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Spatial–temporal analysis of polyethylene glycol-reduced aluminium accumulation and xyloglucan endotransglucosylase action in root tips of common bean (*Phaseolus vulgaris*)

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• **Background and Aims** Aluminium (Al) toxicity and drought are two major limiting factors for common bean (*Phaseolus vulgaris*) production on tropical acid soils. Polyethylene glycol (PEG 6000)-induced osmotic stress (OS) simulating drought stress reduces Al accumulation in the entire root tips of common bean by alteration of cell-wall (CW) porosity, which might be regulated by two genes encoding xyloglucan endotransglucosylase/hydrolase, *PvXTH9* and *PvXTHb*. The aim of this research was to understand the spatial and temporal regulation of both *XTH* genes in PEG-mediated Al accumulation in the root tips.
• **Methods** In this study the spatial and temporal expression patterns of Al-inhibited root elongation, Al accumulation, *XTH* gene expression and xyloglucan endotransglucosylase (XET) enzyme action in the root tips were analysed under PEG-induced OS by a combination of physiological and molecular approaches such as quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and *in situ* fluorescence detection of XET in root tips.
• **Key Results** The results showed that Al accumulation, expression of *XTH* genes and XET action were distinctly reduced in the apical 0–2, 2–7 and 7–12 mm zones under OS, implying a potential regulatory role of *XTH* genes and XET enzyme in CW Al accumulation in these zones.
• **Conclusions** The results provide novel insights into the physiological and molecular mechanisms of CW structure modification as a response of plant roots to OS, which will contribute to mitigate Al and drought stresses, severely limiting crop yields on acid soils.

Key words: aluminium toxicity, cell wall, drought, PEG, root tip, XET.

INTRODUCTION

Soil acidity with pH $\leq$ 5·5 is among the important environmental factors limiting crop production worldwide on approx. 30 % of the world's total land area and more than 50 % of the world's potentially arable lands. Acid soils cover about 43 % of the world's tropical land area, 68 % of tropical America, 38 % of tropical Asia and 27 % of tropical Africa (von Uexküll and Mutert, 1995). When soil pH drops below 5, trivalent aluminium (Al) ions become soluble in soil solution and seriously limit plant growth and development (Kochian *et al*., 2004). In many parts of the tropics and subtropics, drought stress is becoming an additional important limiting factor for crop production because of dry spells during the cropping season becoming increasingly frequent with the change in global climate (Z. B. Yang *et al*., 2013).

Much progress has been made in recent years in our understanding of the physiological and molecular responses of plants roots to combined Al and drought stress in common bean (*Phaseolus vulgaris*) (Z. B. Yang et al., 2010, 2011, 2012, 2013; Butare *et al*., 2011; Araújo *et al*., 2015). Using polyethylene glycol (PEG-6000), we previously revealed that PEG-induced osmotic stress (OS) simulating drought stress (Z. B. Yang *et al*., 2012) enhanced Al resistance by excluding Al from the root apex (Z. B. Yang *et al*., 2010), which is the most Al-sensitive root zone (Ryan *et al*., 1993). The OS-induced reduction of Al accumulation in the root tips mainly results from reduced cell-wall (CW) porosity caused by OS-induced water loss from the apoplast (Z. B. Yang *et al*., 2010). Transcriptome analysis further suggested a role of xyloglucan endo-transglycosylase/hydrolase (XTH) in the modification of CW structure (Z. B. Yang *et al*., 2011). However, the detailed molecular mechanism remains obscure.

The primary CW of flowering plants is mainly composed of cellulose, hemicelluloses (principally xyloglucans) and glycoproteins embedded in a pectic matrix (Carpita and Gibeaut, 1993). The extensibility of the CW structural components determines cell expansion (Cosgrove, 1993; Szymanski and Cosgrove, 2009). XTH plays a crucial role in the regulation of CW extensibility. It acts on xyloglucans either as xyloglucan endotransglucosylase (XET), cutting and rejoining xyloglucan (Rose *et al*., 2002), or as xyloglucan hydrolase (XEH), hydrolysing xyloglucan (Rose *et al*., 2002; Baumann *et al*., 2007; Ibatullin *et al*., 2009). The maintenance of CW extensibility has been suggested to be a prerequisite for root growth under drought conditions (Sharp *et al*., 2004; Yamaguchi and Sharp, 2010). Under water deficit the CW in the apical region (0–3 mm from the tip) of maize maintains a flexible state and

allows continuous cell growth, while the CW in the elongation region (3–9 mm from the tip) becomes inextensible leading to inhibited or ceased cell elongation (Wu and Cosgrove, 2000; Fan and Neumann, 2004; Fan *et al*., 2006). There are conflicting results about the role of XTH in cell elongation under drought. Proteomic analysis by Zhu *et al*. (2007) demonstrated that drought decreased the abundance of the XTH protein in the root apical 0–3 mm section of the maize root, while Wu *et al*. (1994, 1996) indicated that the enhanced CW extensibility in maize root tips under drought is related to elevated XTH enzyme activity, and Pritchard *et al*. (1993) showed that drought has no effect on XTH enzyme activity in maize root tips. In common bean, transcriptome analysis showed that the transcripts level of two genes, *PvXTH9* and *PvXTHb*, which belong to the *XTH* gene family, are repressed in root tips by both PEG-induced OS and drought in soils (Z. B. Yang *et al*., 2011, 2012), while the removal of PEG stress rapidly rescued *XTH* gene expression (Z. B. Yang *et al*., 2011). The consistency of the change of *PvXTH9* and *PvXTHb* gene expression (Z. B. Yang *et al*., 2011) with Al accumulation (Z. B. Yang *et al*., 2010) in the root tips before and after PEG stress suggest the involvement of both *XTH* genes in the regulation of CW porosity and thus Al accumulation (Z. B. Yang *et al*., 2011, 2013).

The root apex is the major site of perception for Al toxicity (Ryan *et al*., 1993). In common bean, both the transition zone (TZ) and the elongation zone (EZ) of root tips are primary targets of Al injury (Rangel *et al*., 2007), and a lower Al accumulation in root tips confers Al resistance (Rangel *et al*., 2007; Z. B. Yang *et al*., 2010). In *Arabidopsis* and tobacco (*Nicotiana tabacum*), the action of XET was detected primarily in the root TZ and EZ where XET regulates root growth mainly through mediating CW extension of epidermis and cortex cells (Vissenberg *et al*., 2000). To better understand the regulation of XTH in PEG-mediated Al accumulation in the root tips and thus Al resistance, a spatial and temporal analysis of Al accumulation, *XTH* gene expression and XET action in the root tips of common bean genotype VAX 1 under OS was performed.

MATERIALS AND METHODS

Plant materials and growing conditions

Seeds of the common bean (*P. vulgaris* L.) genotype VAX 1 (Al-sensitive; Rangel *et al*., 2007) were germinated on filter paper sandwiched between sponges. After 3 d, uniform seedlings were transferred to a continuously aerated simplified nutrient solution containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 (Rangel *et al*., 2007). The pH of the solution was gradually lowered to 4·5 within 2 d. The plants were then transferred to the simplified nutrient solution without or with $AlCl_3$ (25 μM) and/or PEG 6000 (150 g L^{-1}) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), pH 4·5. The osmotic potential of 150 g L^{-1} PEG 6000 solutions was −0·60 MPa, measured with a cryoscopic osmometer (Osmomat 030; Gonotec GmbH, Berlin, Germany). Plants were cultured in a growth chamber under controlled environmental conditions of a 16/8-h light/dark cycle, 27/25 °C day/night temperature, 70 % relative air humidity and a photon flux density of 230 μmol m^{-2} s^{-1} of photosynthetically active radiation at plant height. After the treatment period, root tips or segments were harvested for Al analysis or immediately frozen in liquid nitrogen for RNA isolation.

Measurement of partial elongation of root tips

Assessment of spatial growth patterns of individual root sections was based on the approach developed by Rangel *et al*. (2007). The first 10 mm of the root apex was marked with consecutive black dots at 1-mm intervals using a fine brush and Indian ink (Pelikan, Hannover, Germany), which does not affect root growth. The plants were then transferred to nutrient solution containing 0 or 150 g L^{-1} PEG 6000. The elongation between the dots was measured after 4 h of treatment and then the elongation rates of each root apical section were calculated. The relative elemental growth rates (REGRs) were calculated according to Erickson and Sax (1956), Silk (1984), and Peters and Bernstein (1997).

Isolation of cell-wall material

After pre-treating without or with 150 g L^{-1} PEG for 4, 8 or 24 h, 30 root tips of 1 cm length or root segments at different root apical sections were excised and transferred to 1 mL of 96 % ethanol. CW material was prepared as an alcohol-insoluble residue after repeated washing with ethanol, modified after Schmohl and Horst (2000) and following the procedures described by Z. B. Yang *et al*. (2010).

Cell-wall binding capacity of Al^{3+} in root tips

The isolated CW material was incubated for 30 min in 1 mL of a solution (pH 4·3) containing 300 μM $AlCl_3$ according to Z. B. Yang *et al*. (2010), and then the suspension was centrifuged at 23000 *g* for 10 min. The supernatant was discarded and the pellet was re-suspended and washed with ultra-pure deionized water three times. The residues were then digested with 65 % (v/v) ultra-pure HNO_3 and prepared for Al determination.

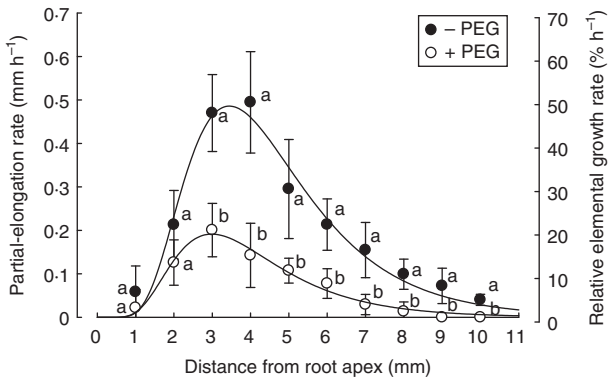

FIG. 1. Effect of PEG-induced osmotic stress (–0·60 MPa OP) on the partial-elongation rates of apical 1-cm root segments in common bean genotype VAX 1. Plants were pre-cultured in a simplified nutrient solution containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 for 48 h for acclimation and pH adaptation, and then treated without or with polyethylene glycol (PEG-6000, 150 g L^{-1}) in the simplified nutrient solution (pH 4·5) for 4 h. Means with different letters are significantly different between PEG treatments for each root segment at $P < 0·05$. Bars represent means ± s.d., $n = 9$.

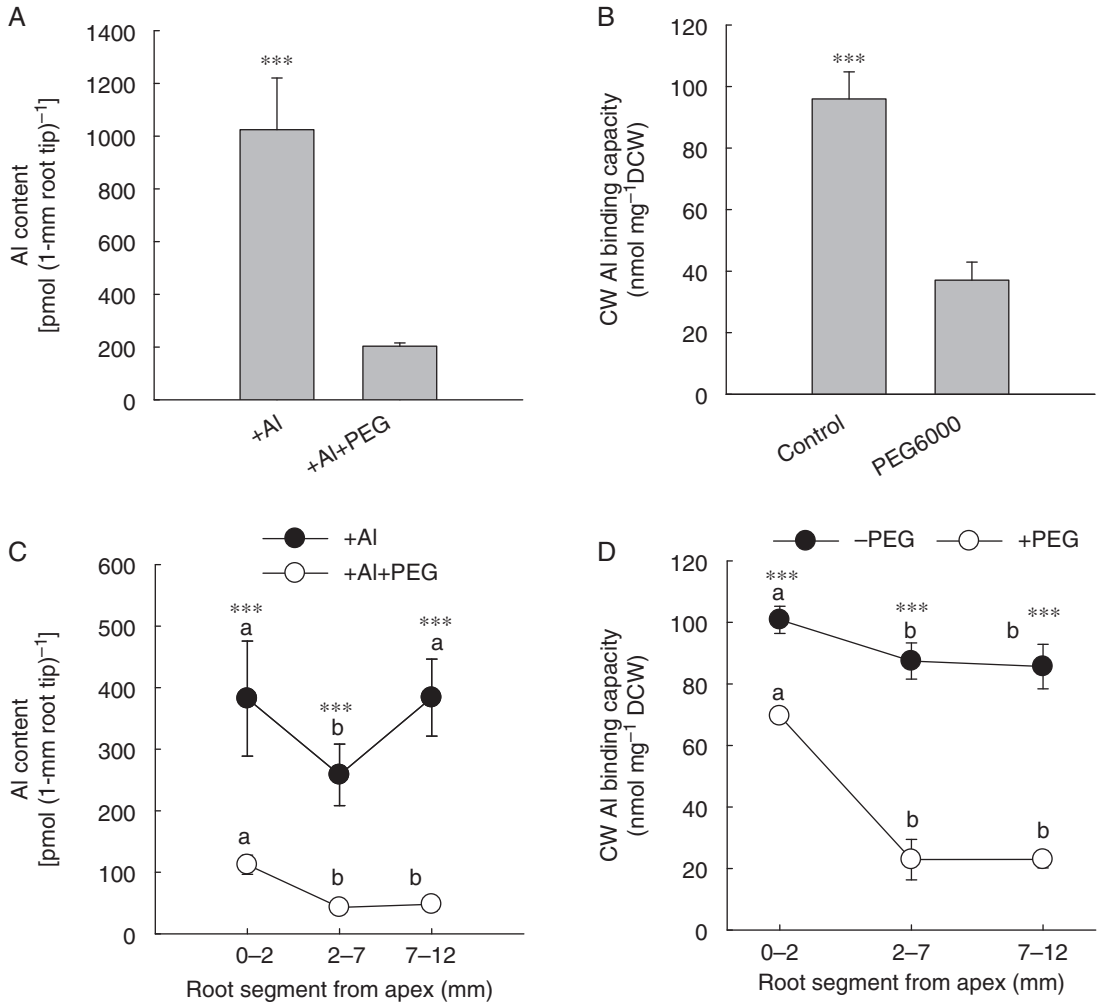

FIG. 2. Al accumulation of 0–12 mm root tips (A) or root-tip segments (C) and Al binding capacity of isolated cell walls (CWs) of entire root tips (B) or root-tip segments (D) under PEG-induced osmotic stress. (A, C) Plants were treated without or with polyethylene glycol (PEG-6000, 150 g L^{-1}) in the absence or presence of 25 μM $AlCl_3$ in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3. After 24 h root tips (12 mm) (A) or root-tip segments (0–2, 2–7 and 7–12 mm from the root tip) were harvested for each sample and Al accumulation was quantified. (B, D) Plants were pre-treated without or with 150 g L^{-1} PEG in the simplified solution. After 24 h PEG treatment, from root tips and root segments CWs were ethanol-isolated as described in the Materials and Methods. For determination of the Al binding capacity, the isolated CWs were treated with 1 ml 300 μM of $AlCl_3$ for 30 min. Means with different letters are significantly different between root segments at $P < 0{\cdot}05$ (Tukey test). ***Significant differences between PEG treatments at $P < 0{\cdot}001$ (*t* test). Bars represent means ± s.d. ($n = 4$). DCW, dried cell wall.

Determination of Al

Root tips or CW material were digested in 500 μL ultra-pure HNO_3 (65 %, v/v) by overnight shaking in a rotary shaker. The digestion was completed by heating the samples in a water bath at 80 °C for 20 min. Then, 1·5 mL of ultra-pure deionized water was added after cooling the samples in an ice-water bath. Al was measured with a Unicam 939 QZ graphite furnace atomic absorption spectrophotometer (GFAAS; Analytical Technologies Inc., Cambridge, UK) or inductively coupled plasma mass spectroscopy (ICP-MS) (7500cx, Agilent Technologies, Santa Clara, CA, USA).

RNA isolation and quantitative real-time PCR

The root tips or root segments from each plant were rinsed with distilled water, harvested and shock-frozen in liquid nitrogen. Total RNA was isolated using Tri Pure isolation reagent (Roche, Basel, Switzerland), and first-strand cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's protocol. Real-time PCR (RT-PCR) was performed using the CFX ConnectReal-Time System (Bio-Rad, Hercules, CA, USA) with FastStart Universal SYBR Green Master(Rox) (Roche). Samples for quantitative RT-PCR (qRT-PCR) were run in three biological and two technical replicates. Relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method according to Livak and Schmittgen (2001). For the normalization of gene expression, *β-tubulin* was used as an internal standard according to Eticha *et al.* (2010), and the non-treated plants of bean genotype VAX 1 were used as the reference sample. The primers for *β-tubulin* and *PvXTH9* and *PvXTHb* were obtained from Eticha *et al.* (2010) and Z. B. Yang *et al.* (2011), respectively. Specifications of the primers of the genes studied are given in

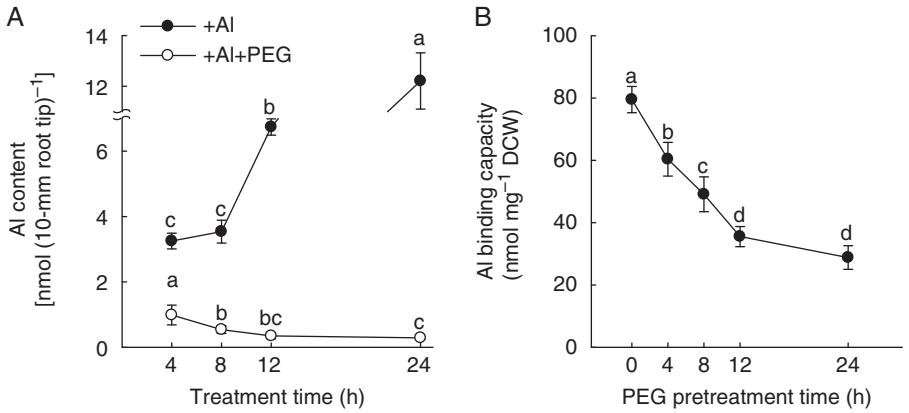

FIG. 3. Al accumulation of intact root tips (A) and Al binding capacity of cell walls (CWs) isolated from the root tips (B) as affected by PEG-induced osmotic stress. (A) Plants were treated without or with polyethylene glycol (PEG-6000, 150 g L^{-1}) in the absence or presence of 25 μM $AlCl_3$ for 4, 8, 12 and 24 h in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3. After the treatment, root tips (10 mm) were harvested for Al determination. (B) Plants were pretreated without or with PEG for 0, 4, 8, 12 and 24 h in the simplified solution. After the PEG treatment, from 30 root tips per sample and treatment CWs were ethanol-isolated as described in the Materials and Methods. For determination of the Al binding capacity the isolated CWs were treated with 1 ml of 300 μM $AlCl_3$ for 30 min. Means with different letters are significantly different between treatment times at $P < 0{\cdot}05$ (Tukey test). Bars represent means ± s.d. ($n = 4$).

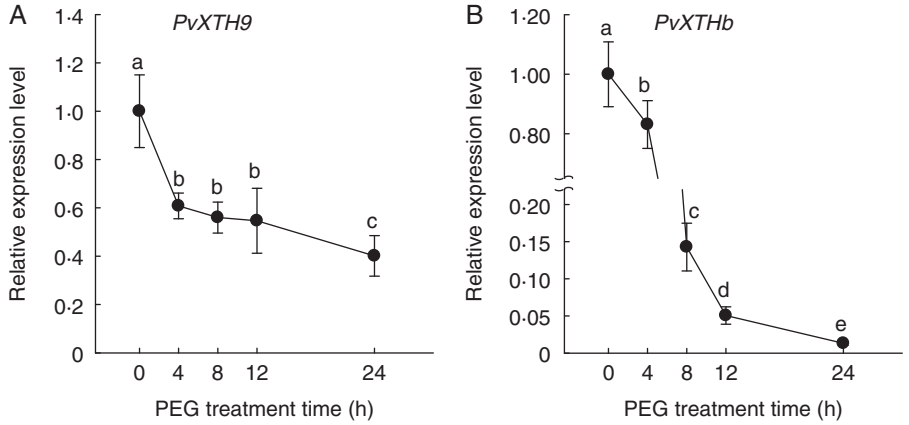

FIG. 4. Relative linear expression of *PvXTH9* (A) and of *PvXTHb* (B) in the root tips as affected by the duration of PEG-induced osmotic stress. Seedlings were treated without or with polyethylene glycol (PEG-6000, 150 g L^{-1}) in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 for 0, 4, 8, 12 and 24 h before the root tips (10 mm) were harvested. qRT-PCR was performed using the *β-tubulin* gene as internal standard. Gene expression of the non PEG-treated root tips at 0 h was set to 1. Bars represent means ± s.d., $n = 3$. Means with different letters are significantly different at $P < 0{\cdot}05$ (Tukey test) between treatment times.

Supplementary Data Table S1. The PCR efficiencies of the primer pairs were in the range 90–110 % as determined by dilution series of the cDNA template. Primer pairs with PCR efficiencies deviating from this range were discarded and new primers of the genes were designed to obtain more reliable quantification.

XET assay

The action of XET was determined according to Vissenberg *et al*. (2000). In brief, after treating the 3-d-old seedlings of common bean genotype VAX 1 without or with PEG 6000 (150 g L^{-1}) or $AlCl_3$ (25 μM) in the simplified nutrient solution (pH 4·5) for 24 h, transverse sections (~100–300 μm) from the central region of root segments (0–2, 2–7 and 7–12 mm from the root apex) were free-hand sectioned with a razor blade, and the cross-sections or the entire root tips (10 mm from the root apex) were incubated in a 6·5 μM sulforhodamine conjugates of xyloglucan oligosaccharides (XGO-SR) mixture containing SR labelled XLLG (nonasaccharide), XXLG (octasaccharide), and XXXG (heptasaccharide) (XLLG-SR > XXLG-SR > XXXG-SR) dissolved in 25 mM MES buffer at pH 5·5 for 1 h. The samples were washed for 10 min in ethanol/formic acid/water (15:1:4, v/v/v) to remove any remaining unreacted XGO-SRs and subsequently incubated in 5 % formic acid overnight to remove apoplastic, non-wall-bound XGO-SRs. Samples were mounted on glass slides and examined with an LSM-700 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) or a fluorescence microscope (Olympus, Tokyo, Japan). The fluorescence intensity across the root sections was analysed using Adobe Photoshop CS software.

Statistical analysis

Statistical analysis was performed using SAS 9·2 (SAS Institute, Cary, NC, USA). Means were compared using a *t*-test

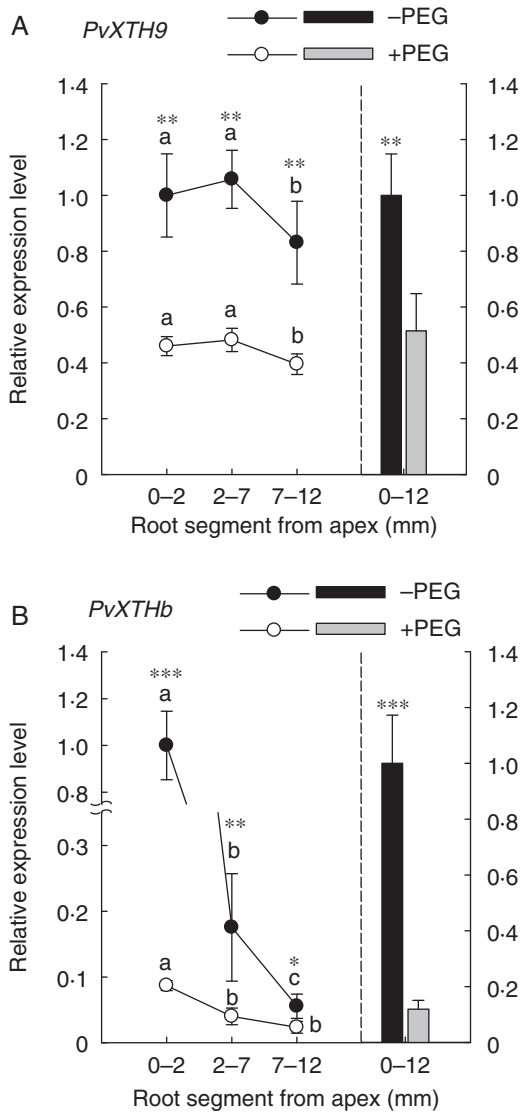

FIG. 5. Relative linear expression of *PvXTH9* (A) and of *PvXTHb* (B) in the entire root tips and root-tip segments under PEG-induced osmotic stress. Seedlings were treated without or with polyethylene glycol (PEG-6000, 150 g L^{-1}) in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 for 24 h before entire root tips (12 mm) or root-tip segments (0–2, 2–7 and 7–12 mm from the apex) were harvested. qRT-PCR was performed using the *β-tubulin* gene as internal standard. The non-PEG-treated control plants at 0–2 mm root-tip segments (the linear graph in A and B) or 0–12 mm root tips (the histogram in A and B) were set to 1. Bars represent means ± s.d., $n = 3$. Means with different letters are significantly different at $P < 0{\cdot}05$ (Tukey test) between root segments. Asterisks indicate significant differences between PEG treatments at $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$ (*t* test).

or Tukey test, depending on the number of treatments being compared.

RESULTS

To better understand the effect of PEG-induced OS on the spatial accumulation of Al along the root apex, we first needed to know how OS affects the partial elongation rate of the apical root segments. Without OS root elongation extended to the entire apical 10 mm (Fig. 1). Beyond 10 mm the elongation rate was not different from zero. The maximum elongation rate was reached at 3–4 mm from the root apex. The elongation of the 0–2 mm root apical zone (including meristem zone and TZ) was maintained during PEG stress (no significant difference between PEG treatments), while the elongation in the EZ (2–10 mm from the root apex) (Rangel *et al.*, 2007) was strongly inhibited by PEG, particularly in the central EZ of 2–7 mm from the root apex, where the maximal inhibition rate by PEG reached 71 % of the control.

Based on Fig. 1, three contiguous sections within the apical 12 mm of the roots were harvested for the various measurements in this study: Section 1 (S1, 0–2 mm from the root apex), in which the elongation rates were maintained during OS; Section 2 (S2, 2–7 mm) exhibited maximum elongation rates in non-stressed roots and maximum inhibition of root elongation by OS; and Section 3 (S3, 7–12 mm) where root elongation declined and stopped in non-stressed roots at 10 mm and in OS-stressed roots already at 8 mm from the root apex.

PEG-induced OS substantially reduced not only the Al accumulation of intact root tips (Fig. 2A) but also the Al binding capacity of CWs isolated from these root tips (Fig. 2B). The three root tip sections differed significantly in Al accumulation in non OS-stressed plants: S2 accumulated less Al than S1 and S3 (Fig. 2C) and the CW Al binding capacity was higher in S1 than in S2 and S3 (Fig. 2D). OS strongly reduced Al accumulation and CW binding capacity in all sections but particularly in S2 and S3, while S1 root section maintained substantial Al accumulation (Fig. 2C) and particularly CW binding capacity (Fig. 2D).

A kinetic study revealed that the Al accumulation of the root tips increased from 4 to 24 h Al treatment without OS, but when the roots were submitted to combined Al and PEG application the Al accumulation of the root tips was increasingly impeded from 4 to 24 h treatment duration (Fig. 3A). With increasing duration of PEG pre-treatment from 4 to 24 h the Al binding capacity of the CWs decreased (Fig. 3B).

The spatial and temporal change of Al accumulation in the root tips induced by OS might be related to the action of XTH, which was studied on the transcriptional level in a first approach. PEG treatment reduced the expression of both *PvXTH9* and *PvXTHb* genes in the root tips progressively with treatment duration up to 24 h (Fig. 4). After 24 h the expression of *PvXTH9* was repressed to 40–50 % and of *PvXTHb* even to a level close to the detection limit.

Al stress did not reduce the expression of *PvXTH9* in the root tips compared with the non-Al-treated control, and it was even slightly enhanced after 4–12 h of Al treatment. Expression of *PvXTHb* in the root tips was not affected by Al up to 12 h of treatment. However, Al treatment reduced *PvXTHb* expression by a factor of 6·4 after 24 h (Supplementary Data Fig. S1).

Without PEG treatment, *PvXTH9* was rather uniformly expressed in all root-tip sections, with only S3 showing a slightly decreased level of expression (Fig. 5A). PEG decreased its expression level uniformly in all sections. In contrast to *PvXTH9*, *PvXTHb* showed a steep decease in expression from S1 to S3 particularly in the absence of PEG (Fig. 5B). The decrease in *PvXTHb* expression under OS differed greatly between the root-tip sections (S1 >> S2 > S3). After 24 h PEG pre-treatment, which slightly and strongly reduced the expression of

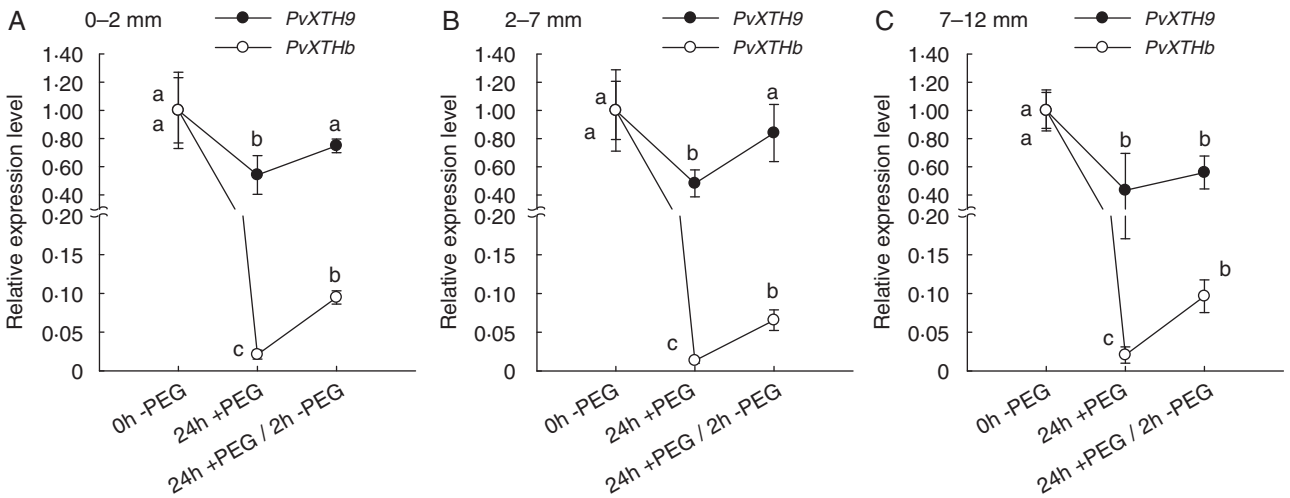

FIG. 6. Relative linear expression of *PvXTH9* and of *PvXTHb* in individual root-tip segments (A, 0–2 mm from the apex; B, 2–7 mm; C, 7–12 mm) under PEG-induced osmotic stress and recovery from the stress after stress retrieval. Seedlings were treated with polyethylene glycol (PEG-6000, 150 g L^{-1}) in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 for 24 h, and then the PEG-treated plants were allowed recover in a solution without PEG for 2 h. Root-tip segments were harvested. qRT-PCR was performed using the *β-tubulin* gene as internal standard. The non PEG-treated root-tip segments at 0 h were set to 1. Bars represent mean ± s.d., $n = 3$. Means with different letters are significantly different at $P < 0{\cdot}05$ (Tukey test) between treatments.

PvXTH9 and *PvXTHb* in all root tip sections, respectively, the removal of PEG stress for only 2 h fully (*PvXTH9* in S1 and S2) or partially (*PvXTH9* in S3 and *PvXTHb* in all sections) restored the expression of the genes to the level of unstressed plants (Fig. 6).

Phylogenetic tree analysis revealed that *PvXTH9* showed a close genetic relationship with *AtXTH9*, while *PvXTHb* showed a close genetic relationship with *AtXTH4* and *AtXTH5* in Arabidopsis (Supplementary Data Fig. S2). *AtXTH9*, *AtXTH4* and *AtXTH5* belong to Group I/II of the XTH gene family, which has been proposed to be essential for XET activity (Baumann *et al*., 2007). Therefore, XET action was analysed in cross-sections of root-tip sections using a histochemical procedure (Fig. 7). In the absence of PEG, XET action differed between the root-tip sections (S1 > S2 > S3, Fig. 7) reflecting *PvXTHb* expression (see Fig. 5B). However, when roots were subjected to PEG stress, XET action was only significantly suppressed in the EZ (S2 and S3) in contrast to *PvXTHb* expression. The removal of PEG stress for 2 h fully restored action of the enzyme. In S3, the action even reached a level higher than in the roots prior to PEG application (Fig. 7). The detection of XET along the 10-mm root apex showed that in the absence of Al, XET action was high in the root cap and meristem zone. Al treatment greatly reduced the action of XET in the EZ (Fig. S1).

DISCUSSION

The maintenance of primary root growth has been considered an important feature of the adaptation of plants to water deficit (Sharp and Davis, 1989; Wu and Cosgrove, 2000; Sharp *et al*., 2004). Elongation is maintained preferentially towards the root apex during water deficit (Sharp *et al*., 1988). Detailed studies indicate that root elongation is maintained in the apical 0–3 mm in maize (Liang *et al*., 1997; Spollen and Sharp, 1991) and 0–4 mm in soybean (Yamaguchi *et al*., 2010) under reduced water supply. In common bean, root elongation was maintained only in the apical 0–2 mm under PEG-induced OS based on the result shown in Fig. 1. In contrast to the apical 2 mm, the elongation rate of root segments in the EZ (2–10 mm from the root apex) was notably suppressed by OS (Fig. 1). In water-stressed maize and soybean primary roots, elongation was progressively inhibited at 3–9 and 4–8 mm behind the root tip, respectively, which were attributed to the reduced CW extendibility due to the accumulation of wall phenolics (Fan *et al*., 2006; Yamaguchi *et al*., 2010). The different root-growth response of the apical and basal sections of the root apex is considered to result from the maintenance of apoplastic acidification (Fan and Neumann, 2004), higher production of hydrogen peroxide (Voothuluru and Sharp, 2013), XET activity (Wu *et al*., 1994, 1996) and *expansin* gene expression (Wu *et al*., 2001), or lower lignification (Yamaguchi *et al*., 2010) in the apical sections.

Numerous studies have provided evidence that the majority of Al in root tips primarily accumulates in the apoplast where Al^{3+} binds strongly to the negative charge provided by the unmethylated pectin of the CW (Blamey *et al*., 1990; Schmohl *et al*., 2000; Eticha *et al*., 2005; Horst *et al*., 2010) or xyloglucan through formation of an Al–xyloglucan complex (Zhu *et al*., 2012) after short-term Al exposure. PEG-induced OS greatly excluded Al from the apoplast, and transcriptome analysis revealed that genes related to CW-structure modification, especially members of the *XTH* gene family including *PvXTH9* and *PvXTHb*, may play an important role in this regulatory process (Z. B. Yang *et al*., 2012, 2013). The spatial pattern analysis revealed that OS reduced Al accumulation in the apical meristem, TZ and EZ (Fig. 2) and this PEG-induced reduction of Al content in the CW is dependent on PEG exposure time (Fig. 3). The PEG-induced OS highly suppressed the expression of both *PvXTH9* and *PvXTHb* along the root tips (Fig. 5), further

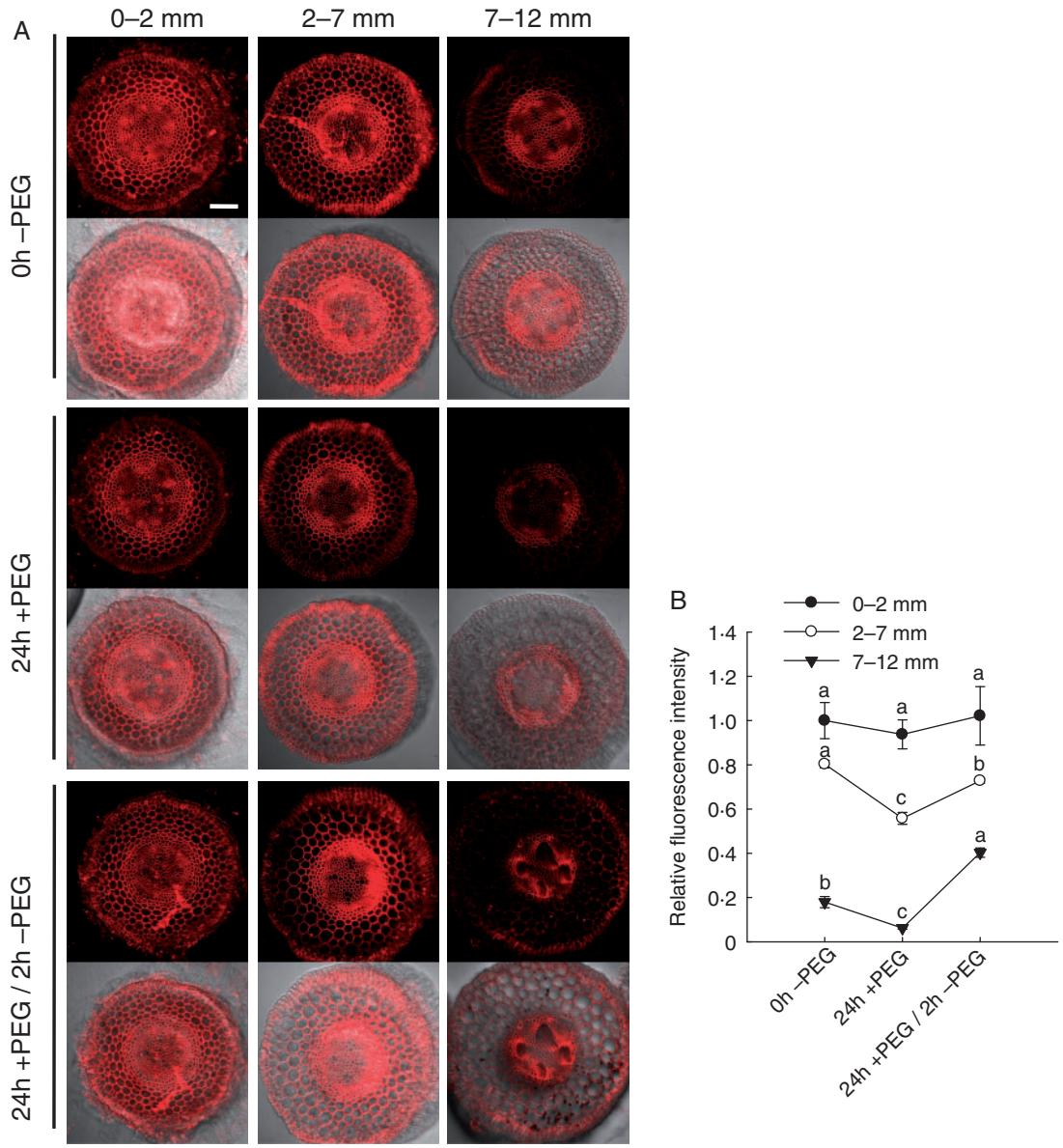

FIG. 7. *In-situ* analysis of XET action in the root-tip sections under PEG-induced osmotic stress. Seedlings were treated with polyethylene glycol (PEG-6000, 150 g L^{-1}) in a simplified solution (pH 4·5) containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3 for 24 h, and then the PEG-treated plants were allowed recover in a solution without PEG for 2 h. The assay of XET action was performed in the cross-sections of different root-tip segments (0–2, 2–7 and 7–12 mm from the apex) (A) according to Vissenberg *et al.* (2000). The upper and lower rows within the PEG treatments represent fluorescence images and merged images, respectively. Bar in A: 100 μm. (B) Quantification of the fluorescence intensity from the cross-sections shown in A. Bars in B represent mean ± s.d., $n = 6$. Means with different letters in B are significantly different at $P < 0{\cdot}05$ (Tukey test) between treatments.

implying the potential role of both genes in the regulation of Al accumulation in the root tips under OS.

XTH genes encode proteins that can potentially have two distinct catalytic activities, with greatly different effects on xyloglucans: XET activity (formally, xyloglucan:xyloglucosyl transferase; EC 2·4·1·207) results in the non-hydrolytic cleavage and ligation of xyloglucan chains, whereas XEH activity (formally, xyloglucan-specific endo-β-1,4-glucanase; EC 3·2·151) yields irreversible chain shortening (Eklöf and Brumer, 2010). XTH enzymes are generally encoded by a large multi-gene family, approx. 20–60 *XTH* genes in diverse land plants (Eklöf and Brumer, 2010). The molecular phylogeny of *XTH* genes and gene products has been divided into three major groups (I, II and III) on the basis of sequence similarity (Campbell and Braam, 1999; Rose *et al.*, 2002; Baumann *et al.*, 2007; Eklöf and Brumer, 2010). Genes belonging to group I/II exhibit exclusively XET activity, while genes from group III mainly show XEH activity. Phylogenetic tree analysis showed that *PvXTH9* and *PvXTHb* have close genetic relationships with *AtXTH9*, *AtXTH4* and *AtXTH5* (Fig. S2), which belong to group I that has been proposed to exhibit exclusively XET activity (Campbell and Braam, 1999; Rose *et al.*, 2002).

In Arabidopsis and tobacco roots, XET action was highest at the epidermis of the proximal end of the TZ to mediate CW extension (Vissenberg *et al*., 2000), while in this study, the highest XET action and *PvXTHb* gene expression were detected in the 0–2 mm root section (Figs 5B and 7), which includes the meristem zone and TZ in common bean (Rangel *et al*., 2007). In Arabidopsis, *AtXTH5* is detected in the root cap and regions of the distal EZ while *AtXTH9* has highest expression in the meristematic zone and extends into the elongation and differentiation zones (Becnel *et al*., 2006). This high XET action and expression of *PvXTHb* in the apical 2 mm section in this study may function in the regulation of primary root morphogenesis through alteration of CW properties (Becnel *et al*., 2006). The *in-situ* assay of the enzyme action in the root tip demonstrated that the action of XET was suppressed by OS in the apical 2–7 and 7–12 mm sections while it was not significantly affected in the apical 0–2 mm section (Fig. 7). In particular, the consistent rapid rescue of XET action (Fig. 7) after release of OS with the recovery of Al accumulation in the root tip (Z. B. Yang *et al*., 2010) provides further evidence that the reduction of Al accumulation in the CW of EZ is dependent on XET action. Also, in this study XET action in the EZ of root tips was greatly reduced by Al stress (Fig. S1), supporting the results reported for Arabidopsis (J. L. Yang *et al*., 2011). Through an analysis of the spatial patterns of root growth in the Al-sensitive genotype VAX 1, Rangel *et al*. (2007) showed that not only the TZ but also the EZ was inhibited by Al. This may imply that the inhibition of root elongation by Al in the EZ may result from the reduced XET action in this zone.

A similar tendency of XET action and *PvXTHb* gene expression in the non-stressed primary root tips was found, while the OS-suppressed expression of *PvXTHb* in the apical 2 mm section is different from XET action (Figs 5B and 7); the detailed function of *PvXTHb* in this region under OS needs to be further studied. The spatial and temporal analysis of the PEG-reduced Al accumulation and XET action in the root tips support the conclusion that the PEG-suppressed XET action in the EZ results in less Al accumulation in the CW through alteration of CW porosity (Z. B. Yang *et al*., 2011) or reduced xyloglucan content of the CW (Zhu *et al*., 2012). However, according to previous studies the physical stress resulting from water loss of the apoplast also contributes to reduced Al accumulation in the CW of bean root tips (Z. B. Yang *et al*., 2010). This might explain why Al accumulation in the 0–2 mm root section *in vivo* (Fig. 2C) and Al binding in the ethanol-isolated CW of this section *in vitro* (Fig. 2D) remain suppressed by OS, although XET action in this section is not affected by OS (Fig. 7). The reduced OS-induced suppression of Al accumulation in the isolated CWs (Fig. 2D) than in the intact root section (Fig. 2C) at the apical 0–2 mm might be due to physical destruction of the CW structure with higher Al accumulation (Z. B. Yang *et al*., 2010).

In conclusion, the results provide new understanding of the spatially variable pattern of Al accumulation, XET action and *XTH* gene expression in the root tips, and reveal a potential role of *PvXTH9* and *PvXTHb* and XET in OS-reduced Al accumulation. The results contribute to better focus the breeding of common bean for higher yield capacity on acid Al-toxic and drought stress-prone soils through physiological and molecular modulation of CW structure and functions.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: list of main genes and specific primer pairs used for quantitative gene expression analysis. Figure S1: expression of *PvXTH9* and *PvXTHb* and *in-situ* XET action in the root tips under Al stress. Figure S2: sequence alignment tree of *PvXTH9* and *PvXTHb* (expressed sequence tags) with the known *XTH* genes in legumes.

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