RESEARCH PAPER

Xyloglucan endotransglucosylase-hydrolase30 negatively affects salt tolerance in Arabidopsis

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Abstract

Plants have evolved various strategies to sense and respond to saline environments, which severely reduce plant growth and limit agricultural productivity. Alteration to the cell wall is one strategy that helps plants adapt to salt stress. However, the physiological mechanism of how the cell wall components respond to salt stress is not fully understood. Here, we show that expression of XTH30, encoding xyloglucan endotransglucosylase-hydrolase30, is strongly up-regulated in response to salt stress in Arabidopsis. Loss-of-function of XTH30 leads to increased salt tolerance and overexpression of XTH30 results in salt hypersensitivity. XTH30 is located in the plasma membrane and is highly expressed in the root, flower, stem, and etiolated hypocotyl. The NaCl-induced increase in xyloglucan (XyG)-derived oligosaccharide (XLFG) of the wild type is partly blocked in xth30 mutants. Loss-of-function of XTH30 slows down the decrease of crystalline cellulose content and the depolymerization of microtubules caused by salt stress. Moreover, lower Na+ accumulation in shoot and lower H2O2 content are found in xth30 mutants in response to salt stress. Taken together, these results indicate that XTH30 modulates XyG side chains, altered abundance of XLFG, cellulose synthesis, and cortical microtubule stability, and negatively affecting salt tolerance.

Keywords: Arabidopsis, cellulose, microtubule, salt stress, XLFG, XTH30, xyloglucan.

Introduction

Salt stress severely reduces plant growth and limits agricultural productivity (Zhu, 2002, 2016). Plants have evolved various strategies to sense and achieve protection against salt stress (Shi et al., 2000; Munns and Tester, 2008). They sense and transmit the stress signal from cell surface to nucleus, then regulate downstream genes to adaptively respond to salt stress (Julkowska and Testerink, 2015; Zhu, 2016).

The plant cell wall is a structural layer outside the cell membrane that provides the cell with both structural support and protection. One of the important plant adaptations to salt stress...
is differential regulation of growth, accompanying dynamic changes of the plant cell wall (Tenhaken, 2014; Cosgrove, 2015; Wang et al., 2016). The plant cell wall has a dynamic architecture with cellulose microfibrils embedded in an amorphous matrix of pectin and hemicellulose polysaccharides as well as structural proteins, and in some cells also lignin (Mutwil et al., 2008; Scheller and Ulvskov, 2010). Several genes that are involved in cellulose synthesis, CesA1, CesA6, and CesA8, have been implicated in salt tolerance (Chen et al., 2005; Zhang et al., 2016a). Knocking out either CesA6 or CesA1 confers salt stress sensitivity (Zhang et al., 2016a). Companion of cellulose synthase proteins (CC1 and CC2) interact with CesAs and microtubules, and mutations of CC1 and CC2 led to salt-sensitive phenotypes by altering microtubule and cellulose synthase complex (CSC) behavior (Endler et al., 2015).

Xyloglucan (XyG) is an important hemicellulose polymer of the primary cell wall in dicotyledons and non-commelindoid monocotyledons. XyG plays a vital role in loosening or stiffening the cell wall by binding to cellulose microfibrils with hydrogen bonds during cell elongation (Hayashi and Kaida, 2011; Park and Cosgrove, 2015; Pauly and Keegstra, 2016). XyG has a common backbone of (1–4)-linked β-D-glucopyranosyl residues (β-D-Glup), which can be connected with α-D-xlyopyranosyl (α-D-Xylp) residues at O-6 (Cosgrove, 2005). A standard nomenclature using a single letter residue, while X, L, and F indicate Glc residues, α-D-2O-D-β-D-Glu residue, and α-D-Fucp-(1–2)-β-D-Galp-(1–2)-α-D-Xylp side chains, respectively (Fry et al., 1993). XyG chains can be cleaved or rejoined by xyloglucan endotransglucosylase–hydrolase (XTH) (Nishitani and Tominaga, 1992). Many members of the XTH family in plants have been identified including 33 members in Arabidopsis (Yokoyama and Nishitani, 2001), 29 in rice (Yokoyama et al., 2004), and 22 in barley (Strohmeier et al., 2004). It has been shown that XTHs play a vital role in plant development. During cell wall expansion, ArXTH27 is highly expressed and can modulate the generation of tracheary elements in Arabidopsis rosette leaves (Matsui et al., 2005).ArXTH21 plays a vital role in the growth of the primary roots by affecting cellulose deposition (Liu et al., 2007). ArXTH28 is involved in automatic self-pollination (Kurasawa et al., 2009). In addition to regulating developmental growth, XTHs are also involved in the response of plants to abiotic stress. Loss-of-function mutation in AtXTH15 and AtXTH31 enhances aluminum tolerance (Zhu et al., 2012; Shi et al., 2015). Constitutive expression of a Capsicum annum XTH, CaXTH3, in Arabidopsis or tomato improved salt and drought tolerance (Cho et al., 2006; Choi et al., 2011). Overexpression of Populus euphratica XTH, PeXTH, in tobacco enhanced salt tolerance by the development of leaf succulence (Han et al., 2013). However, the function of XTHs in salt stress and the mechanisms by which XTHs respond to salt stress are still unclear.

In this study, Arabidopsis xyloglucan endotransglycosylase–hydrolase30 (XTH30) was identified as responding to salt stress. XTH30 loss-of-function mutations led to salt tolerance and gain-of-function resulted in salt sensitivity. Moreover, XTH30 modulated XyG structure, cellulose content and depolymerization of microtubules in response to salt stress.

Materials and methods

Plant materials and growth conditions

Arabidopsis (L.) Heynh. ecotype Columbia (Col-0), xth30-1 (CS16544) and xth30-2 (CS16543) mutant were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous xth30 mutants were identified by T-DNA insertion-based PCR using the specific primers listed in Supplementary Table S1 at JXB online. The xth30 mutants (xth30-1 and xth30-2) were crossed with transgenic Arabidopsis expressing green fluorescent protein (GFP)-tagged α-tubulin 6 isoform (35S:GFP-TUA6) for the observation of cortical microtubules.

Seeds were sterilized and sown on a solid medium containing half-strength Murashige and Skoog salts including vitamins and 1% (w/v) sucrose at 4°C for 2 d and then grown in a growth chamber (22°C, 100–200 μmol m⁻² s⁻¹, 14 h light/10 h dark, 60% humidity).

Phenotypic analysis

For a salt sensitivity assay on plates, 5-day-old seedlings grown on 1/2 MS medium were transferred onto 1/2 MS medium with mannitol or salt added as described and allowed to grow for an additional 7 d. Seedlings were photographed and freshly weighed.

For the hydroponic salt sensitivity assay, 3-week-old seedlings were treated with nutrient solution as described in Fang et al. (2016) containing 125 mM NaCl and allowed to grow for an additional 9 d. After recovery with normal nutrient solution for 3 d, the seedlings were photographed and the survival rate was calculated.

For a heavy metal stress (Zn²⁺ and Cd²⁺) tolerance test, 5-day-old seedlings grown on 1/2 MS medium were transferred onto 1/2 MS medium with 50 μM CdCl₂ and 500 μM ZnSO₄, then allowed to grow for an additional 7 d. For ABA treatments, 5-day-old seedlings grown on 1/2 MS medium were transferred onto 1/2 MS medium with 20 μM ABA, then allowed to grow for an additional 7 d. Seedlings were photographed after various treatments.

Determination of Na⁺ content

Shoots and roots of Arabidopsis seedlings were harvested separately, and Na⁺ content was measured according to the methods described by Wang et al. (2015). Briefly, dry samples were digested in 1 ml of nitric acid at 90°C for 12 h, diluted to 5 ml with distilled water, and then analysed using an inductively coupled plasma–optical emission spectrometry instrument (Pekin Elmer, USA).

Hypocotyl length measurements

For isoxaben and oryzalin treatments, seeds were sown on 1/2 MS medium plates supplemented with or without 2 nM isoxaben or 400 nM oryzalin (Sigma–Aldrich, USA) and grown for 6 d in the dark. For salt treatments, 2-day-old etiolated seedlings were transferred to 1/2 MS plates containing 100 mM NaCl for another 5 d. Phenotypic analysis

Seed germination assays

More than 100 seeds were sown on 1/2 MS medium containing 1% sucrose with or without 125 mM NaCl. The plates were stratified at 4°C for 48 h, and placed at 22°C under light conditions. Radicle emergence of >1 mm was counted every day for germination analysis.
Real-time PCR analysis
Total RNA was extracted from seedlings or etiolated hypocotyls using the RNasy Plant Mini Kit (Qiagen, USA). RNA was first treated with DNase I (Sigma-Aldrich), and first-stand cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed using SYBR Select Master Mix (Applied Biosystems, USA) on diluted (5 times) cDNA using the StepOne Plus Real-Time PCR System (Applied Biosystem 7500). The specific primers are listed in Supplementary Table S1. The relative expression levels were determined using the 2^-ΔΔCT method as described in (Yan et al., 2015).

Determination of H2O2 level
For quantification of H2O2, 3-week-old hydroponically grown seedlings were treated with nutrient solution containing 125 mM NaCl for 6 d, and shoots were harvested. H2O2 content was determined by a horseradish peroxidase-based spectrophotometric assay following the protocol described by Pierce™ Quantitative Peroxide Assay Kit (Aqueous) (Thermo Fisher Scientific, USA).

Antioxidant enzyme assay
Three-week-old hydroponically grown seedlings were treated with nutrient solution containing 125 mM NaCl for 6 d, and shoots were harvested. The samples were homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM sodium ascorbate. The homogenate was centrifuged at 12 000 g for 30 min at 4 °C; and then the supernatant was used for measuring the activities of antioxidant enzymes as previously described (Huang et al., 2013).

Subcellular localization of XTH30
The XTH30 coding sequence was fused to yellow fluorescent protein (YFP) at the N-terminus driven by 35S promoter (XTH30-YFP). Four-week-old Nicotiana benthamiana leaves were co-infiltrated with Agrobacterium tumefaciens strain GV3101 carrying the 35S::XTH30-YFP fusion construct and the AtpH2PA-mCherry marker (pm-rk) as described by Nelson et al. (2007). The plasmolysed tobacco cells were observed by treatment with 300 mM mannitol for 10 min. The microscopy was performed using a Zeiss LSM710 device as previously described (Zhu et al., 2016).

Plasma membrane isolation
The leaves of OK#1 seedlings were harvested and homogenized in a solution (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol) and centrifuged at 6000 g for 10 min at 4 °C. The supernatant was centrifuged at 100 000 g for 45 min, the pellet was used as total microsomal membrane proteins, and the supernatant was regarded as cytosolic proteins.

XTH30 promoter::GUS construct and GUS activity
A 2000 bp promoter region of XTH30 was amplified by PCR using the specific primers listed in Supplementary Table S1. The PCR product was cloned into the pGWB3 vector using the BP and LR clonase reaction (Invitrogen). The recombinant plasmid was sequenced and introduced into Col-0 by Agrobacterium tumefaciens-mediated transformation. Positive transformants were selected on 1/2 MS medium containing 50 μg ml−1 hygromycin (Omega Scientific, USA). Various tissues were submerged in X-Gluc buffer (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mg ml−1 5-bromo-4-chloro-3-indolyl β-D-GlcUA [X-Gluc] (Sigma-Aldrich), 0.4% Triton X-100, 5 mM potassium ferrocyanide, and 5 mM potassium ferrocyanide) and incubated at 37 °C overnight in the dark, followed by washing with 70% ethanol to remove chlorophyll as described in Lee et al. (2015).

Cell wall preparation
For quantification of the crystalline cellulose fraction and XyG contents, 2-day-old etiolated seedlings were transferred to 1/2 MS plates containing 100 mM NaCl for another 5 d. The fresh samples were harvested into 96% ethanol and incubated for 30 min at 100 °C to inactivate cell wall-degrading enzymes. The tissue was homogenized using a Retsch MM200 mixer mill and centrifuged. The pellet was washed with 100% ethanol and twice with a mixture of chloroform and methanol (2:1), followed by four successive washes with 100% ethanol and acetone. The pellet was air-dried overnight and is referred to as alcohol-insoluble residue (AIR).

Crystalline cellulose content
The crystalline cellulose content was determined as described (Fang et al., 2016). In Brief, the pellet was air-dried overnight. The starch in the samples was degraded with α-amylase, amyloglucosidase, and pullulanase (Megazyme, Brae, Ireland). Destarched AIR (2 mg) was hydrolysed in 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h. Then the TFA-insoluble material was washed with water and further hydrolysed with 72% (v/v) sulfuric acid containing 10 mg myo-inositol for 1 h at room temperature. The sulfuric acid was then diluted to 1 M with water and the samples further incubated at 100 °C for 3 h; they were neutralized with barium carbonate (BaCO3) to provide the crystalline cellulose fraction and analysed by high performance anion exchange chromatography (HPAEC) on an ICS-5000 instrument (Thermo Fisher Scientific) equipped with a CarboPac PA20 (3 mm×150 mm, Thermo Fisher Scientific) analytical anion exchange column, PA20 guard column (3 mm×30 mm), borate trap, and a 500 pulsed amperometric detector. The myo-inositol was used as an internal standard.

MALDI-TOF mass spectrometry analysis of XyG oligosaccharides
To analyse the XyG, we used the rapid phenotypeing method using enzymatic oligosaccharide fingerprinting as used in Lerouxel et al. (2002). Two-day-old seedlings grown in the dark on 1/2 MS medium were transferred to 1/2 MS medium with or without 100 mM NaCl, and then grown for 5 d. The hypocotyls were stored at 100% ethanol. After ethanol removal and rehydration, XyG oligosaccharides were generated by treating with 1 unit of xyloglucanase (Megazyme) or endoglucanase (Megazyme) in 50 mM sodium acetate buffer (pH 5.0) overnight at 37 °C. Matrix-assisted laser-desorption ionization time of flight mass spectrometry of the XyG oligosaccharides was recorded with an Applied Biosystems 4800 MALDI/TOF mass spectrometer using super-DHB (Sigma-Aldrich) as matrix.

Xyloglucan content quantified by iodine staining
XyG content was measured using iodine staining as in Kooiman (1960) and Vuttipongchakij et al. (2012) with minor modifications. In brief, 2-day-old etiolated seedlings were transferred to 1/2 MS plates containing 100 mM NaCl for another 5 d in dark. The AIR was extracted from hypocotyl. Hemicellulosic material was isolated from de-starched AIR by incubating with 4 M KOH containing 1% sodium borohydride overnight. The supernatant were neutralized with glacial acetic acid on ice and dialysed in dialysis tubing (Spectra/Por6 3000 MWCO; Spectrum, USA) against water. The dialysed material was freeze-dried on ice and dialysed in dialysis tubing (2:1), followed by four successive washes with 100% ethanol and acetone. The pellet was air-dried overnight and is referred to as alcohol-insoluble residue (AIR).

Microtubule observation
Both wild type and xth30 mutant seeds expressing 35S::GFP-TUA6 were grown on 1/2 MS medium for 3 d and then transferred to 1/2 MS
medium with 100 mM NaCl or 10 µM oryzalin. After the indicated treatments, the cortical microtubules in etiolated hypocotyls were observed under a confocal microscope (Zeiss LSM710) with an excitation at 488 nm and emission at 533 nm.

Results

The expression of XTH30 is strongly induced by salt stress

XTH30 expression was examined in order to investigate whether it responds to salt stress. As shown in Fig. 1A, the expression of XTH30 was strongly induced at 6 h, maximal at 12 h, and then decreased at 24 h after 150 mM NaCl treatment. The XTH30 expression in various tissues was also investigated. The results showed that XTH30 accumulated at high levels in root, stem, and flower but at low levels in rosette leaves and cauline leaves (Fig. 1B). Besides that, the 2.0 kb promoter region of XTH30 was fused to a β-glucuronidase (GUS) reporter gene. GUS activity was strongly detected in root and etiolated hypocotyl but weakly observed in leaves, which was further increased by NaCl treatment (Fig. 1C). These results are consistent with the qRT-PCR results.

XTH30 negatively affects salt stress tolerance

To determine the role of XTH30 in plants under salt stress, two independent mutants of XTH30 with T-DNA insertion (xth30-1

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**Fig. 1.** Expression pattern of XTH30 in Arabidopsis. (A) Expression of XTH30 in Arabidopsis seedlings in response to salt stress. Fourteen-day-old seedlings were treated with 150 mM NaCl for different time as indicated. (B) Expression of XTH30 in rosette leaves, stem, roots, cauline leaves, and flowers. (C) XTH30 promoter::GUS expression patterns under salt stress in various developmental stages. XTH30 promoter::GUS expression of two lines (no. 3 and no. 6) in 3-day-old seedling (a), 5-day-old seedling (b), leaves (c), 7-day-old etiolated hypocotyl (d), and roots (e) exposed to salt stress. Seedlings in (a, b) were treated with 150 mM NaCl for 1 h. Seedlings in (c–e) were treated with 150 mM NaCl for 12 h. Error bars represent SD (n=3) in (A, B). ACTIN 2 acts as a reference standard. The asterisk in (A) indicates a significant difference compared with the control using unpaired Student’s t-test (*P<0.05). Experiment in (B) was performed at least three times with similar results. (This figure is available in color at JXB online.)
and xth30-2) were used to perform salt sensitivity assays. The T-DNA insertion in both xth30-1 and xth30-2 is located in the third intron of XTH30 genome and was confirmed by PCR using specific primers as indicated in Supplementary Fig. S1. When 5-day-old seedlings of the wild type and xth30 mutants were transferred to 1/2 MS medium with 125 mM NaCl, xth30 mutants showed more tolerance than the wild type (Fig. 2A). To completely investigate the function of XTH30, we expressed XTH30 in the xth30-1 knock-out background (com#1 and com#3) as well as in Col-0 (OX#1 and OX#5) (Supplementary Figs S2, S3). As shown in Fig. 2C, E, com#1 and com#3 were more sensitive to salt stress than xth30-1 and performed similarly to the wild type, and both OX#1 and OX#5 lines showed hypersensitivity to salt stress compared with the wild type. Hydroponically grown seedlings of xth30-1 and xth30-2 were also used to confirm the role of XTH30. The xth30 mutants wilted less and showed a much higher survival rate than the wild type under 125 mM NaCl treatment (Supplementary Fig. S4). These results indicate that XTH30 negatively affects salt stress tolerance. No growth phenotype difference was observed between xth30 mutants and the wild type under heavy metal stresses (CdCl2 and ZnSO4) or the plant hormone abscisic acid (ABA) treatment (Supplementary Fig. S5).

To investigate the role of XTH30 in more detail, the seedling fresh weight of xth30, com, OX, and wild type was measured. As shown in Fig. 2B, D, F, compared with wild type, xth30 showed a higher seedling fresh weight, com showed similar performance, and OX showed a lower fresh weight. Salt stress results

**Fig. 2.** XTH30 negatively affects salt stress tolerance. (A) Analysis of salt sensitivity in xth30 mutants. Five-day-old seedlings of xth30-1, xth30-2, and Col-0 grown on 1/2 MS medium were transferred to 1/2 MS medium with or without 125 mM NaCl. Photographs were taken after 7 d of treatment. (B) Fresh weight of seedlings tested in (A). (C) Analysis of salt sensitivity in complementation of XTH30 in xth30-1 (com#1 and com#3). Five-day-old seedlings of xth30-1, complementation of XTH30 in xth30-1 (com#1 and com#3), and Col-0 grown on 1/2 MS medium were transferred to 1/2 MS medium with or without 125 mM NaCl. Photographs were taken after 7 d of treatment. (D) Fresh weight of seedlings tested in (C). (E) Analysis of salt sensitivity in XTH30 overexpressors (OX#1 and OX#5). Five-day-old seedlings of XTH30 overexpressors (OX#1 and OX#5) and Col-0 grown on 1/2 MS medium were transferred to 1/2 MS medium with or without 125 mM NaCl. Photographs were taken after 7 d of treatment. (F) Fresh weight of seedlings tested in (E). Data in (B, D, F) are means ±SD (n=3). The asterisks indicate a significant difference between various genotypes and wild type using unpaired Student’s t-test (*P<0.05, **P<0.01). (This figure is available in color at JXB online.)
in both osmotic and ionic stress in plant (Munns and Tester, 2008). Next we determined whether salt tolerance of xth30 mutants is a specific response to the sodium ions. As shown in Supplementary Fig. S6, seedlings of xth30-1 and xth30-2 respond to Na+ and Li+, but not K+, Cl−, and osmotic stress, which was a similar behavior to sos mutants (Shi et al., 2000; Zhu, 2002). No significant difference was observed between the wild type and xth30 mutants in seed germination under salt stress (Supplementary Fig. S7) or in growth under normal conditions (Supplementary Fig. S8).

One of the most damaging effects of high salinity is the accumulation of salt in plants, and so Na+ content was investigated. The results showed that under 125 mM NaCl treatment, Na+ accumulated more in the wild type than in xth30 mutants in shoots, and there was no difference in Na+ accumulation between the wild type and xth30 mutants in roots (Supplementary Fig. S9). Salt stress leads to the overproduction of reactive oxygen species (ROS), which are highly reactive and toxic (Ma et al., 2012; Ben Rejeb et al., 2015). To understand the role of XTH30 in salt stress, we investigated H2O2 production in xth30 mutants and wild type seedlings under salt stress. When seedlings were grown on the 1/2 MS medium, the wild type and xth30 mutants showed a similar basal level of H2O2 (Supplementary Fig. S10). H2O2 levels increased both in the wild type and xth30 mutants after 125 mM NaCl treatment, and a relatively much smaller H2O2 increase was observed in xth30 mutants (Supplementary Fig. S10). We also measured the activities of antioxidant enzymes (catalase (CAT) and peroxidase (POD)) that are responsible for removing H2O2. As shown in Supplementary Fig. S11, the activities of CAT and POD increased both in the wild type and xth30 mutants exposed to NaCl treatment, and a greater increase was observed in xth30 mutants than in the wild type.

**XTH30 is located in the plasma membrane**

XTHs were shown to be localized to endoplasmic reticulum, cell wall, or plasma membrane (Genovesi et al., 2008; Zhu et al., 2012; Han et al., 2013). To understand the functions of XTH30, its subcellular localization was investigated. As shown in Fig. 3, XTH30–YFP protein was observed around the cell periphery consistent with plasma membrane-localized marker (pm-rk) (Nelson et al., 2007), suggesting that XTH30 was localized in plasma membrane (Fig. 3Aa–d). To clearly determine the plasma
membrane location of XTH30, plasmolysis was performed. The results showed that XTH30 was localized in the plasma membrane but not in the cell wall (Fig. 3Ae–f). In order to further confirm the localization of XTH30, the plasma membrane and cytoplasmic fractions were isolated from XTH30 overexpressor (OX#1). The isolated plasma membrane fractions were confirmed by the marker, H+-ATPase. Consistent with the results of XTH30 expression in tobacco cell, XTH30–YFP–HA was only detected in plasma membrane (Fig. 3B).

**XTH30 affects the xyloglucan oligosaccharide composition during salt stress**

To further understand the role of XTH30 in plants under salt stress, etiolated hypocotyl of Arabidopsis seedling was used. It is widely used to study plant primary cell wall because no cell division occurs in the dark and growth is due to cell elongation only (Refrégier et al., 2004; Endler et al., 2015; Xiao et al., 2016). As shown in Fig. 4, the etiolated hypocotyl in xth30 mutants was longer than that in the wild type under salt stress. This indicated that XTH30 negatively modulated the salt tolerance during cell elongation in etiolated Arabidopsis seedlings.

XTHs can cleave or rejoin XyG chains, resulting in the modification of content or structure of XyG (Nishitani and Tominaga, 1992), and XyG is shown to cross-link adjacent cellulose microfibrils and modulate cell wall extensibility in response to environmental variation (Hayashi et al., 2010), which prompted us to investigate the XyG content. Iodine staining (Kooiman, 1960; Gille et al., 2011; Vuttipongchaikij et al., 2012) was used to measure the amount of XyG in de-starched alkaline extracts from xth30 mutants and wild-type etiolated hypocotyls. Unexpectedly, the extractable XyG content in xth30 mutants was similar to that in the wild type after 100 mM NaCl treatment (Fig. 5A). These results suggest that XTH30 does not affect XyG content.

Next we analysed the XyG structure from etiolated hypocotyls. Xyloglucan was treated with xyloglucanase, which attacks unconnected Glc residues and produces an oligosaccharide mixture that includes GXXG, XXXG, XXLG, XXFG, XXFG+Ac, XLFG, and XLFG+Ac (Gille et al., 2011). As shown in Fig. 5B, salt stress decreased the relative level of XXFG and XXFG+Ac, but increased the level of XXXG, XLFG, and XLFG+Ac in the wild type. More importantly, the increase of XLFG caused by salt stress in the wild type was partly blocked in xth30 mutants (Fig. 5B). To confirm the effect of XTH30 on XLFG content, another enzyme, endoglucanase (Sechet et al., 2016), was used. As expected, similar changes were also observed by digesting with endoglucanase (Fig. 5C). These results indicate that XTH30 plays an important role in the accumulation of the largely connected XyG oligosaccharides XLFG in response to salt stress.

**XTH30 affects cellulose synthesis under salt stress**

XyG affects cellulose biosynthesis (Xiao et al., 2016). To explore whether cellulose synthesis is also affected in xth30 mutants, we first used the cellulose synthesis inhibitor isoxaben (Lei et al., 2014; Zhang et al., 2016b). We grew the xth30 mutants and the wild type on 1/2 MS medium supplemented with isoxaben, and found that the xth30 mutants were less sensitive to growth inhibition by isoxaben than was the wild type (Fig. 6A, B). These results suggest that XTH30 might be involved in cellulose synthesis. Next, we...
estimated the crystalline cellulose content. Salt stress significantly decreased the crystalline cellulose content in the wild type (Fig. 6C), which is consistent with a previous report (Endler et al., 2015). The cellulose synthesis genes (*CesA1*, *CesA3*, *CesA6*) were then analysed by qRT-PCR. The results showed that salt stress increased *CesA3* and *CesA6* expression in both the wild type and *xth30* mutants, which was not affected by the loss of *XTH30* (Supplementary Fig. S12). However, the decrease of crystalline cellulose content resulting from salt stress was much smaller in *xth30* mutants (Fig. 6C). These suggest that XTH30 affects cellulose synthesis under salt stress.

**Discussion**

As a structural layer outside the cell membrane, the plant cell wall not only provides the cell with structural support but also acts as the first line of defense against various stresses. The cell wall is flexible so that it can be rapidly remodeled when subjected to developmental, biotic, or abiotic stimuli. Maintaining the synthesis/deposition of cellulose is vital for the tolerance to salt stress. Several genes involved in cellulose synthesis (such as *CesA1* and *CesA6*) as well as companions of cellulose synthase proteins (CC1 and CC2) have been found to function...
in salt stress responses, and knockout of these genes resulted in salt stress sensitivity (Endler et al., 2015; Zhang et al., 2016a). However, the mechanisms of cell wall function in plant response to stresses are largely unknown. In this study, we revealed that an XTH, XTH30, negatively affected salt tolerance. XTH enzymes play a role in cell wall loosening and therefore can affect cell expansion. Unlike mutants of other members of the XTH family, such as XTH21, XTH27, XTH28, and XTH31 (Matsui et al., 2005; Liu et al., 2007; Kurasawa et al., 2009; Zhu et al., 2012), xth30 mutants do not have an obvious growth phenotype (Supplementary Fig. S8). However, xth30 mutants showed a very different performance from the wild type when subjected to salt stress. We found that XTH30 negatively affected salt tolerance based on the following evidence. First, knock-out mutations in XTH30 led to a salt-tolerant phenotype during growth of seedlings and etiolated hypocotyls (Figs 2A, B, 4; Supplementary Figs S4, S6). Second, overexpression of XTH30 enhanced sensitivity to salt stress (Fig. 2E, F). Cho et al. (2006) found that CaXTH3 participated in the protection of Arabidopsis mesophyll cells from salt stress via strengthening the cell wall layers. PeXTH positively modulated salt tolerance in tobacco by mainly affecting the development of leaf succulence (Han et al., 2013). XTH30 is another XTH member that is found to respond to salt stress (Cho et al., 2006; Choi et al., 2011; Han et al., 2013). However, these XTH genes (CaXTH3 and PeXTH) act as positive

**Fig. 7.** Knockout of XTH30 alleviates depolymerization of cortical microtubules under salt stress. (A) Seedlings germinated and grown for 6 d on 1/2 MS with or without 400 nM oryzalin in dark. (B) Quantification of hypocotyl elongation of seedlings in (A). Error bars represent SD, n>15 seedlings per biological replicate. Similar results were observed in three independent experiments. (C) Confocal images of GFP–TUA6 labeled cortical microtubules. Three-day-old etiolated seedlings were exposed to 10 μM oryzalin for 5 min. The epidermal cells in etiolated hypocotyls were observed. (D) Quantification of hypocotyl elongation of seedlings in (C). (E) Confocal images of GFP–TUA6 labeled cortical microtubules. Three-day-old etiolated seedlings were exposed to 100 mM NaCl for 12 h. The epidermal cells in etiolated hypocotyls were observed. (F) Quantification of hypocotyl elongation of seedlings in (E).
During cell elongation (Hayashi et al., 2011; Zhang et al., 2011; Park and Cosgrove, 2015), XyG often undergoes turnover by wall-localized enzymes. XyG is the most abundant hemicellulose in the primary cell wall in dicots. XyG often undergoes turnover by wall-localized enzymes during cell elongation (Hayashi et al., 1984). In this study, we observed that XTH30 did not affect the extractable XyG content and the abundance of XyG oligosaccharides, i.e., XXXG, XXLG, and XXFG, under salt stress (Fig. 5). However, we found that loss-of-function of XTH30 obviously suppressed the strong increases in the abundance of XyG side chain, found that loss-of-function of XTH30 affected cellulose biosynthesis genes (CesA1, CesA3, CesA6) in response to salt stress (Fig. 6; Supplementary Fig. S12). It is possible that XTH30 somehow directly affects the synthesis/deposition of cellulose at the plasma membrane in response to salt stress. Cellulose microfibrils are typically transversely organized and can align with cortical microtubules that are tethered to the plasma membrane (Park and Cosgrove, 2015). Microtubule depolymerization and reorganization are believed to be essential for plant survival under salt stress (Wang et al., 2011; Zhang et al., 2012; Dou et al., 2018). XTH30 affected the cortical microtubule depolymerization in response to salt stress (Fig. 7), which contradicted the view that microtubules mass was inversely correlated with the cellulose crystallinity and CSC velocity (Fujita et al., 2011). However, there are also some studies indicating that inhibiting cellulose synthase activity with isoaxaben could influence microtubule organization (Paredez et al., 2008; Scheible et al., 2011). XyG side chains in the xyloglucan xylosyltransferase mutant xct affected cellulose synthase activity and cellulose content in primary cell walls, and disrupted microtubule stability (Xiao et al., 2016).

Interestingly, knock-out mutations of XTH30 led to lower Na+ accumulation in shoots (Supplementary Fig. S9) and showed similar Na+ accumulation in roots compared with the wild type, which suggests that XTH30 affected Na+ transportation from root to shoot. Based on the lower Na+ content and higher antioxidant defense ability, knock-out mutations of XTH30 led to a lower level of H2O2 than that of the wild type under salt stress (Supplementary Figs S10, S11), which alleviated the damage of salt stress to plants. The mechanism needs to be further explored.

Thus, our findings suggest that the modified XyG side chain detected in xth30 mutants potentially affects cellulose synthesis and cortical microtubule stability through its effects on the interactions with components of the cell wall under salt stress (Supplementary Fig. S13).

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Summary of T-DNA lines used in this study.
Fig. S2. The expression of XTH30 in wild type, xth30-1, and complementation of XTH30 in xth30-1.
Fig. S3. The expression of XTH30 in wild type and XTH30 overexpressors.
Fig. S4. Sensitivity of hydroponically grown seedlings of xth30 mutants and wild type to salt stress.
Fig. S5. XTH30 does not affect the tolerance to heavy metal stress (Zn2+ and Cd2+) and ABA.
Fig. S6. xth30 mutants are resistant to Na+ and Li+.
Fig. S7. XTH30 does not affect the germination of seeds exposed to salt stress.
Fig. S8. The phenotypes of wild type and xth30 mutants.
Fig. S9. Effect of XTH30 on Na+ accumulation in hydroponically grown seedlings exposed to salt stress.
Fig. S10. Accumulation of H2O2 in wild-type and xth30 mutant seedlings.
Fig. S11. Effect of XTH30 on the activities of antioxidant enzymes exposed to salt stress.
Fig. S12. The effect of XTH30 on the expressions of CesA1, CesA3, and CesA6 in response to salt stress.
Fig. S13. A potential model for XTH30 regulation in salt stress responses.
Table S1. Primer sequences used in this study.

Accession numbers

Sequences data from this article relate to the following IDs: ACTIN 2, At3G18780; CesA1, At4g32410; CesA3, At5g05170; CesA6, At5g64740; XTH30, AT1G32170.

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