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Over-expression of *AtEXLA2* alters etiolated arabidopsis hypocotyl growth

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• **Background and Aims** Plant stature and shape are largely determined by cell elongation, a process that is strongly controlled at the level of the cell wall. This is associated with the presence of many cell wall proteins implicated in the elongation process. Several proteins and enzyme families have been suggested to be involved in the controlled weakening of the cell wall, and these include xyloglucan endotransglucosylases/hydrolases (XTHs), yieldins, lipid transfer proteins and expansins. Although expansins have been the subject of much research, the role and involvement of expansin-like genes/proteins remain mostly unclear. This study investigates the expression and function of AtEXLA2 (At4g38400), a member of the expansin-like A (EXLA) family in arabidposis, and considers its possible role in cell wall metabolism and growth.
• **Methods** Transgenic plants of *Arabidopsis thaliana* were grown, and lines over-expressing *AtEXLA2* were identified. Plants were grown in the dark, on media containing growth hormones or precursors, or were gravistimulated. Hypocotyls were studied using transmission electron microscopy and extensiometry. Histochemical GUS (β-glucuronidase) stainings were performed.
• **Key Results** *AtEXLA2* is one of the three EXLA members in arabidopsis. The protein lacks the typical domain responsible for expansin activity, but contains a presumed cellulose-interacting domain. Using promoter::GUS lines, the expression of *AtEXLA2* was seen in germinating seedlings, hypocotyls, lateral root cap cells, columella cells and the central cylinder basally to the elongation zone of the root, and during different stages of lateral root development. Furthermore, promoter activity was detected in petioles, veins of leaves and filaments, and also in the peduncle of the flowers and in a zone just beneath the papillae. Over-expression of *AtEXLA2* resulted in an increase of >10 % in the length of dark-grown hypocotyls and in slightly thicker walls in non-rapidly elongating etiolated hypocotyl cells. Biomechanical analysis by creep tests showed that *AtEXLA2* over-expression may decrease the wall strength in arabidopsis hypocotyls.
• **Conclusions** It is concluded that *AtEXLA2* may function as a positive regulator of cell elongation in the dark-grown hypocotyl of arabidopsis by possible interference with cellulose metabolism, deposition or its organization.

Key words: *Arabidopsis thaliana*, cell development, cell elongation, plant cell wall, expansins, expansin-like, extensiometry, hypocotyl growth, gravitropism.

INTRODUCTION

Plant growth and shape result from carefully orchestrated cell divisions and mainly from the highly controlled and sometimes massive expansion of these newly formed cells. During expansion, the walls are centres of high activity. Primary walls of most dicotyledonous plants consist of xyloglucan (XyG)-tethered cellulose microfibrils embedded in a matrix of highly hydrated pectins and structural proteins (Carpita and Gibeaut, 1993). As plant cell walls can be considered as rather rigid boxes encapsulating the protoplast, their controlled weakening is crucial to allow turgor-driven expansion. It is well established that a wide variety of enzymes and factors influence and regulate the wall's yielding properties, allowing an accurate control of the expansion process (Cosgrove, 2005). These include peroxidases (Passardi *et al.*, 2006), β(1→4)-glucanases (Labrador and Nevins, 1989), xyloglucan endotransglucosylase/hydrolases (XTHs; Nishitani and Vissenberg, 2007), expansins (McQueen-Mason *et al.*, 1992), yieldins (Okamoto-Nakazato *et al.*, 2001) and lipid transfer proteins (LTPs; Nieuwland *et al.*, 2005). For several of these enzyme families, enzymatic activities have been determined *in vitro*, but for yieldins and LTPs the mode of action still remains speculative.

Expansins were first discovered in studies of acid-stimulated growth as proteinaceous factors that have the capacity to induce extensibility and stress relaxation in cucumber hypocotyl walls in a pH-dependent manner (McQueen-Mason *et al.*, 1992). This necessity for an acidic cell wall environment coupled to the cell wall-loosening properties links the acid growth theory, auxin and expansins (e.g. reviewed in Rayle and Cleland, 1992). Expansins show specific binding to cellulose, with a significant increase in binding strength when the cellulose is coated with hemicelluloses, suggesting possible interactions of expansins with XyG. Further experiments showed, however, that expansins do not possess hydrolytic activity and that they do not change the mass distribution of cucumber xyloglucans.

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These data led to the currently accepted model for expansin action: *in vitro* cell wall extension and *in vivo* cell expansion involve the disruption of the hydrogen bonds between cellulose microfibrils and tethering glucans in the wall by the expansins (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2000*a, b*; Li *et al*., 2003; Sampedro and Cosgrove, 2005). This loosening would allow slippage of the cell wall polymers (creep) during turgor-driven cell wall extension, but prevents an irreversible weakening of the cell wall, which could result in detrimental lysis.

Expansins are classified into α- and β-expansins based on their sequence (Shcherban *et al*., 1995; Li *et al*., 2003; Choi *et al*., 2006). Expansins are small proteins (250–275 amino acids) composed of two compact domains [domain 1 (D1) and domain 2 (D2)] preceded by a signal peptide. Domain 1 is the N-terminal domain, homologous to the catalytic domain of proteins in the glycoside hydrolase family 45 (GH45). The C-terminal domain 2 is homologous to group-2 grass pollen allergens and has a sandwich fold. This is the cellulose-binding domain (CBD) (Cosgrove, 2000*b, c*; Sampedro and Cosgrove, 2005; Kerff *et al*., 2008). According to the recent nomenclature (Kende *et al*., 2004), only proteins with homology to both expansin domains should be designated expansins.

The effect of expansins on cell walls *in vitro* suggests the well-known cell wall-loosening function, yet roles in several other processes are also described. These include leaf abscission (Belfield *et al*., 2005), plant root colonization by *Trichoderma reesei* (Brotman *et al*., 2008), fruit ripening (Civello *et al*., 1999; Brummell *et al*., 2002; Fonseca *et al*., 2005), waterlogging-induced elongation (Cho and Kende, 1997; Caturla *et al*., 2002), cotton fibre elongation (Orford and Timmis, 1998), increasing cell wall flexibility upon cell wall folding during de- and rehydration in *Craterostigma plantagineum* (Jones and McQueen-Mason, 2004), root hair initiation (Cho and Cosgrove, 2002), pollen tube penetration (Cosgrove *et al*., 1997), hypocotyl and coleoptile elongation (Rochange *et al*., 2001; Gao *et al*., 2008), root elongation (Lee *et al*., 2003) and leaf development (Sloan *et al*., 2009).

Further attempts were made to better understand the working mechanism of expansins. Recently, the idea that expansins would bind not only to cellulose but also to a wider range of cell wall polymers was suggested (Nardi *et al*., 2013). By using a putative carbohydrate-binding module (CBM) from strawberry expansin 2 (CBM-FaExp2), the capacity to bind to xylan and pectin with different affinities was demonstrated. By this binding, the CBM-FaExp2 interfered with the activity of other cell wall-degrading enzymes, such as polygalacturonase, endoglucanase, pectinase and xylanase (Nardi *et al*., 2013). Recently Goh and co-workers (2014) suggested that the specific effects of ectopic expansin expression depend on the degree of induction and the endogenous developmental pattern of organ growth. Transgenic arabidopsis plants with inducible cucumber expansin expression showed that arabidopsis was very sensitive to higher levels of expansin gene induction, even resulting in growth suspension, while the small increase in expansin expression led to an increase in lamina and petiole growth (Goh *et al*., 2014). These authors postulated that expansin only unlocks the molecular structure of the cell wall and that the observed growth depends on other apoplastic factors (Caderas *et al*., 2000; Rochange *et al*., 2001; Sloan *et al*., 2009).

It is striking that dicotyledonous plants with type I cell walls, such as *Arabidopsis thaliana*, also contain a minority of β-expansins besides α-expansins. These β-expansins are thought to bind to type II cell walls that are mainly found in monocots and grasses. For arabidopsis, 26 α-expansins and six β-expansins are described; for the monocot rice these numbers are 33 and 19, respectively (http://www.bio.psu.edu/expansins/). Blasting sequenced plant genomes with the known α- and β-expansin sequences revealed the additional presence of two families of expansin-like genes, which are significantly similar to the already known expansins. In arabidopsis, three expansin-like A (EXLA) members and one expansin-like B (EXLB) member were found (Sampedro and Cosgrove, 2005). Both expansin domains are present in EXLA and EXLB, but their amino acid sequences are different from those of α- and β-expansins (Li *et al*., 2002, 2003).

Despite the vast number of publications on α- and β-expansins, the exact functions of EXLA and EXLB in *A. thaliana* remain largely unclear (Choi *et al*., 2008; Dal Santo *et al*., 2013). However, different expansin-like proteins isolated from fungi or bacteria, such as swollenin expansin-like proteins from *Trichoderma asperellum* (Wang *et al*., 2011) or a ceratoplatanin (CP) protein from the fungus *Ceratocystis platani*, are of interest to different research groups due to their cellulose biomass bioconversion abilities. CP has expansin-like activity and weakens filter paper in a concentration-dependent manner, and it represents a novel one-domain expansin-like protein with a hypothesized function in fungal interaction with the plant (Baccelli *et al*., 2014). Another example is bacterial Hc-EXLX2 from *Hahella chejuensis* (Lee *et al*., 2010), which enhanced the activity of cellulase and thereby increased the accessibility of other cell wall-degrading enzymes (Lee *et al*., 2010).

Here we investigate the expression and function of AtEXLA2 (At4g38400), a member of the EXLA family in *A. thaliana*, and discuss its possible role in cell wall metabolism and growth.

MATERIALS AND METHODS

Constructs

To generate the promoter::reporter constructs, a fragment 1044 bp upstream of the translation initiation site of At4g38400 was amplified by PCR from genomic DNA that was isolated as described by Edwards *et al*. (1991). Primers used for amplifying the promoter region were promEXLA2For (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCGTCGACCAATGGATTGTCGGTG-3′) and promEXLA2Rev (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTCTTGAAATTGAAGAATAACTAAAAAAAC-3′). The resulting product was gel-purified using the Ultra-Sep Gel Extraction Kit (Omega Bio-Tek), subsequently recombined with pDONR207 (Invitrogen) using GATEWAY™ technology (Invitrogen) and transformed into $CaCl_2$-competent *Escherichia coli* MC1061 (Hoffman-La Roche Ltd). ENTRY-clones were screened by colony PCR, and selected clones were verified by bidirectional dideoxy-sequencing (VIB-Flemish Institute of Biotechnology) using primers AttL1 (5′-TCGCGTTAACGCTAGCATGGATCTC-3′) and AttL2 (5′-GTAACATCAGAGATTTTGAGACAC-3′).

Sequence-verified ENTRY clones were recombined with destination vectors pGWB3 and pGWB4 (Nakagawa *et al*., 2007), leading to insertion of the promoter region upstream of the *uidA* or *GFP* sequence, respectively. After transformation in *E. coli* and colony PCR using primers promEXLA2for, GUSrev (5′-ATTGCCCGGCTTTCTTATA-3′) and GFPrev (5′-CCCTCGCCGGACAGCTGAA-3′), the final constructs were purified from *E. coli* using the Nucleospin® Plasmid QuickPure kit (Macherey-Nagel, Germany). The final constructs were then electroporated into *Agrobacterium tumefaciens* (strain C58 Rif^R harbouring pMP90; Koncz and Schell, 1986). Transformants were screened by colony PCR using the primers mentioned above and used for flower dip transformation of wild-type *A. thaliana* (Col-0, N1093) plants as described by Clough and Bent (1998) using a transformation buffer containing 5 % sucrose, $MgCl_2 \cdot 6H_2O$ (4 mM) and 0·02 % (v/v) Silwet L-77 (polyalkyleneoxide-modified heptomethyltrisiloxane) (GE Specialty Materials, Switzerland).

For the over-expression construct, the *AtEXLA2* coding region was amplified from the cDNA clone RAFL04-09-M02 (pda00182) (Seki *et al*., 1998, 2002; Sakurai *et al*., 2005) using primers 35SEXLA2For (5′-GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGCTGCAAGGCTTTCTCTTCCTC-3′) and 35SEXLA2Rev (5′-GGGGACCACTTTGTACAAGAAAGCT GGGTTTCAGTTCCAGATGTGATCGTCGC-3′). The product was recombined with pDONR207, and colonies were screened as described above. Correct ENTRY clones were recombined with pH2GW7·0 (Karimi *et al*., 2002, 2007) and screened by colony PCR using primers 35SEXLA2For and OVrev (5′-GCTCAACACATGAGCGAAAC-3′). Transformation of agrobacterium and arabidopsis was as described above, except that the corresponding primers were used.

Plant growth conditions

Wild-type (Col-0) and mutant seeds (SALK_147678) of *A. thaliana* were obtained from the Nottingham Arabidopsis Stock Centre (Scholl *et al*., 2000). For plant growth on plates, seeds were surface sterilized for 5 min in 6 % commercial bleach followed by three rinses in sterile water before they were placed in rows on half-strength MS Petri plates containing 2·2 g L^{-1} Murashige and Skoog salts including vitamins (Duchefa, The Netherlands), 1 % (w/v) sucrose (Duchefa), pH 5·7 (KOH) and 8 g L^{-1} plant agar (Duchefa). Plates were subsequently stratified for 4 d at 4 °C in the dark. Plates were then placed in a growth chamber (1·5E + 15 photons cm^{-2} s^{-1}; 16 h light/8 h dark; 24 °C). Images of plates were taken at the time points as mentioned in the Results, using a digital camera (Canon, 50D), and measurements were performed using ImageJ (Abramoff *et al*., 2004).

For measurements of dark-grown hypocotyls, seeds were sown on solid ES medium (Estelle and Sommerville, 1987) and stratified for 3 d at 4 °C. The synchronous germination was induced by exposure to fluorescent white light (150 µmol m^{-2} s^{-1} True Light; Philips, Eindhoven, The Netherlands) for 4 h at 21 °C. The transfer to light is referred to as 'induction' and considered as time zero for all the experiments. Darkness was obtained by wrapping the Petri dishes in four layers of aluminium foil. Covered plates were afterwards placed vertically at 21 °C in an environmentally controlled growth cabinet (cooled incubator BRC120, Bioconcept-Firlabo, Beun De Ronde, Belgium). Images of plates were taken at the time points as mentioned in the Results under green safe light to avoid any white light inhibition, using a digital camera (Canon, 50D). Manipulation of seedlings had no effect on hypocotyl growth or final length. Hypocotyl length was measured as the distance between the top of the root hairs around the collet, and the base of 'V' made by the petioles of the cotyledons (Scheres *et al*., 1994). The length of the hypocotyls was measured using ImageJ software (Abramoff *et al*., 2004).

For experiments on medium containing hormones or precursors, stocks of the studied compounds were made in dimethylsulphoxide (DMSO) and added to the medium when it had reached a temperature of <50 °C after autoclaving.

For the gravitropism experiment, half-strength MS medium (without sucrose) was used. Seeds were prepared as mentioned above and, after 3 d of stratification at 4 °C, plates were placed vertically in the growth chamber ($1{\cdot}5 \times 10^{15}$ photons cm^{-2} s^{-1}; 16 h light/8 h dark; 24 °C). Subsequently, after 3 d of growing, plates were turned 90 ° (Ishikawa and Evans, 1997; Hou *et al*., 2004; Young *et al*., 2006; Miller *et al*., 2007). This moment of turning is considered as time zero for this experiment. Plates with seedlings were collected every hour till 10 h after the turning and stained in β-gluronidase (GUS) solution on the plates in order to keep the real position of the root. Images of seedlings were taken under bright field illumination using a Zeiss Axioskope (Zeiss, Jena, Austria) equipped with a Nikon DXM 1200 or Nikon DS-Fi1 digital camera (Nikon Instruments Inc., Melville, NY, USA).

To prepare leaf series, seeds of the wild type and two over-expressing lines were sown in the soil. Seeds were stratified for 3 d at 4 °C in order to obtain synchronous germination. Subsequently, trays with seeds were placed in a growth chamber (1·5E + 15 photons cm^{-2} s^{-1}; 16 h light/8 h dark; 24 °C) and plants were collected at different developmental stages (14, 20, 26 and 32 d old). Cotyledons and leaves were dissected from the stem under a binocular microscope (Nikon SMZ-1B). Images of leaf series were taken using a Nikon AZ100 Multizoom equipped with a Nikon DS-Ri1 digital camera and in DIC (differential interference contrast) mode. The area and roundness of the leaves and length of the petioles were measured using ImageJ (Abramoff *et al*., 2004).

Identification of over-expression lines

Homozygous plants were grown for 2 weeks on half-strength MS medium before harvesting the plant material. Total RNA was extracted from leaves and roots using the Concert™ Plant RNA reagent (Invitrogen) and concentrations were determined on a nanodrop (model ND1000, Montchanin, DE, USA). A 1 µg aliquot of RNA was subsequently treated with RQ1 RNase-free DNase I (Promega, USA) according to the manufacturer's instructions in a total volume of 10 µL at 37 °C for 27 min and heat-inactivated at 65 °C for 15 min. This reaction was subsequently used for cDNA synthesis with a mixture of random hexamers and $oligod(T)_{18}$ primers using Superscript II reverse transcriptase (Invitrogen). For qPCR analysis, a capillary-based LightCycler (Roche Diagnostics, Belgium) was used

in combination with SYBR green containing Maxima^{TM} SYBR Green Master Mix (Fermentas, catalogue no. K0252) (ROX was omitted). As template 12·5 ng (5 µL of 1/20 diluted cDNA) was used in a reaction of 25 µL. Before quantification, the efficiency of primers was determined over six concentrations ranging from 0 to 50 ng. Expression ratios were calculated according to Pfaffl (2001). Primers used to amplify reference genes were Forwβ-actRT (5′-GCGATGAAGCTCAATCCAA A-3′), Revβ-actRT (5′-GTCACGACCAGCAAGATCAAG-3′), ForwEF-1αRT (5′-ATGCCCCAGGACATCGTGATTTCAT-3′) and RevEF-1αRT (5′-ACCTAGCCTTGGAGTATTTGG GGG-3′). *AtEXLA2* transcripts were detected using the primers FEXLA2RT (5′-CTTGTCCTTAGCAGCAGAGCC-3′) and REXLA2RT (5′-GGTACAAGAGCTTTATCGCC-3′).

GUS staining

Histochemical GUS stainings were performed according to a modified protocol of Jefferson *et al*. (1987). Plant tissues were submerged in a staining solution [potassium phosphate buffer 200 mM (pH 7·0), potassium ferrocyanide 1 mM, potassium ferricyanide 1 mM, disodium EDTA 10 mM, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide 1 mM] and incubated overnight at 37 °C. To improve the penetration of the staining solution in tissues containing a cuticle, samples were briefly incubated in 70 % acetone prior to staining. After staining, tissues were rinsed 3 × 10 min in H_2O, subsequently fixed in ethanol:acetic acid (3:1, v/v) and cleared in 8 M NaOH. Images were taken under bright field illumination using a Zeiss Axioskope equipped with a Nikon DXM 1200 or Nikon DS-Fi1 digital camera (Nikon Instruments Inc.). Representative images of at least three independent transgenic lines are shown.

Transmission electron microscopy

Seedlings of 35S::*AtEXLA2* 6_2 and wild-type Col-0 were grown for 4 d in the dark as described above before being prepared as described by Roland *et al*. (1982). The hypocotyls were first cut into three pieces (upper, middle and basal sections) and the sections were marked with an aqueous solution of ruthenium red so that the orientation would be known during embedding. Subsequently samples were fixed for 2 h in 3 % glutaraldehyde in 0·1 M Na-cacodylate buffer (pH 7·4) and washed three times for 10 min in the same buffer without fixative. At 1 h post-fixation with 1 % OsO_4 in the same buffer, they were rinsed three times for 10 min in the buffer only. Hypocotyl sections were extracted for 10 h in 40 % methylamine under continuous mild stirring and rinsed three times for 10 min in distilled water. Samples were then dehydrated for 10–15 min in a graded ethanol series (30, 50, 70, 80, 95 % and twice in 100 %) and embedded in Spurr resin following the manufacturer's instructions (SPURR: Modified SPURR Embedding Kit). Polymerization was performed by heating the samples at 70 °C for 8 h. Ultrathin transverse sections (90–100 nm) were made at the level of the apical hook, middle and the basal part of the hypocotyl. Observations were made with a Tecnai G2 SpiritBio Twin microscope equipped with a Veleta camera under a power of 120 kV.

Extensiometry

The biomechanics of hypocotyls was studied using the constant-load (creep) method. Etiolated 4-day-old wild-type and 35S::*AtEXLA2* 6_2 arabidopsis seedlings were transferred individually into 1·5 mL Eppendorf test tubes, frozen by plunging the closed tubes into liquid nitrogen, stored at −20 °C and used for extensiometry within 2 weeks after freezing. For heat inactivation, distilled water (1·5 mL, 22 °C) was added to Eppendorf test tubes with frozen sampes, and the tubes were transferred to a pre-heated (90 °C) thermomixer (Eppendorf Thermomixer® comfort) and incubated with shaking at 100 rpm for 3 min. Then, the heat inactivation was rapidly stopped by transferring the tubes to a 1000 mL beaker with water at 22 °C. Finally, the frozen–thawed heat-inactivated arabidopsis seedlings were transfered to small Petri dishes filled with distilled water and kept in a refrigerator at 4 °C for 1–2 h before being used in creep tests.

In vitro extension of frozen–thawed arabidopsis hypocotyls was measured with a custom-built constant-load extensiometer described in Suslov and Verbelen (2006) using the same procedure as in Miedes *et al*. (2013). A 5 mm long hypocotyl segment (starting from 1·5 mm below the cotyledons) was secured in the extensiometer and pre-incubated in a buffer (20 mM MES-KOH, pH 6·0 or 20 mM Na-acetate, pH 5·0) in the relaxed state for 2 min, after which it was extended in the same buffer under a constant load (400, 500, 600 or 700 mg) for 15 min. The creep rate was calculated with the formula given in Vandenbussche *et al*. (2011).

The cross-sectional area of the hypocotyl cell walls used in extensiometry was determined by measuring their dry weight per unit length (Cleland, 1967) as detailed in Miedes *et al*. (2013).

RESULTS

AtEXLA2 *expression patterns*

In order to investigate the potential functions of *AtEXLA2*, a detailed expression analysis was performed using the arabidopsis eFP browser (Winter *et al*., 2007; Supplementary Data Figs S1 and S2) and promoter*AtEXLA2*::reporter lines. Both *uidA* and *GFP* were used as reporters, but only GUS lines were analysed in detail.

Staining with GUS was observed in seeds before and during testa rupture, suggesting a potential role for *AtEXLA2* during embryonic growth or seed germination (Fig. 1A). In very young seedlings (2 d old), it was obvious that *AtEXLA2* expression was present both in the hypocotyl and in the root. *AtEXLA2* is strongly expressed throughout the hypocotyl, while weak expression is seen only in the tip of the cotyledon, presumably around the central hydathode. In young roots, expression was strongest in the root tip, with a maximum in the central cylinder starting at the onset of the elongation zone. In the differentiation zone of the root, expression was limited to the central cylinder (Fig. 1B). In roots of 8-day-old seedlings *AtEXLA2* expression mimicked that of younger roots, with the addition of clear expression in all stages of lateral root primordia development (Fig. 1C–G). During the initial lateral primordia divisions, expression was present throughout the new primordia (Fig. 1C, D); in older stages *AtEXLA2* expression was confined to the central cylinder of the new lateral root

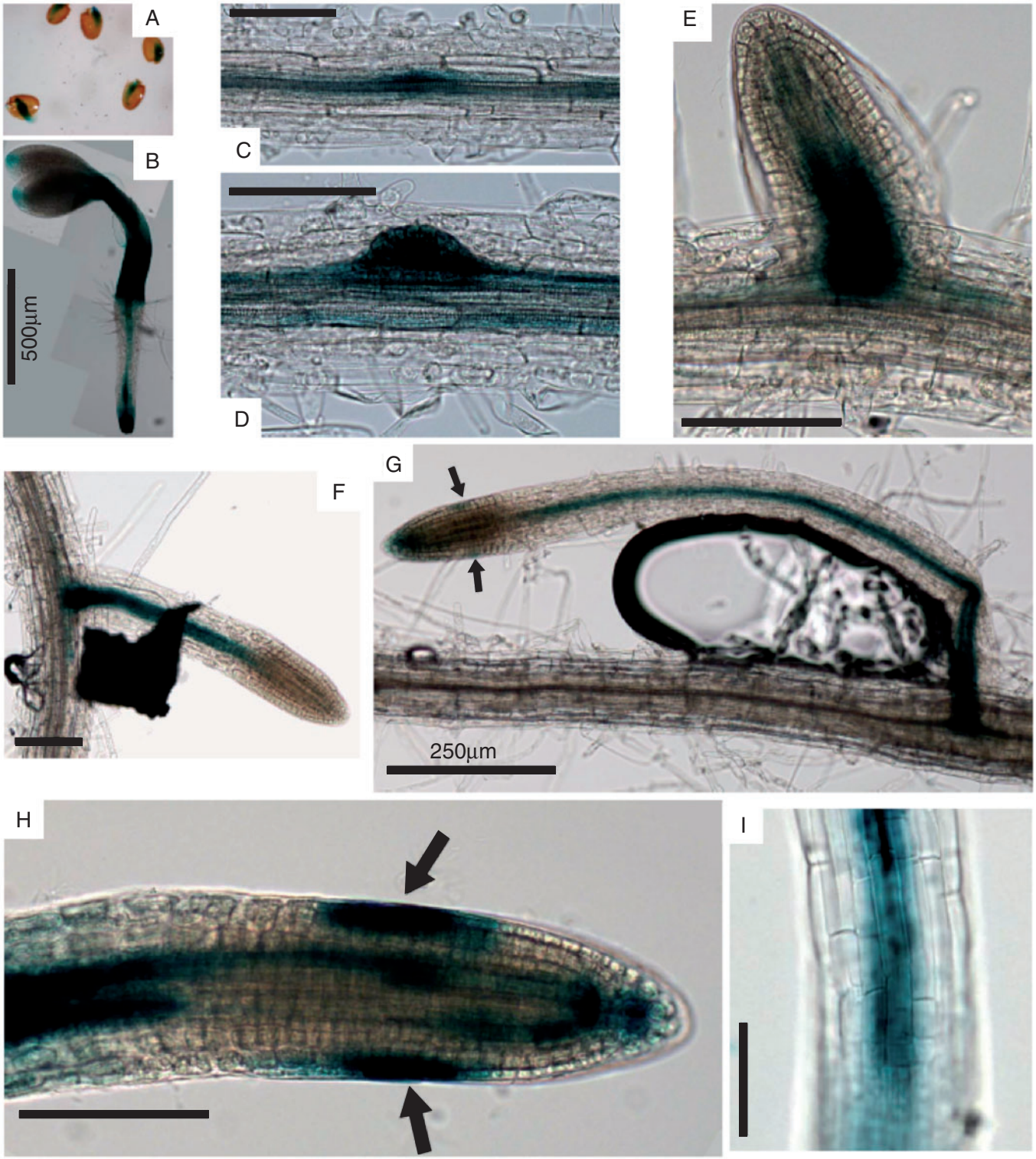

FIG. 1. GUS staining in seedlings and roots of promoter*AtEXLA2*::*uidA* plants. (A) Seedlings emerging from seeds. (B) Two-day-old seedling. (C–G) Staining at different developmental stages of lateral root development. (G) Magnification of the lateral root tip with staining in the central cylinder, elongating lateral root cap cells (see arrows), columella and quiescent centre cells. (H) Primary root grown on ES medium showing the increase in expression in the central cylinder when cell elongation commences. Arrows indicate staining in elongating lateral root cells as in (G). (I) Close-up of the elongation zone with expression in the central cylinder. Scale bars represent 100 µm, unless indicated otherwise.

(Fig. 1E–G). Weak GUS staining in the central cylinder was present in the meristem from the quiescent centre on, and became much stronger as soon as cells entered the elongation zone (Fig. 1H, I). As primary and lateral roots matured, the oldest cells belonging to the lateral root cap (which also undergo a phase of cell elongation) and to the columella showed strong GUS staining (see arrow in Fig. 1G, H).

It was reported before that over-expression of expansins affects different aspects of plant growth (Choi *et al*., 2006); therefore, the expression of *AtEXLA2* was also investigated in the above-ground parts of seedlings and adult plants. Cotyledons showed strong GUS staining throughout the whole petiole and in the vasculature (Fig. 2A). An additional site of GUS staining was localized at the central hydathode of the cotyledon (Fig. 2A), while the other cotyledon cells showed no *AtEXLA2* expression at all. In older soil-grown plants, GUS staining was found in a pattern similar to that in the cotyledons. The first two leaves showed a similar pattern, with staining concentrated in or around the veins of the petiole and leaf blade and the most intense staining in the midvein of the leaf and the petiole (Fig. 2B). In the third and the fourth leaf, staining was not visible in the veins but was concentrated around the hydathodes, a pattern that was also present in the cauline leaves (results not shown). In flowers, staining was mainly present in the veins of filaments (Fig. 2C). Also the peduncle and the zone just underneath the papillae showed GUS staining (Fig. 2D).

Sensitivity of AtEXLA2 *expression for auxin*

As expansins are accepted to be involved in acid growth and since cell wall acidification by plasma membrane-localized H^+-ATPases can be induced by auxin, we checked whether the *AtEXLA2* promoter is auxin responsive. We performed an *in silico* analysis of the promoter sequence (Fig. 3), revealing sequence similarities to a part of an auxin-inducible element of *Glycine max* and of an auxin-responsive element of *Brassica oleraceae*, but not to auxin-specific *cis*-elements. In addition, some potential *cis*-elements related to abscisic acid (ABA) and methyl jasmonate were detected. A further analysis using Genevestigator (Zimmermann *et al*., 2004) indicated that the expression of At4g38400 changes after auxin and ABA application. To check the validity of the *in silico* results, the effects of different compounds on the GUS expression pattern were studied. No visible differences in GUS staining patterns were found when either indole acetic acid (IAA), ABA, naphthalene acetic acid (NAA), 1-naphthylphthalamic acid (NPA), 1-aminocylcopropane-1-carboxylicacid (ACC), L-α-(2-aminoethoxyvinyl)glycine (AVG), GA_3 or jasmonic acid was added to seedlings (results not shown).

Comparison of AtEXLA2 *gene structure with* AtEXPA, AtEXPB, AtEXLA *and* AtEXLB *family members*

A graphical display of all the expansin and expansin-like genes of arabidopsis reveals that most *AtEXPA* genes have two introns, all *AtEXPB* genes have three introns, *AtEXLA1* and *2* have two introns, *AtEXLA3* has only one intron, and *AtEXLB1* has three introns (Fig. 4). In this figure, those regions of the exons that code for the catalytic site of expansins (D1) and the CBD (D2) are indicated with red and green boxes, respectively. This analysis indicates that only the CBD is present in *AtEXLA2* (see red arrow), while the catalytic site is clearly absent.

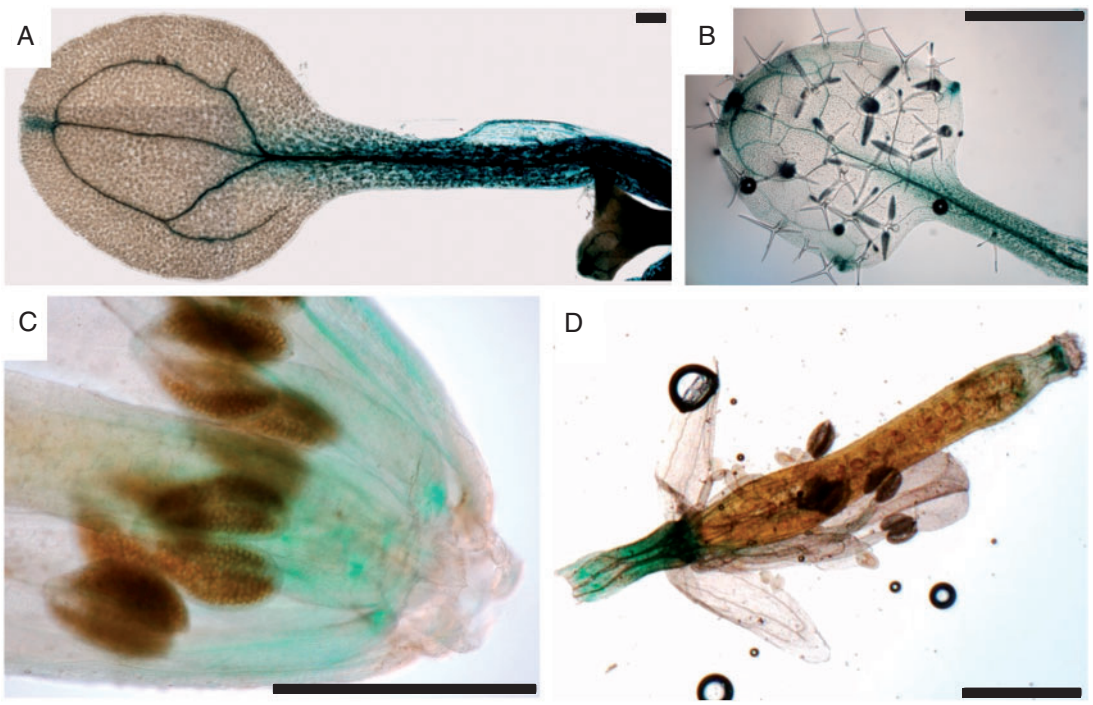

FIG. 2. GUS staining in cotyledons, leaves and inflorescences of promoter*AtEXLA2*::*uidA* plants. (A) Cotyledon, (B) young leaf, (C) inflorescence, (D) detail of stamen. Scale bars represent 1 mm.

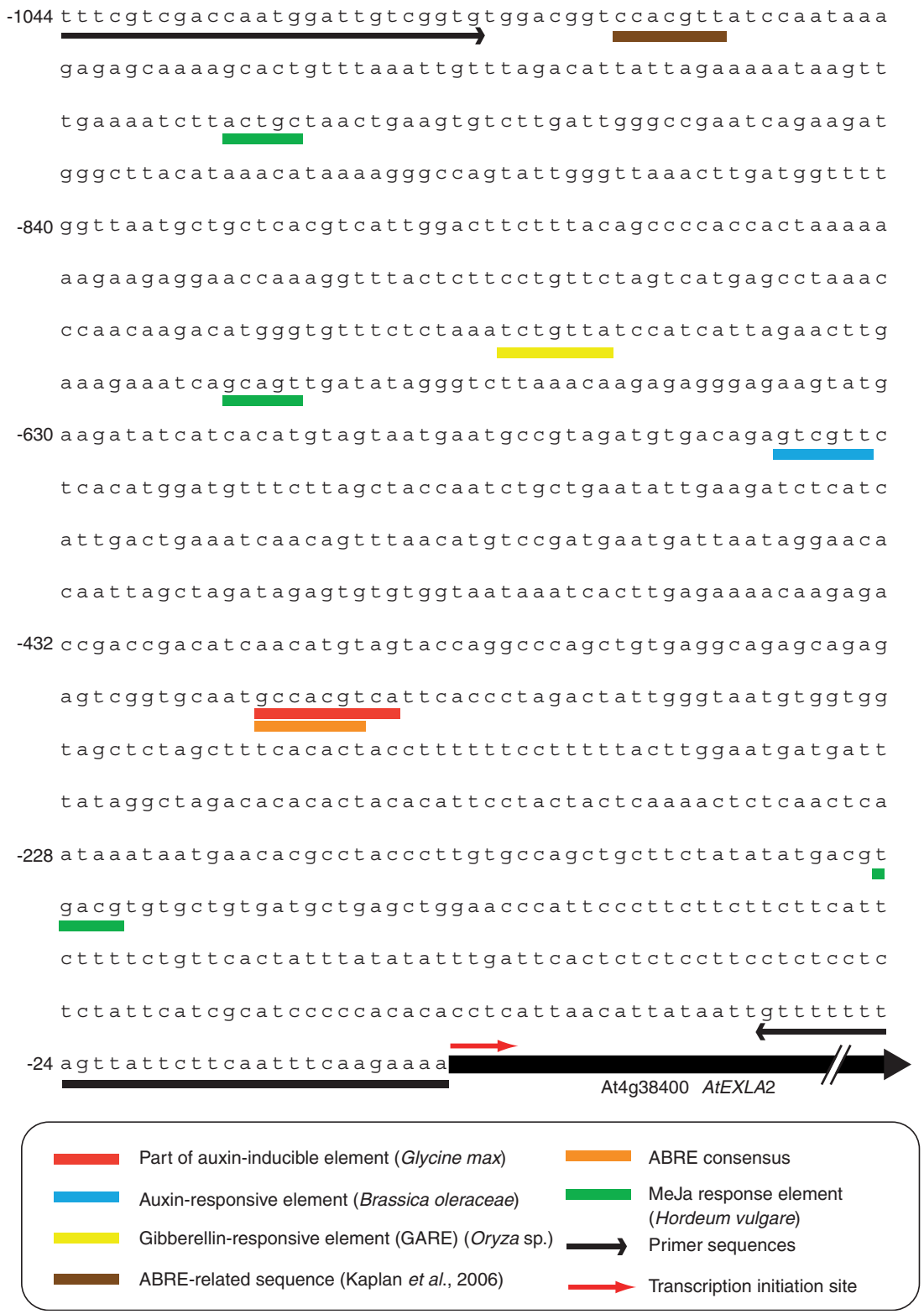

FIG. 3. *In silico* analysis of the promoter region of At4g38400. *In silico* promoter analysis of *AtEXLA2* (At4g38400) using PlantCARE (Rombauts *et al.*, 1999; Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999). The sequences underlined with arrows represent the primers used for PCR amplification of the promoter. *Cis*-elements are colour coded.

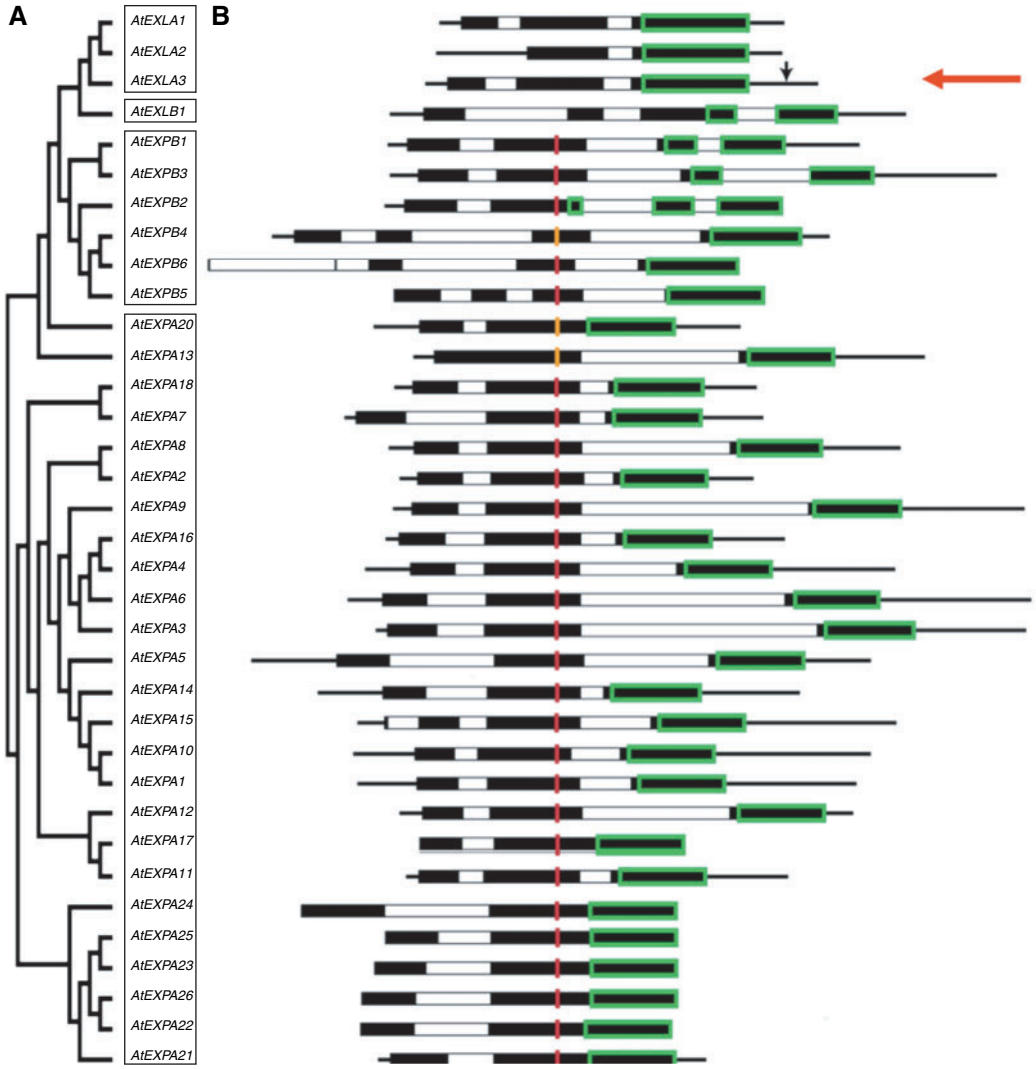

FIG. 4. Phylogenetic and structural analysis of *Arabidopsis thaliana* expansins. (A) Phylogenetic tree of *A. thaliana* expansin proteins. The different expansin subfamilies are boxed. (B) Graphical representation of the gene structure of the corresponding expansins from (A). White and black bars depict introns and exons, respectively; black lines represent 3′- and 5′-untranslated regions. The regions coding for the presumed carbohydrate-binding modules are boxed in green, and the coding region for the presumed catalytic residues (HFD) are shown in red. Where the catalytical triad differed by only one amino acid, it is shown in orange. The T-DNA insertion in *AtEXLA2* is indicated by a black arrow. Sequences are aligned to the catalytical triad where possible.

Effect of altered AtEXLA2 *expression levels on development*

To investigate the effects of lowered or absent *AtEXLA2* expression levels on plant development, the only available T-DNA insertion line (SALK_147678) in *AtEXLA2* was obtained and homozygous plants were selected by PCR screening. Unfortunately sequencing of a PCR product covering the T-DNA border indicated that the T-DNA insertion was localized 127 bp after the stop codon (see the vertical black arrow in Fig. 4). Semi-quantitative PCR of *AtEXLA2* expression levels did not show visible differences in the Salk line compared with the wild type. The presence of the At4g38400 transcript at wild-type levels in the homozygous Salk line was confirmed with qPCR (results not shown).

To overcome the lack of a suitable knock-out plant and to avoid the potential problems with redundancy, plants over-expressing *AtEXLA2* were generated. The AtEXLA2-coding region was placed downstream of the *Cauliflower mosiac virus* (CaMV) 35S promoter in pH2GW7, 0 (Karimi *et al.*, 2002, 2007) and transformed to wild-type (Col-0) plants. Homozygous T_3 plants harbouring a single insertion event were selected, and *AtEXLA2* expression levels were determined in 7-day-old seedlings using qPCR. Two lines with high over-expression levels compared with the wild type (339 × in line_6_2 and 73 × in line_3_5, respectively) were selected for further experiments.

The strong expression of *AtEXLA2* in the root (Fig. 1) suggested an involvement in root development. Therefore, the effect of *AtEXLA2* over-expression on root growth was studied. After 3 and 7 d of growth, roots of the two over-expressing lines were significantly longer than those of the wild type (Fig. 5).

As well as being expressed in the root, GUS staining patterns suggested prominent expression of *AtEXLA2* in the

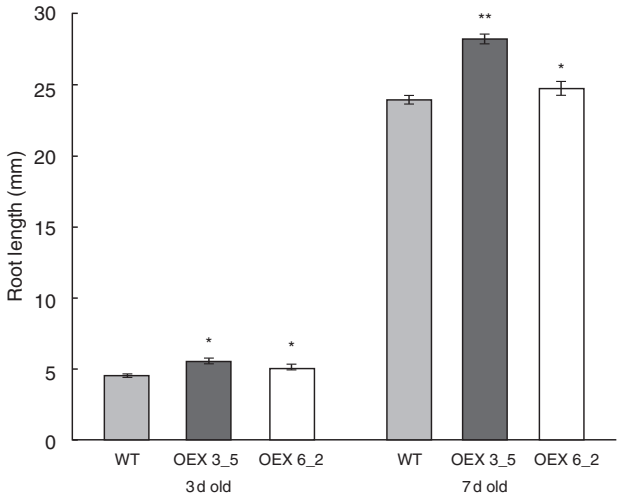

FIG. 5. Root length in the wild type (WT) and two 35S::*AtEXLA2* lines. The WT (Col-0) and two CaMV35S::*AtEXLA2* lines (line 6_2 and 3_5) were grown in the light on half-strength MS medium for 3 and 7 d and their root length was scored (mean ± s.e.). Asterisks indicate significant differences.

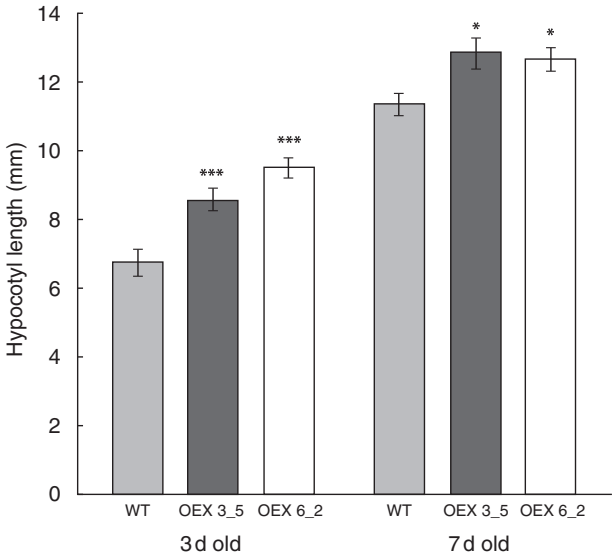

FIG. 6. Dark-grown hypocotyl length in the wild type (WT) and two 35S::*AtEXLA2* lines. The WT (Col-0) and two CaMV35S::*AtEXLA2* lines (line 6_2 and 3_5) were grown in the dark on ES medium for 3 and 7 d and their hypocotyl length was measured (mean ± s.e.). Asterisks indicate significant differences.

above-ground parts of the plant, including the hypocotyl and cotyledons. The length of 3- and 7-day-old dark-grown hypocotyls was determined. Under standard conditions, hypocotyls of over-expressing plants were significantly longer than in wild-type plants at both day 3 (142 and 128 % for lines 6_2 and 3_5, respectively) and day 7 (111 and 113 % for lines 6_2 and 3_5, respectively) [*t*-test, $P < 0{\cdot}01$ (day 3) and $P < 0{\cdot}05$ (day 7); Fig. 6]. The leaf area of cotyledons and real leaves (14, 20, 26 and 32 d old) was studied in the two over-expression lines, but did not show differences compared with the wild type (results not shown). Also no significant differences were found in vein pattern or petiole length (results not shown).

Plant roots and stems bend in response to gravity in a process called gravitropism (Blancaflor, 2002; Miller *et al.*, 2007). Promoter*AtEXLA2*::reporter lines have shown GUS staining in the elongation zone and lateral root cap cells (Fig. 1H, I), both zones being involved in the auxin-dependent gravitropic reaction. The expression pattern of *AtEXLA2* genes was examined during gravitropism by turning the Petri plates 90 ° and checking the GUS signal every hour until 10 h after the turning. The GUS expression pattern did not change when compared with the normal growth situation (results not shown).

Effect of AtEXLA2 *over-expression on cell wall thickness*

To study the effect of *AtEXLA2* on cell wall thickness, 4-day-old etiolated hypocotyls were sampled and the thickness of the outer epidermal cell wall was measured at the bottom, middle and upper part of the hypocotyl. At this moment, cells at the base have undergone rapid elongation so that their walls are at their thinnest, cells in the middle part are undergoing fast elongation and cells in the upper part are still in the slow growth phase where a thick cell wall is deposited and just before the rapid elongation will start. From Fig. 7 it becomes clear that in the upper part, cell walls are thicker in over-expression line 6_2 than in the wild type, while they are equally thin in the other two zones.

Effect of AtEXLA2 *over-expression on cell wall extensibility*

Cell wall extensibility plays a crucial role in the control of plant cell growth at the biophysical level. Thus the cell well extensibility was estimated in frozen–thawed wild-type and 35S::*AtEXLA2* 6_2 hypocotyls using the creep test (Fig. 8). Four-day-old growing hypocotyls were chosen, because they were sufficiently long for clamping in the extensiometer. At this stage, wild-type and 35S::*AtEXLA2* 6_2 hypocotyls had growth rates that did not differ significantly. In an attempt to predict the effect of different turgor values on the wall deformation, the hypocotyls were extended under a range of loads. The wall cross-sectional area in the 5 mm long sub-apical zone of hypocotyls extended in the creep test was similar in the wild-type and 35S::*AtEXLA2* 6_2 plants (results not shown) when estimated from the wall dry weight per unit length (Miedes *et al.*, 2013). This zone includes the middle part of hypocotyls analysed in Fig. 7, so the results on the wall cross-sectional area obtained by the two alternative methods are consistent. Equal cell wall cross-sectional areas in the wild-type and 35S::*AtEXLA2* 6_2 plants indicate that each constant load generates a similar stress in their hypocotyl cell walls.

The creep rates of hypocotyls were compared at pH 5, pH 6 and pH 5 with heat inactivation. A pH 5 buffer was used because expansins are activated at pH $\leq 5{\cdot}5$ (McQueen-Mason *et al.*, 1992). They lose their activity at pH 6, but this pH value is optimal for different classes of cell wall-loosening proteins such as XTHs (Maris *et al.*, 2011). The heat treatment was used to inactivate essentially all cell wall-loosening proteins to reveal their contribution to wall creep.

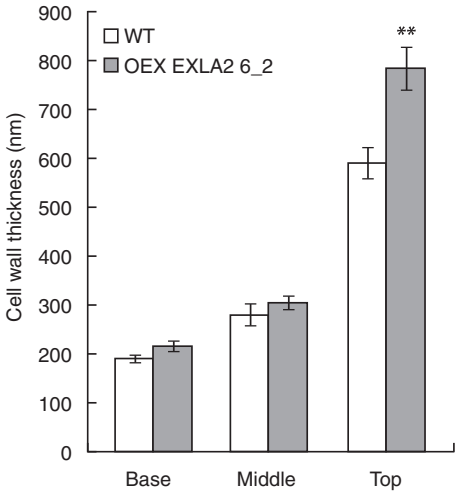

FIG. 7. Cell wall thickness in the wild type (WT) and 35S::*AtEXLA2*. Ultrathin sections were prepared at the base, middle and top of 4-day-old etiolated hypocotyls of the WT and 35S::*AtEXLA2* line 6_2. Samples were studied with a transmission electron microscope and the thickness of the external epidermal cell wall was measured. Asterisks indicate significant differences.

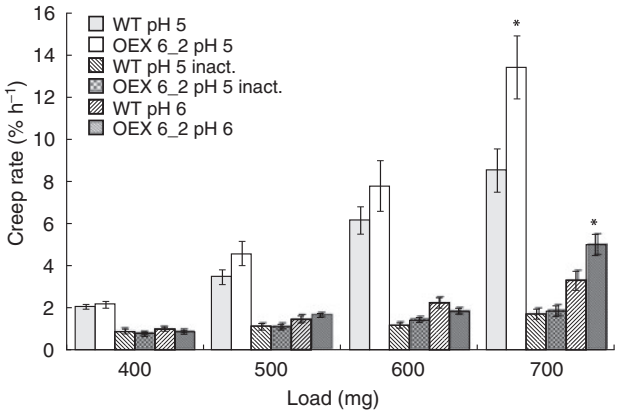

FIG. 8. Creep rate of frozen–thawed arabidopsis hypocotyls. The creep rate of frozen–thawed wild type (WT) and 35S::*AtEXLA2* 6_2 (OEX 6_2) hypocotyls was measured under four different loads at pH 5 and pH 6 without heat inactivation, and pH 5 after heat inactivation (inact.) (mean ± s.e., $n = 8–9$). Asterisks indicate significant differences between the over-expression line and the WT under the same load and pH value. In all cases, the creep rate at pH 5 was significantly higher than that at pH 6 and pH 5 after heat inactivation (not shown on the plot).

Hypocotyls of wild-type and 35S-*AtEXLA2* 6_2 plants demonstrated a strong acid-induced creep in a pH 5 buffer under all the loads studied (Fig. 8). This presumably expansin-mediated effect was completely eliminated by a heat treatment. The creep rate of heat-inactivated hypocotyls extended at pH 5 dropped to or below that found at pH 6 without heat inactivation. No significant differences were found in the creep rate of the wild type and the over-expression line under 400–600 mg loads, consistent with their *in vivo* growth rates. A marked proportion of hypocotyls broke under a 700 mg load (Table 1), and this was accompanied by a very different trend for *in vitro* cell wall extension. The creep rate of 35S::*AtEXLA2* 6_2 hypocotyls was significantly higher than in the wild type at both pH 5 and pH 6 under a 700 mg load (Fig. 8). Interestingly, hypocotyls of the over-expression line broke twice as often as wild-type hypocotyls under the maximal load used (Table 1). These results show that *AtEXLA2* over-expression did not affect the ability of the wall to extend under moderate uniaxial stress values. On the other hand, the biomechanics under the maximal load suggests that the over-expression decreases the wall strength. The high creep rate of 35S::*AtEXLA2* 6_2 hypocotyls at pH 5 and pH 6 under a 700 mg load could precede their failure or just be an indicator that their ultimate strength is approaching. This burst increase in the creep of the over-expression line is not seen after a heat treatment, presumably because of failure of hypocotyls (Table 1) that would demonstrate a high creep rate without heat inactivation.

DISCUSSION

Plant growth is the result of an increase in the number of cells and, more importantly, the volume of these newly formed cells through cell expansion. For the latter process, the initially deformation-resistant cell walls need to be modified. The ability of cell walls to undergo irreversible turgor-driven expansion is largely determined by the physical properties of the cell wall and by apoplastic enzymes modifying and/or restructuring its components (Cosgrove *et al*., 2000*a, b, c*; Thompson and Fry, 2001). For several protein families, the ability to induce cell wall extension *in vitro* or/and *in vivo* has been experimentally demonstrated (Labrador and Nevins, 1989; Okamoto-Nakazato *et al*., 2001; Nieuwland *et al*., 2005; Passardi *et al*., 2006; Van Sandt *et al*., 2007). In the same year as XTHs were discovered (Fry *et al*., 1992), the existence of another class of proteins with cell wall-loosening capacities, i.e. eexpansins, was proven (McQueen-Mason *et al*., 1992). Phylogenetic and sequence analyses have shown that expansin sequences are present in early diverging land plants, such as the liverwort *Marchantia* and the water fern *Marsilea*. These data point to a likely early evolutionary origin in the algal lineage (Cosgrove, 2000*c*). Moreover, expansin-coding sequences are even found in non-plant species. However, the gene structure from expansins of non-plant species such as a mycetozoan slime mould (*Dictyostelium discoideum*), fungi, mussels and a Solonaceous nematode (*Globodera rostochiensis*) (Xu *et al*., 2001; Li *et al*., 2002; Darley *et al*., 2003; Qin *et al*, 2004; Kudla, 2005; Brotman *et al*., 2008) differs from that in plants, suggesting that expansins have evolved separately in plant and non-plant species (Choi *et al*., 2006). Taken together with the vast number of expansins in some species (mostly due to gene duplication), this clearly indicates that expansins fulfil a crucial function during plant life.

Several characteristics are conserved in the expansin and expansin-like genes. First of all, the overall gene structure is conserved in expansin gene families. All expansins contain four conserved introns, except α-expansins, which lack the second and fourth intron. The signal peptide is also conserved in all expansin(-like) proteins. The N-terminal part of the protein contains the presumed catalytic domain that contains several conserved cysteines that are responsible for the secondary structure of the catalytic domain. In expansin-like A and B subfamilies, several of these conserved cysteines are not present, while

expansin-like A proteins have two cysteines in their C-terminal domain. The catalytic site itself is believed to be represented by the amino acids HFD (histidine–phenyalanine–aspartate), and, strikingly, is absent in members of the expansin-like A and B groups (Fig. 4). This strongly suggests that expansin-like proteins cannot show classical expansin activity. However, this does not preclude the potential presence of an activity which differs from that described for α- and β-expansins. The C-terminal part of expansins has been described as a putative CBD, based on the similarity to the CBD of cellulase (Cosgrove, 2000*c*). From a similar analysis, it seems that AtEXLA2 (At4g38400) only contains the CBD and not the catalytic site that is typical for α- and β-expansins.

The *in silico* expression pattern analysis of the At4g38400 gene using the arabidopsis eFP browser (Winter *et al*., 2007; Supplementary Data Figs S1 and S2), suggests enriched expression in roots, hypocotyls and leaves, and this was fully confirmed in independent promoter*AtEXLA2*::reporter lines, indicating that the cloned promoter region was representative. In the root, *AtEXLA2* expression was strongest in the elongation zone, quiescent centre, columella cells and stele, while in older roots clear expression was observed at all stages of lateral root primordia development. The expression analysis clearly shows that *AtEXLA2* expression is detected in elongating cells of the root, hypocotyl and petioles, which suggests the involvement of *AtEXLA2* in the expansion process. Furthermore, microarray data clearly show that expression levels of *AtEXLA2* are differentially regulated during the process of dark-grown hypocotyl elongation. In the early phase of hypocotyl elongation at 48 h post-induction (hpi) *AtEXLA2* is 1·7-fold up-regulated compared with 45 hpi. Later during hypocotyl elongation, *AtEXLA2* levels keep rising, with a 3·0- and 4·2-fold increase at 52 and 55 hpi, respectively, compared with the onset of elongation at 45 hpi (Pelletier *et al*., 2010). These transcriptomic data also point towards a role for *AtEXLA2* during hypocotyl elongation as its expression rises at the moment when rapid elongation of etiolated hypocotyl cells is seen. As suggested by these data, *AtEXLA2* over-expression indeed caused a small but significant growth-promoting effect in the root and etiolated hypocotyl, indicating that increased AtEXLA2 stimulates elongation in the early stages of root and etiolated hypocotyl growth since differences are already apparent in 3-day-old organs.

Strong *AtEXLA2* expression was also localized in the central cylinder of the root, leaves and the filament, suggesting that AtEXLA2 could play a role in secondary cell wall deposition in xylem cells. Over-expression of *AtEXLA2* would increase the CBDs present in cells, which could interfere somehow with the massive cellulose deposition that is typical for secondary wall formation. This mechanism could explain the decrease in cell wall strength as a result of *AtEXLA2* over-expression (Table 1).

It is generally believed that expansins facilitate *in vitro* cell wall creep and that they therefore are key regulators of cell expansion. In this light, it is important to mention that numerous papers have indeed pointed to the correlation between expansin expression and cellular elongation (e.g. Cho and Kende, 1997; Cho and Cosgrove, 2000; Lee *et al*., 2003), but several other studies have also stressed that this correlation is far from absolute (Caderas *et al*., 2000; Rochange *et al*., 2001; Choi *et al*., 2003; Sloan *et al*., 2009). Using transgenic arabidopsis plants with inducible over-expression of cucumber expansin, Goh *et al*. (2014) have recently proposed that the effects of expansins on growth largely depend on the developmental pattern, organ and the level of changes in expansin expression, and that the effects can vary from growth promotion even to an inhibition. These findings indicate that the levels of expansin in the cell wall are crucial for the resulting phenotypic effect. Under natural conditions, expansins are present in the cell wall at very low concentrations of around one protein per 5000 parts of cell wall (data from cucumber hypocotyls) (McQueen-Mason and Cosgrove, 1995; Cosgrove, 2000*b*). It is thus not surprising that abnormally high concentrations of these proteins can lead to unexpected phenotypes. From our results, it is clear that expansin-like proteins do not induce drastic changes in cell wall mechanics, as expansins do, so they could have a different mechanism of action.

Besides possible effects of (unnatural) changes in protein abundance in the cell wall, the effects of the over-expression combined with the expression patterns point to a pivotal role for the CBD, especially in the absence of a conventional expansin active site. It is noteworthy that the structure of the EXLA CBD differs slightly from that of α- and β-expansins by harbouring an additional tryptophan and two cysteines. In this light, it is important to mention that over-expression of a CBD-truncated form of swollenin, an expansin-like protein from *Trichoderma reesei*, did not increase cucumber root colonization, while over-expression of the native form did (Brotman *et al*., 2008). Also the bacterial CBD of *Clostridium cellulovorans* has been shown to affect elongation of roots, pollen tubes

TABLE 1. *Frequency of hypocotyl failures in the creep test under different loads*

	400 mg	500 mg	600 mg	700 mg
WT pH 5	0·0 %* (8/8)†	0·0 % (8/8)	0·0 % (8/8)	27·3 % (8/11)
OEX 6_2 pH 5	0·0 % (8/8)	0·0 % (8/8)	20·0 % (8/10)	20·0 % (8/10)
WT pH 5 inactivated	0·0 % (8/8)	11·1 % (8/9)	27·3 % (8/11)	27·3 % (8/11)
OEX 6_2 pH 5 inactivated	0·0 % (8/8)	11·1 % (8/9)	11·1 % (8/9)	55·6 % (8/18)
WT pH 6	0·0 % (9/9)	0·0 % (9/9)	25·0 % (9/12)	25·0 % (9/12)
OEX 6_2 pH 6	0·0 % (9/9)	0·0 % (9/9)	18·2 % (9/11)	25·0 % (9/12)

*The frequency of hypocotyl failures expressed as a percentage of total measurements.

†The first number in parentheses indicates the number of successful creep measurements without hypocotyl failures, and the second number after a slash refers to the total number of creep measurements.

OEX6_2 represents 35S::*AtEXLA2* line 6_2.

and root hairs *in vitro* (Shpigel *et al*., 1998). Bacterial CBDs have been extensively studied with the aim of changing plant fibre properties and cell wall digestibility (Levy *et al*., 2002). The effects of over-expression of several CBDs on plant development and cell wall structure has been investigated, and these studies have indicated that the relationship between increased CBD levels and phenotypes is quite ambiguous. For example, over-expression of a tandem fusion protein of CBM29 (carbohydrate-binding module 29) resulted in a reduced stem elongation phenotype (Obembe *et al*., 2007), while over-expression of a bacterial CBM3 stimulated growth in potato plants (Safra-Dassa *et al*., 2006). Over-expression of the CBD of the fungus *Aspergillus japonicus* in arabidopsis also resulted in smaller and thinner plants (van der Valk *et al*., 2003). Further, it has been shown that some bacterial CBDs are capable of modifying plant cell wall structure *in planta* (Shpigel *et al*., 1998; Levy *et al*., 2002). CBDs are believed to exert their effect on cell walls by influencing normal cell wall polymer organization. *In vivo*, CBDs are thought to bind to nascent cellulose chains, inhibiting their aggregation into crystalline cellulose microfibrils by shielding the potential hydrogen-bonding sites of the cellulose. Moreover, Shpigel *et al*. (1998) have reported that increased CBD levels compete for cellulose-binding sites with xyloglucan and result in a lower degree of xyloglucan–cellulose association. Alternatively, CBDs could also bind to cellulose after its synthesis and crystallization, leading to non-hydrolytic fibre disruption (Levy *et al*., 2002). Also, several other cellulose-binding compounds influence cellulose crystallization, such as fluorescent brighteners (e.g. calco-fluor white) or carboxymethyl cellulose, resulting in cell wall changes in bacteria. The CBD from *Clostridium cellulovorans*, for example, led to an increased cell wall biosynthesis in *Acetobacter xylinum* (Shpigel *et al*., 1998). These documented effects on cellulose and/or cellulose/xyloglucan could explain the observed decrease in the wall strength (Table 1) as a result of *AtEXLA2* over-expression.

In bacterial cellulases, CBDs are responsible for the concentration of the catalytic domain at the substrate surface. For some of these cellulases with multiple CBDs, it has been demonstrated that the individual CBDs display different affinities towards specific cell wall polymers. This indicates that the substrate specificity of CBDs largely determines its effect on the cell wall. As most CBDs (or CBMs in general) have been determined based on sequence similarity, their substrate specificities have not yet been proven. Therefore, it is of major importance to determine empirically the binding preferences of the many different CBDs.

Taken together, all these data point to a potential influence of CBDs on cell walls. Cellulose has been proposed as the substrate that is recognized by the CBD of expansins (Cosgrove, 2000*b, c*). However, the CBD of AtEXLA2 displays some differences when compared with the well-studied α- and β-expansins, potentially reflecting a different substrate specificity. Only when these unknown specificities are elucidated can their exact function and effects in the cell wall be properly interpreted. From the present data, an increase in typical expansin activity by over-expressing *AtEXLA2* cannot explain the observed effect on growth. Based on our results, we hypothesize that AtEXLA2 functions in cell wall remodelling, although its exact mechanism of action remains to be determined. It would be very interesting to test whether members of the expansin-like A or B families have any effects on cell wall extension in *in vitro* cell wall extension assays under a range of protein concentrations and to examine the specificity of their CBDs in binding to cellulose and other cell wall polymers.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: expression patterns of *AtEXLA2* in different above-ground organs of arabidopsis revealed by the eFP browser (Winter *et al*., 2007). Figure S2: expression patterns of *AtEXLA2* in the arabidopsis root revealed by the eFP browser (Winter *et al*., 2007)

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