum} plants could tolerate a large variation in total hydroxyproline content and soluble extensin concentration without apparent phenotypic effects. A similar approach where the \textit{Ext1.2A} gene was expressed in transgenic \textit{N. tabacum} (Jamet \textit{et al.}, 2000), again found no phenotypic changes, although the authors stated that examining stressed plants might be more informative.

It has been shown in this work that increasing plant extensin levels results in an enhanced increase in stem thickness, with an indirect effect on stem height. A number of studies have shown direct correlations between high extensin levels and the cessation of cell elongation: the removal of cell wall protein increased extensibility in \textit{Avena sativa} coleoptiles, treatment of \textit{Allium cepa} roots with a peptidylprolyl hydroxylase inhibitor caused a reduction in HRGP levels leading to early elongation in developing root cells. The current work supports this idea as transgenics expressing high levels of extensin have significantly shorter inflorescences (although the effect is not large). This suggests that extensin synthesis causes the cessation of elongation itself, possibly through the formation of a cross-linked network. While \textit{in vivo} cross-linking of extensins has yet to be convincingly demonstrated, \textit{in vitro} studies have shown that peroxidases are able to cross-link extensin precursors. Cooper and Varner (1984) first reported that cell wall preparations from \textit{D. carota} roots contained extensin precursors which could be cross-linked in solution by the action of horseradish peroxidase into high molecular weight polymers. Further work by Schnabelrauch \textit{et al.} (1996) showed that different isoforms of tomato peroxidase were also able to cross-link extensin precursors \textit{in vitro}, and more recent \textit{in vitro} work by Alconada Magliano and Casal (1998) has shown that extracellular peroxidase isoforms from \textit{Sinapis alba} are able to cross-link carrot extensin precursors into higher molecular weight species.

It should be borne in mind, however, that despite hydroxyproline levels being over 4-fold higher than wild type in some transgenic lines, the phenotypic effect is relatively small – stem height is only reduced by 5–8%.
It is entirely possible that the functionality of extensin proteins is dependent on the extent of their cross-linking, and that if cross-linking in vivo is promoted, perhaps by increasing levels of hydrogen peroxide, drastic effects on plant morphology in the extensis overexpressors will be observed. This possibility is currently being investigated.

The results shown in Fig. 6 imply that reduced stem elongation is also accompanied by an increase in either stem density or stem diameter. The Atext1 over-expressing lines have stems with a higher fresh mass mm⁻¹ than wild-type control lines, this difference being significant at the 99.9% level in line 5.5. However, in order to extend these data, additional experiments need to be performed to examine the morphology of the inflorescence stem during development.

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