


PagWOX11/12a activates PagCYP736A12 gene that facilitates salt tolerance in poplar

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Summary

The WUSCHEL-related homeobox (WOX) transcription factors WOX11 and WOX12 regulate adventitious rooting and responses to stress. The underlying physiological and molecular regulatory mechanisms in salt stress tolerance remain largely unexplored. Here, we characterized the roles of *PagWOX11/12a* from 84K poplar (*Populus alba* × *P. glandulosa*) and the underlying regulatory mechanism in salt stress. *PagWOX11/12a* was strongly induced by salt stress in roots. Overexpression of *PagWOX11/12a* in poplar enhanced salt tolerance, as evident by the promotion of growth-related biomass. In contrast, salt-treated *PagWOX11/12a* dominant repression plants displayed reduced biomass growth. Under salt stress conditions, *PagWOX11/12a*-overexpressed lines showed higher reactive oxygen species (ROS) scavenging capacity and lower accumulation of hydrogen peroxide (H₂O₂) than non-transgenic 84K plants, whereas the suppressors displayed the opposite phenotype. In addition, *PagWOX11/12a* directly bound to the promoter region of *PagCYP736A12* and regulated *PagCYP736A12* expression. The activated *PagCYP736A12* could enhance ROS scavenging, thus reducing H₂O₂ levels in roots under salt stress in *PagWOX11/12a*-overexpressed poplars. The collective results support the important role of *PagWOX11/12a* in salt acclimation of poplar trees, indicating that *PagWOX11/12a* enhances salt tolerance through modulation of ROS scavenging by directly regulating *PagCYP736A12* expression in poplar.

Keywords: cytochrome P450

monooxygenase gene, *PagWOX11/*

12a, *PagCYP736A12*, root growth,

ROS, salt stress.

Introduction

Salt stress is a major environmental factor that adversely affects plant growth and production globally (Hasegawa, 2013). To cope with long-term ionic stress, plants, especially perennials, have evolved a range of intrinsic mechanisms and adaptive strategies. Transcriptional regulation is important in controlling plant signalling pathways to regulate many biological processes in response to various stress conditions. These responses involve specific transcription factors (TFs), such as the WUSCHEL-related homeobox (WOX), MYB, NAC and WRKY families (Ding *et al.*, 2015; Fang *et al.*, 2017; Liu *et al.*, 2014a, 2014b; Mao *et al.*, 2020; Zhao *et al.*, 2009). Expression of some TFs is induced by salt stress, which activates downstream stress-responsive genes that regulate the levels of reactive oxygen species (ROS) involved in plant developmental and physiological processes (Mittler *et al.*, 2004; Suzuki *et al.*, 2012). However, high concentrations of ROS can lead to cell injury and the oxidative destruction of cells (Dietz *et al.*, 2016; Huang *et al.*, 2016).

The WOX protein family of novel plant-specific TFs plays crucial roles in meristem initiation, stem cell maintenance and organ formation (Der Graaff *et al.*, 2009). The homeobox genes *WOX11* and *WOX12* are members of the WOX family. They are reportedly

involved in root development. For example, rice *OsWOX11* is a key regulator of crown root emergence, and the interaction between *ERF3* and *WOX11* is required to regulate *RR2* expression in the cytokinin signalling pathway during crown root development (Zhao *et al.*, 2009, 2015). *Arabidopsis* *AtWOX11* and *AtWOX12* have been reported to be involved in the first-step cell fate transition for callus initiation during *de novo* root organogenesis (Liu *et al.*, 2014c). Poplar *WOX11* and *WOX11/12* are essential for adventitious root development. Overexpression of *WOX11* (*PeWOX11a* and *PeWOX11b*) or *WOX11/12a* gene increases the number of adventitious roots in transgenic cuttings (Liu *et al.*, 2014a; Xu *et al.*, 2015). A few studies have reported that *WOX11* can regulate root growth in response to abiotic stress. Rice *WOX11* expression is decreased by microcystin-LR treatment, leading to significantly inhibited rice root growth (Chen *et al.*, 2013). *OsWOX11* is responsive to drought stress, and *OsWOX12A* and *OsWOX12B* are differentially regulated by drought, cold and high salt stress; overexpression of *OsWOX11* can enhance drought tolerance by promoting root hair development and growth (Cheng *et al.*, 2014, 2016). Furthermore, Jiang *et al.* (2017) reported that the transcriptional regulatory network of *WOX11* is involved in the control of redox metabolic pathways in rice under drought stress. Recently, we isolated a homolog of

Arabidopsis *WOX11* and *WOX12*, *PagWOX11/12a*, from 84K poplar (*Populus alba* × *P. glandulosa*). *PagWOX11/12a* is a copy of the two *PagWOX11/12* genes (*PagWOX11/12a* and *PagWOX11/12b*) in the 84K genome. *PagWOX11/12a* is strongly expressed under drought stress and can promote root elongation and biomass growth in transgenic poplars. In addition, *PagERF35* was identified as an upstream regulator that directly activates the expression of *PagWOX11/12a* in response to drought stress (Wang et al., 2020a). However, whether *WOX11* is involved in the response to salt stress remains unclear. If it is, the regulatory mechanisms and downstream target genes of *WOX11* in salt stress need to be further investigated.

Recent studies have demonstrated that in addition to TFs, cytochrome P450 monooxygenase (*CYP*) genes are involved in diverse biological processes, including plant development and abiotic stress responses (Kurotani et al., 2015; Li and Wei, 2020; Ma et al., 2014). For instance, *PtCYP714A3* from *P. trichocarpa* was observed to be markedly induced by salt stress, and transgenic rice plants displayed enhanced salt tolerance and reduced shoot growth (Wang et al., 2016). *Panax ginseng* *PgCYP736A12* is up-regulated by NaCl, H₂O₂ and salicylic acid, and overexpression of *PgCYP736A12* in *Arabidopsis* was observed to slightly reduce plant height and enhance tolerance to phenylurea herbicide (Khanom et al., 2019). In addition, *PgCYP736B*, the homolog of *PgCYP736A12*, conferred enhanced salt tolerance by modulating H₂O₂ accumulation, carotenoid levels and abscisic acid biosynthesis in *Arabidopsis* (Balusamy et al., 2019). *SoCYP85A1* from *Spinacia oleracea* contributes to drought tolerance by promoting root development and regulating ROS production and stress-responsive gene expression (Duan et al., 2017). The wheat *TaCYP81D5* gene is involved in the response to salt stress by regulating ROS scavenging during seedling and reproductive stages (Wang et al., 2020b). Finally, *Arabidopsis* *WRKY33* regulates *CYP94B1* expression and enhances suberin deposition in the endodermal cells of roots, which confers salt tolerance (Krishnamurthy et al., 2020). The foregoing highlights the need to clarify the regulation of *CYP* genes in the response to abiotic stress.

In the present study, we demonstrated that *PagWOX11/12a* is involved in the salt acclimation of poplars using both its overexpression and down-regulation of transgenic poplars. In addition, we defined a molecular mechanism by which *PagWOX11/12a* enhances salt tolerance and ROS scavenging in roots by directly up-regulating *PagCYP736A12* expression. This study provides novel insights into the physiological roles of the *WOX* gene in response to salt stress.

Results

PagWOX11/12a responses to salt stress

PagWOX11/12a expression was significantly induced by salt stress and was transiently increased in the roots under salt stress, reaching a maximum 4.5-fold increase 9 h after salt stress treatment (Figure 1a). β-Glucuronidase (GUS) staining of *ProPagWOX11/12a::GUS* transgenic poplar plants revealed that *PagWOX11/12a* was mainly expressed in the root tips under control culture conditions. However, GUS staining was intense in whole roots treated with salt for 6 and 9 h (Figure 1b). The strong GUS activity was significantly increased by nearly 13-fold in the roots at 9 h after salt stress (Figure 1c). The results indicate that *PagWOX11/12a* can be strongly induced by salt stress in poplar roots.

PagWOX11/12a positively regulates root growth under salt conditions

To investigate whether *PagWOX11/12a* regulates root growth under salt stress conditions, transgenic poplar plants overexpressing (OE) *PagWOX11/12a* (OE1 and OE3) and plants with dominant repression (DR) of *PagWOX11/12a* (DR7 and DR9) were chosen for subsequent studies (Wang et al., 2020a). Under different concentrations of NaCl treatment, the development of roots was retarded in each poplar genotype, and the growth was significantly different between transgenic and 84K plants, especially at high salt concentrations (Figure 2a). The main root (MR) number was markedly higher in OE lines but lower in DR lines than in 84K plants. In addition, MR development appeared completely suppressed in DR plants. Therefore, root biomass accumulation was reduced to a greater extent in 84K and DR plants than in OE lines under salt stress (Figure 2b, c). The relative fresh weight (FW) and dry weight (DW) in OE lines was significantly higher but were obviously lower in DR plants than in 84K plants treated with NaCl.

PagWOX11/12a alleviates inhibition of biomass accumulation under salt conditions

To further investigate the function of *PagWOX11/12a* in response to salt stress in soil conditions, transgenic and 84K plants were cultured in soil under NaCl treatment. Under control growth conditions, although the transgenic lines and 84K plants grew well, the stem height was significantly higher in OE plants and obviously lower in DR plants than in 84K plants (Figure 3a, c). After salt stress treatment, the differences in plant height among different genotypes were more significant. Most leaves of 84K plants wilted and fell off, whereas those of OE plants remained turgid, except for the older leaves. In contrast, some DR plants died at this stage (Figure 3b, c). Although the growth rate of all genotypes was inhibited under salt stress conditions, the OE lines had significantly higher growth rates than the other plants (Figure 3d). In addition, the relative FW of shoots and roots was significantly higher in OE plants than in 84K plants under salt stress. In contrast, the shoot and root biomass of DR plants were markedly lower than the values of 84K plants (Figure 3e). Accordingly, the relative DW of shoots and roots showed the same trend (Figure 3f). These results suggest that the biomass of OE plants was less inhibited by salt stress. Consistent with the results obtained in half-strength Murashige and Skoog (1/2 MS) solid medium, the biomass accumulation of 84K and DR plants was decreased to a greater extent than that in OE plants under salt conditions. The findings suggest that *PagWOX11/12a* overexpression can increase salt tolerance in transgenic poplar plants.

PagWOX11/12a regulates genes involved in oxidation–reduction process under salt stress

To identify the target genes of *PagWOX11/12a* in response to salt stress, RNA-sequencing (RNA-seq) analysis was performed using the roots of transgenic OE, DR and 84K plants. Based on the Gene Ontology term enrichment analysis, we found 236 up-regulated differentially up-regulated genes (DEGs) of 'OE vs 84K' shared by control growth and salt stress conditions. Of these, 28 DEGs (11.86%) were mainly enriched in the oxidation–reduction process (Table S2). In contrast, 223 down-regulated DEGs of 'DR vs 84K' were shared by control growth and salt stress conditions. Of these, 29 DEGs (13%) were involved in the oxidation–reduction process (Table S3). We further compared the two sets

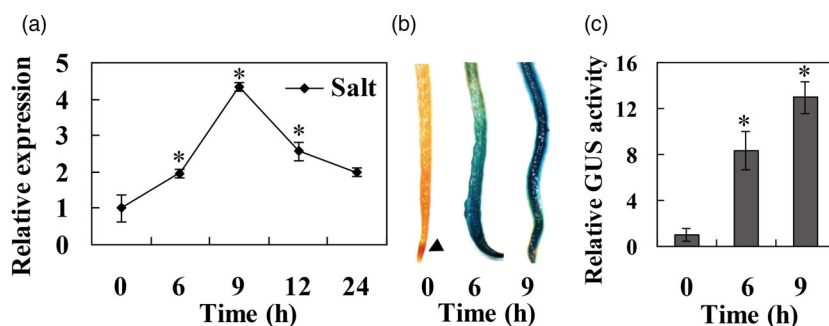


Figure 1 Expression profiles of *PagWOX11/12a* under salt stress. (a) RT-qPCR analysis of *PagWOX11/12a* expression in total roots of non-transgenic 84K poplar under salt stress. Four-week-old non-transgenic 84K poplar plants were subjected to 200 mM NaCl for the indicated times. The root tissues were collected from 10 individuals and used for RT-qPCR analysis. The bars represent means \pm SD ($n = 4$). (b, c) Histological staining and GUS activity of the root tips of *ProPagWOX11/12a::GUS* transgenic poplar plants under salt stress. Four-week-old transgenic poplar plants were subjected to 200 mM NaCl for the indicated times. At least 20 roots were harvested from three biological replicates (more than 10 individuals each) for GUS staining and activity analysis. The values of GUS activity under control growth conditions were normalized to 1. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 6$) and the asterisks indicate significant differences with respect to the values for 0 h ($*P < 0.05$).

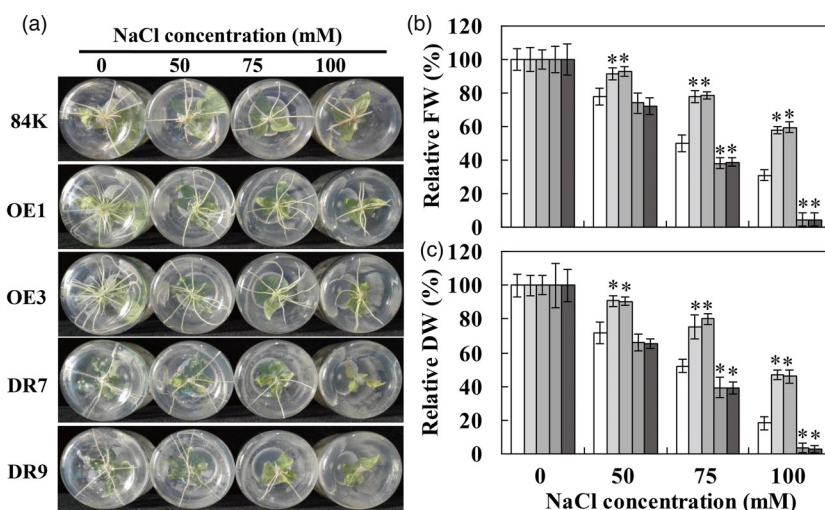


Figure 2 *PagWOX11/12a* enhances salt tolerance of root growth and biomass in poplar. (a) Root phenotypes of non-transgenic 84K and transgenic (OE and DR) poplar plants. (b, c) Relative quantification of root biomass (fresh weight [FW] and dry weight [DW]) of non-transgenic 84K and transgenic poplar plants. The cutting-propagated non-transgenic 84K and transgenic (OE and DR) poplar plants were cultivated on 1/2 MS solid medium supplemented with 0, 50, 75 and 100 mM NaCl for 20 days. The root biomass of individual plant was measured. The values for each plant under control growth conditions were normalized to 100%. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 6$) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants ($*P < 0.05$).

of DEGs (Figure S1) and found 17 DEGs in common, including two DEGs enriched in pathways related to the oxidation–reduction process (Table S4). Finally, we searched for *cis*-acting elements in the 1-kb region upstream of the transcription starting sites of the DEGs and found that only one DEG (*PagCYP736A12*) harboured a WOX-binding motif ('TTAATGC') in its promoter region (Figure S2). It encodes a cytochrome P450 family protein with extensive homology to CYP736A12 from *P. trichocarpa* (XP_002310014.2). Phylogenetic analysis indicated that *PagCYP736A12* exhibited a close phylogenetic relationship with PtCYP736A12 and PpCYP736A12 (Figure S3). The findings indicate that *PagCYP736A12* is a downstream target gene of *PagWOX11/12a* in poplar.

PagWOX11/12a overexpression decreases oxidative damage

To investigate whether overexpression of *PagWOX11/12a* would decrease oxidative damage in the roots of transgenic poplar plants under salt stress, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) staining was performed. The H_2O_2 levels in root tips of all poplars were not visibly different under control growth conditions. In contrast, the accumulation of H_2O_2 in root tips was obviously lower in transgenic OE plants, but was significantly higher in DR lines than in 84K plants under salt stress (Figure 4a). Quantification of H_2O_2 content yielded similar results (Figure 4b). In addition, there were no differences in malondialdehyde (MDA)

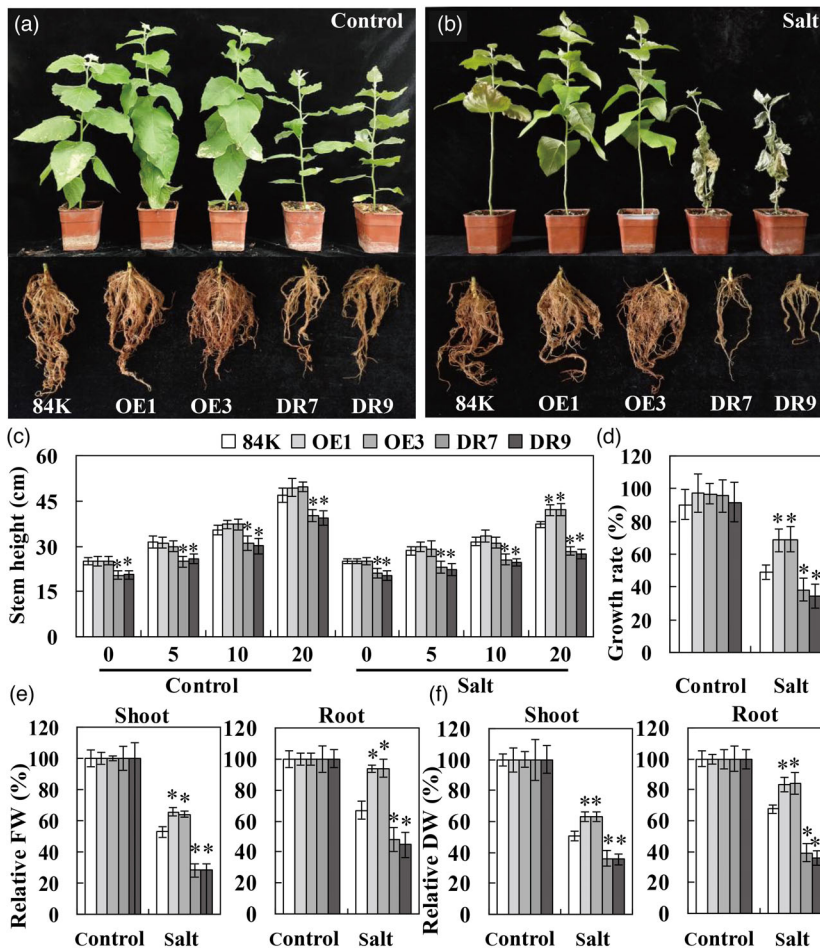


Figure 3 *PagWOX11/12a* enhances salt tolerance of shoot and root biomass in poplar. (a, b) Shoot and root phenotypes of non-transgenic 84K and transgenic (OE and DR) plants. (c) Plant height of non-transgenic 84K and transgenic plants. (d) Growth rate of non-transgenic 84K and transgenic plants. (E, F) Relative quantification of shoot and root biomass (fresh weight [FW] and dry weight [DW]) of non-transgenic 84K and transgenic plants. Four-week-old non-transgenic 84K and transgenic plants cultivated *in vitro* were grown in soil for 20 days and irrigated subsequently with 0 (control) or 200 mM NaCl (salt treatment) every 2 days for 20 days. The plant height of individual plant was periodically measured. The growth rate, shoot and root biomass of individual plant were measured after 20 days of culture. The values for each plant under control growth conditions were normalized to 100%. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 10$) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants ($*P < 0.05$).

content in roots between the transgenic lines and 84K plants under control growth conditions. However, the MDA content in OE lines was significantly lower than that in 84K plants after salt stress, whereas DR plants displayed higher MDA levels than 84K and OE plants (Figure 4c). These results suggest that transgenic plants overexpressing *PagWOX11/12a* accumulated less ROS under salt stress.

PagWOX11/12a overexpression enhances ROS scavenging ability

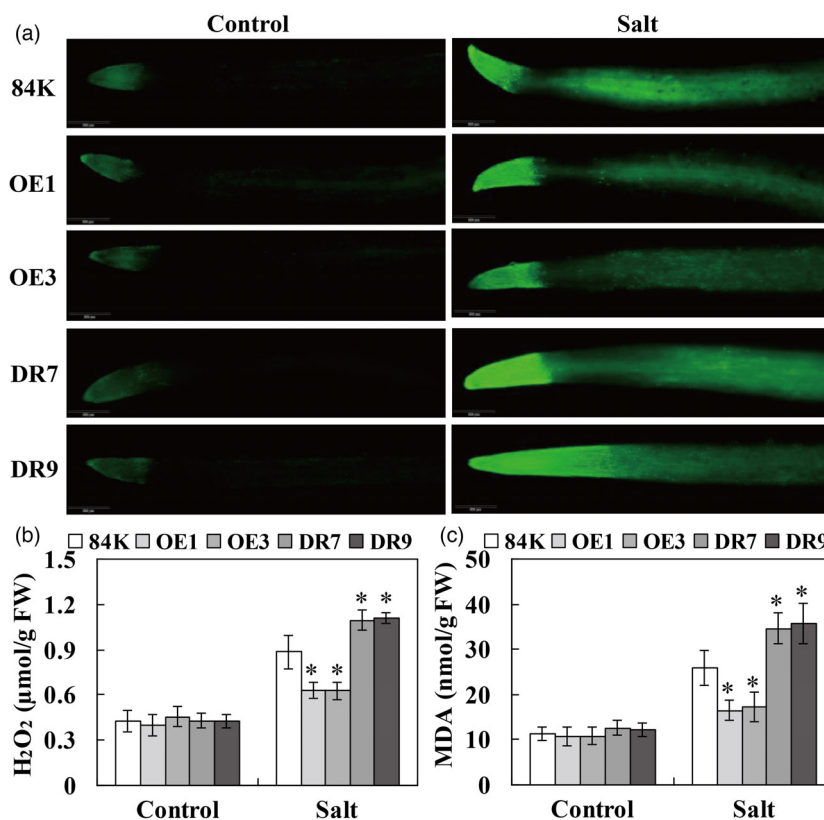
Transgenic poplar plants overexpressing *PagWOX11/12a* showed less oxidative damage, perhaps due to their improved antioxidant capacity. To confirm this, the non-enzymatic and enzymatic antioxidants in the ascorbate–glutathione (AsA–GSH) cycle and superoxide dismutase (SOD) activity between 84K and transgenic poplar plants were examined (Figure 5). No differences were apparent in the antioxidant compounds (AsA and GSH) and enzyme activity levels of ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and SOD in the roots of the transgenic lines (OE and DR) and 84K plants under control growth conditions. However, the antioxidant compounds and enzyme activities were significantly higher in the OE lines, but lower in the DR lines compared with the values in 84K plants under salt stress. In addition, there were no significant differences in Na^+ and K^+ concentrations between 84K and transgenic plants under control and salt stress conditions (Figure S4). However, the Na^+ content was increased and K^+ content was decreased in both 84K and

transgenic plants under salt stress. These results indicate that OE lines can increase their antioxidant capacity to decrease oxidative damage under salt stress, rather than maintain K^+/Na^+ homeostasis.

PagWOX11/12a directly regulates *PagCYP736A12* expression

To investigate whether *PagWOX11/12a* is involved in the salt stress response through direct regulation of *PagCYP736A12* expression by binding to its promoter, yeast one-hybrid (Y1H), chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays were conducted. Yeast transformants harbouring the *PagWOX11/12a* and WOX11-binding motif or *PagCYP736A12* promoter fragment grew well on SD–Leu–Trp–His medium containing 3-amino-1,2,4-triazole (3-AT) (Figure 6a). In addition, *PagWOX11/12a*–green fluorescent protein (GFP) was used in the ChIP assay. The promoter fragment of *PagCYP736A12* containing the WOX11-binding motif was detected and was highly enriched in the ChIP sample compared with that in the mock. The enrichment was significantly higher under salt stress than under control growth conditions, indicating that salt stress enhanced this induction (Figure 6b). Luciferase luminescence was observed when 35S::*PagWOX11/12a* and Pro::LUC were co-transformed into tobacco leaves, but no luminescence was evident when the tobacco leaves were co-transformed with vector controls (62-SK+0800-LUC) and negative controls (62-SK+Pro::LUC and 35S::*PagWOX11/12a*+0800-LUC). The relative LUC/Renilla luciferase

Figure 4 Overexpression of *PagWOX11/12a* decreased oxidative damage levels under salt stress. (a) Representative DCFH-DA staining of H₂O₂ production in the root tips of non-transgenic 84K and transgenic plants. (b, c) H₂O₂ and MDA content in the fresh roots of non-transgenic 84K and transgenic plants. The cutting-propagated non-transgenic 84K and transgenic (OE and DR) poplar plants were cultivated and salinity treated with 0 and 75 mM NaCl as described in Figure 2. At least 20 roots were collected from three biological replicates (more than 10 individuals each) for DCFH-DA staining and analysis of H₂O₂ and MDA contents. FW, fresh weight. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 6$) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants ($*P < 0.05$).



(REN) activity driven by the *PagCYP736A12* promoter co-transformed with the *PagWOX11/12a* effector was significantly higher than that of the vector controls and negative controls (Figure 6c).

In support of their close relationship, we found that the expression of both *PagWOX11/12a* and *PagCYP736A12* was strongly induced by salt stress. The highest levels in OE and 84K roots were observed at 75 mM NaCl (Figure S5A). Notably, the two genes had similar expression patterns in OE and 84K roots under different salt concentrations (Figure S5A) and in 84K roots treated with 200 mM NaCl for 0, 6, 9, 12 and 24 h (Figure 1a and Figure S5B). Furthermore, *PagCYP736A12* expression was strongly enhanced in OE lines, but was suppressed in DR lines under control and salt conditions (Figure S5C). Taken together, these results confirm that *PagWOX11/12a* directly binds to the promoter of *PagCYP736A12*, activating its transcription in response to salt stress.

PagCYP736A12 reduces H₂O₂ level and enhances salt tolerance

To determine whether *PagCYP736A12* can enhance salt tolerance in poplar, transgenic poplars overexpressing *PagCYP736A12* were generated, and the expression levels of *PagCYP736A12* in transgenic lines were confirmed (Figure S6). The transgenic lines (OX1 and OX2) with high expression levels were selected for further study. DCFH-DA staining results showed that the H₂O₂ levels of all poplars were not significantly different under control growth conditions. However, the accumulation of H₂O₂ in OX plant roots was obviously lower than that in 84K plants under salt stress (Figure 7a). Similarly, quantification of H₂O₂ and MDA content showed no significant differences between transgenic lines and 84K plants under control growth conditions. However,

the content was significantly lower in OX lines than in 84K plants when exposed to salt stress (Figure 7b, c).

OX and 84K plants showed the same phenotype in a greenhouse under control growth conditions. The stem height and root biomass were not significantly different between OX and 84K plants. After 10 days of salt stress, the older leaves of 84K plants wilted and fell off, whereas those of OX plants remained turgid (Figure 8a). Furthermore, the stem height and root biomass were significantly higher in OX lines than in 84K plants (Figure 8b, c). Taken together, these results showed that *PagCYP736A12* overexpression, similar to the *PagWOX11/12a* OE plants, could enhance salt tolerance by decreasing ROS levels and thus accumulate more biomass than 84K plants under salt stress.

Discussion

WOX11 genes act as developmental regulators that mediate root formation and growth in plants (Liu *et al.*, 2014c; Xu *et al.*, 2015; Zhao *et al.*, 2009, 2015). Recently, it was reported that the *WOX11* and *WOX12* genes are involved in salt and/or drought stress responses (Cheng *et al.*, 2014, 2016; Wang *et al.*, 2020a). In this study, we assessed the role and underlying mechanism of *PagWOX11/12a* involved in salt tolerance in poplar (Figure 9). *PagWOX11/12a* responds to salt stress and enhances poplar rooting in response to stress conditions. At the same time, *PagWOX11/12a* directly activates *PagCYP736A12* expression, and the latter is involved in ROS scavenging to reduce ROS levels in roots under salt stress. Therefore, *PagWOX11/12a* plays a role in salt tolerance by regulating both the root system and physiological processes. Our study provides insight into the regulation of physiological processes by *PagWOX11/12a* under salt stress.

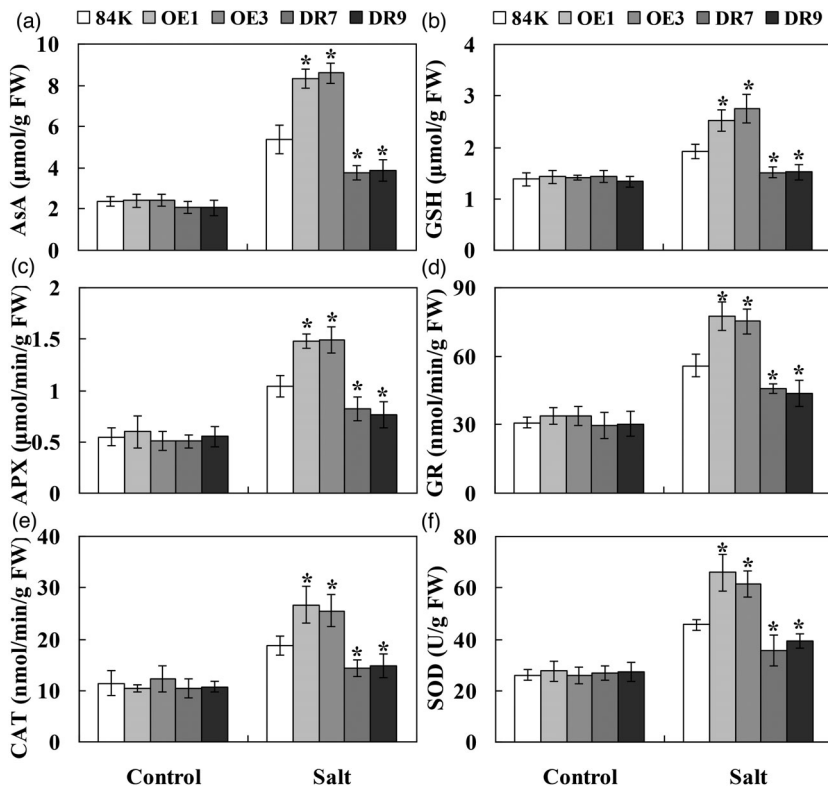


Figure 5 Overexpression of *PagWOX11/12a* enhanced ROS scavenging ability under salt stress. The cutting-propagated non-transgenic 84K and transgenic (OE and DR) poplar plants were cultivated and salinity treated with 0 and 75 mM NaCl as described in Figure 2. The fresh roots were collected and used for analysis of the non-enzymatic [AsA (a) and GSH (b)] and enzymatic [APX (c), GR (d) and CAT (e)] antioxidants in the AsA–GSH cycle and the SOD activity (f). FW, fresh weight. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 6$) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants ($*P < 0.05$).

PagWOX11/12a is involved in tolerance to salt stress

Our previous study showed that *PagWOX11/12a* responds to drought stress and enhances drought tolerance in transgenic poplars (Wang *et al.*, 2020a). In this study, *PagWOX11/12a* was strongly induced by salt stress (Figure 1), and transgenic poplars overexpressing *PagWOX11/12a* displayed promoted growth in biomass compared to 84K plants under salt stress. In contrast, the suppressed *PagWOX11/12a* plants exhibited inhibited root and shoot growth, and high sensitivity to salt stress, resulting in decreased stem and root growth (Figures 2 and 3). These results strongly suggest that salt-induced *PagWOX11/12a* plays an important role in salt tolerance. It should be noted that the small seedlings were used in the NaCl (salt) treatment. Therefore, although statistically significant, the phenotypic differences were relatively small. Therefore, further studies are needed to determine the highest concentration of NaCl that the OE lines could be tolerant to and field trials are necessary to analyse the growth of transgenic poplars in saline regions. The results of these future studies will conclusively indicate the practical use of this gene.

Excess ROS, such as those produced by salt stress, lead to oxidative conditions that are harmful to cells (Kuge *et al.*, 2010; Wang *et al.*, 2008). Generally, H_2O_2 is a prominent ROS species and is a marker of ROS production (Bhattacharjee, 2012; de la Garma *et al.*, 2015). Therefore, maintaining the steady-state level of ROS by scavenging pathways is important to protect against oxidative damage caused by adverse environments (Jiang and Zhang, 2002; Miller *et al.*, 2010; Mittler *et al.*, 2004). In addition, MDA content is a valid indicator of cytomembrane oxidative damage in monitoring ROS levels (He *et al.*, 2018; Shi *et al.*, 2013). In this study, transgenic lines overexpressing *PagWOX11/12a* displayed lower H_2O_2 and MDA levels in roots than those in 84K plants under salt stress, whereas lines with *PagWOX11/12a*

repressors exhibited severe ROS damage (Figure 4). These results suggest that the increased salt stress tolerance of the transgenic poplar plants overexpressing *PagWOX11/12a* was attributed to their low ROS levels.

Both SOD and the AsA–GSH cycle are crucial in scavenging ROS (Hernández *et al.*, 2015; Shi *et al.*, 2013). SOD is the first line of plant ROS defence. The enzyme catalyses the conversion of superoxide ($O_2^{\cdot-}$) to oxygen (O_2) and H_2O_2 (Huang *et al.*, 2016). H_2O_2 is then reduced to H_2O by CAT and APX in the AsA–GSH cycle (Hernández *et al.*, 2015). Transgenic poplar plants overexpressing *PagWOX11/12a* exhibited increased capacity to maintain ROS homeostasis by increasing both antioxidant compounds and antioxidant enzyme activity, but not enhanced capacity to control K^+/Na^+ under NaCl stress (Figure 5 and Figure S4). In contrast to the suppressed plants, low levels of antioxidant compounds and antioxidant enzyme activity were maintained under salt stress. These findings indicate that *PagWOX11/12a* may activate an antioxidant pathway to modulate ROS accumulation.

PagWOX11/12a regulates ROS scavenging by activating expression of *PagCYP736A12*

A recent transcriptomic analysis revealed considerable downstream genes of WOX11 in rice, which were demonstrated to be involved in drought stress response and redox homeostasis (Jiang *et al.*, 2017). The findings indicated that WOXs may regulate genes involved in ROS. In this study, RNA-seq analysis revealed that many DEGs were enriched in the oxidation–reduction process under salt stress (Table S2, S3). Seventeen DEGs were shared by *PagWOX11/12a* overexpressors and suppressors. The 17 DEGs included two involved in the oxidation–reduction process (Table S4). Interestingly, one of the DEGs, *PagCYP736A12*, contains the DNA sequence ‘TTAATGC’ in its promoter, which can be recognized by the WUSCHEL-related

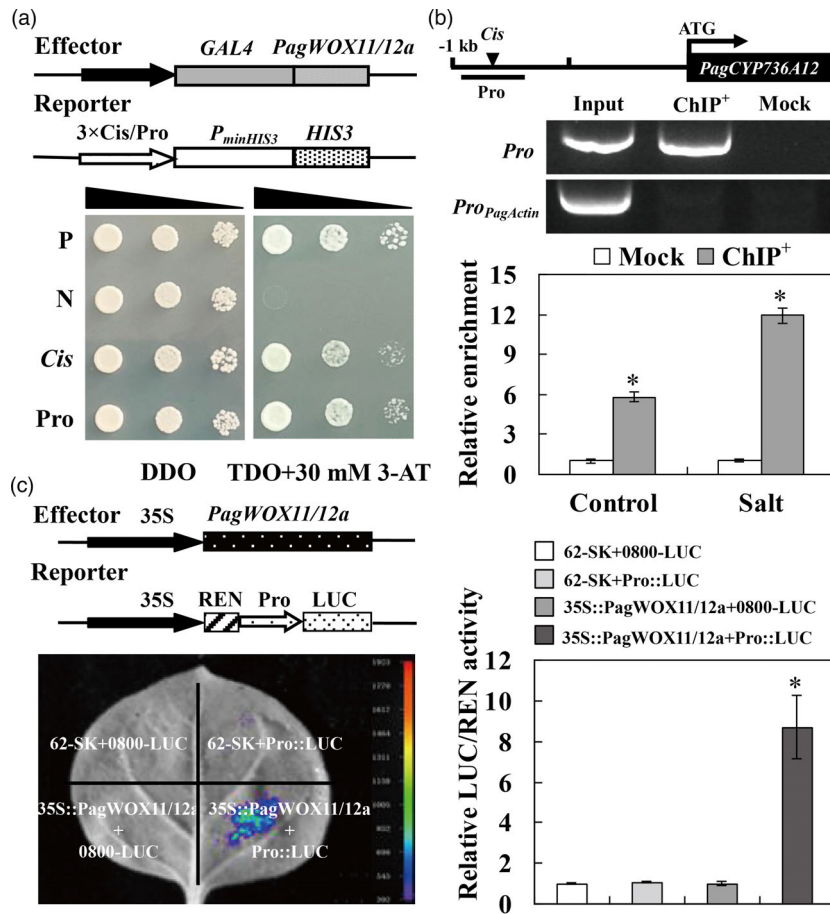


Figure 6 Identification of the targeted gene *PagCYP736A12* of *PagWOX11/12a*. (a) Y1H analysis of the interaction between *PagWOX11/12a* and the *PagCYP736A12* promoter. A diagram of the reporter and effector vectors is shown at the top. The reporter and effector constructs were co-transformed into yeast Y187 cells, and the positive transformants were determined by spotting serial dilutions of yeast onto SD/–Leu/–Trp (DDO) and SD/–His/–Leu/–Trp (TDO) media supplemented with 3-AT. P: positive control (p53HIS2 + pGAD-53); N: negative control (p53HIS2 + pGAD-*PagWOX11/12a*). (b) ChIP analysis of *PagWOX11/12a* binding to the *PagCYP736A12* promoter. Simplified gene structure indicating the locations of the amplified promoter region (Pro) containing the ‘TTAATGC’ element. qPCR analysis of the abundance of the *PagCYP736A12* promoter sequence. Transiently transgenic poplars were grown on 1/2 MS solid medium under normal conditions for 48 h or cultured on 1/2 MS medium for 36 h, and then subjected to 200 mM NaCl for 12 h. Input, chromatin preparation before immunoprecipitation; ChIP⁺, immunoprecipitated with anti-GFP antibody; Mock, immunoprecipitated without anti-GFP antibody. (c) Dual-luciferase reporter analysis of *PagWOX11/12a* activating the *PagCYP736A12* promoter in *N. benthamiana* leaves. Schematic of the reporter and effector constructs is shown at the top. The effector and reporter vectors were instantaneously co-transformed into leaves of *N. benthamiana* and cultured in a greenhouse under control conditions for 48 h. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 6$) and the asterisks indicate significant differences ($*P < 0.05$).

homeobox TFs (Dai *et al.*, 2007; Kamiya *et al.*, 2003; Lohmann *et al.*, 2001; Yadav *et al.*, 2011; Zhao *et al.*, 2009). Previous studies have shown that *CYP* genes confer tolerance to salt, drought and oxidative stress when overexpressed in transgenic plants (Balusamy *et al.*, 2013, 2019; Duan *et al.*, 2017; Mao *et al.*, 2013; Wang *et al.*, 2016; Wang *et al.*, 2020b; Zhou *et al.*, 2020), and that *PagCYP736A12* is induced by salt and H₂O₂ stresses (Khanom *et al.*, 2019). Therefore, we investigated whether *PagWOX11/12a* could directly regulate *PagCYP736A12* using Y1H, ChIP and luciferase reporter assays. The results showed that *PagWOX11/12a* bound to the promoter and activated the expression of *PagCYP736A12* (Figure 6). The similar expression patterns of *PagWOX11/12a* and *PagCYP736A12* in both OE and 84K roots under salt stress support this mode of regulation (Figure S5A). In addition, we found that transgenic poplars overexpressing *PagCYP736A12* showed increased salt tolerance

(Figures 7 and 8), phenocopying the *PagWOX11/12a* OE plants. Moreover, overexpression of *PagCYP736A12* in poplar led to lower H₂O₂ and MDA levels in roots, resulting in higher plant height and root biomass under salt stress (Figures 7 and 8). These results suggest that *PagCYP736A12* confers salt tolerance by regulating H₂O₂ levels. Collectively, these results strongly support the hypothesis that *PagWOX11/12a* directly regulates *PagCYP736A12* expression to improve salt tolerance by maintaining low ROS levels.

In summary, this work demonstrates that *PagWOX11/12a* enhances salt tolerance by regulating the expression of *PagCYP736A12* as a direct downstream gene, which in turn maintains ROS homeostasis in roots. In addition, transgenic poplars with these two genes exhibited increased root biomass under salt stress. This study is the first to report the role of *WOX11/12* gene in salt tolerance in woody plant species. Further

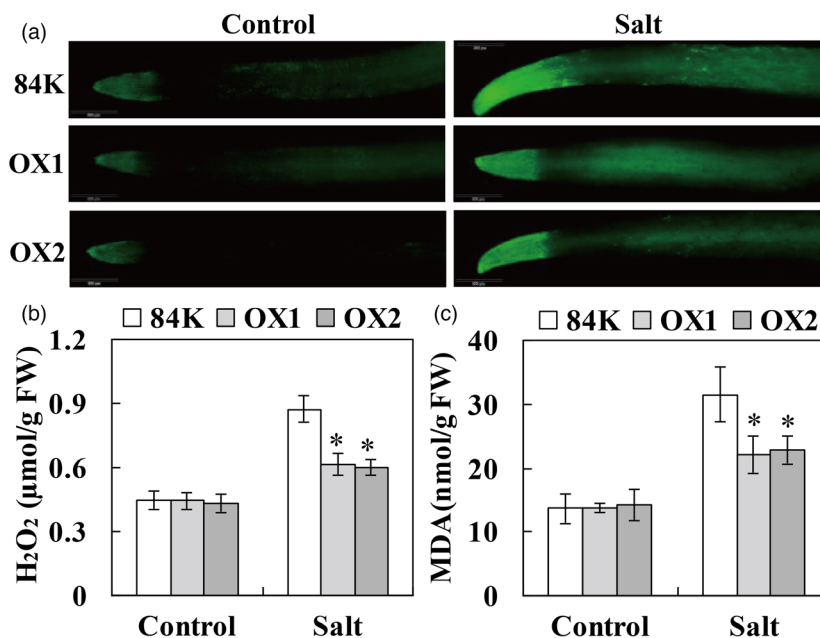


Figure 7 Overexpression of *PagCYP736A12* decreased oxidative damage levels under salt stress. (a) Representative DCFH-DA staining to detect H₂O₂ in the root tips of non-transgenic 84K and transgenic plants. (b, c) H₂O₂ and MDA contents in the fresh roots of non-transgenic 84K and transgenic plants. The cutting-propagated non-transgenic 84K and transgenic poplar lines overexpressing *PagCYP736A12* (OX1 and OX2) were cultivated and salinity treated with 0 and 75 mM NaCl as described in Figure 2. At least 20 roots were collected from three biological replicates (more than 10 individuals each) for DCFH-DA staining and analysis of H₂O₂ and MDA content. FW, fresh weight. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD (n = 6) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants (*P < 0.05).

research is needed to assess the performance of *WOX11/12* transgenic poplars in saline regions.

Experimental procedures

Plant materials and stress treatments

Hybrid poplar '84K' (*Populus alba* \times *P. glandulosa* cv.) was used for gene cloning, expression analysis and genetic transformation. The generation of transgenic 84K poplars expressing the *GUS* gene under the control of the *PagWOX11/12a* promoter (*Pro_{PagWOX11/12a}::GUS*), overexpressing (OE) and dominant repressors (DR) of *PagWOX11/12a* driven by the CaMV 35S promoter were described in our previous study (Wang et al., 2020a). *Pro_{PagWOX11/12a}::GUS* transgenic poplar plants were used for the spatial expression analysis of *PagWOX11/12a*. Two representative OE transgenic lines (OE1 and OE3) with high *PagWOX11/12a* expression levels and two DR transgenic lines (DR7 and DR9) with low expression levels were used for further functional analysis. Tissue cultures of transgenic and non-transgenic 84K poplars were grown on 1/2 MS solid medium (pH 5.8–6.0) in a phytotron (25 °C, 16/8 h light/dark and 50 μ m²/s light intensity). Seeds of *Nicotiana benthamiana* were surface-sterilized and sown onto 1/2 MS solid medium. One-week-old seedlings were transplanted into pots filled with soil and perlite (3:1) in a growth chamber at 22 °C with a 16/8 h light/dark photoperiod and 70%–75% relative humidity. They were grown for 20 days and then used for luciferase reporter assays.

To analyse *PagWOX11/12a* gene expression under salt stress, 4-week-old non-transgenic 84K and *Pro_{PagWOX11/12a}::GUS* poplar

plants grown on 1/2 MS solid medium were subjected to a hydroponic solution of 200 mM NaCl for 0, 6, 9, 12 and 24 h for salt treatment or to fresh hydroponic solution as a control. Root tissues from 10 individuals were collected at each indicated time point, transferred to the staining solution and frozen immediately in liquid nitrogen for expression analyses. The treatment experiments were repeated three times.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR) analysis

The procedures for RNA isolation, quality examination, first-strand cDNA synthesis and RT-qPCR assays were identical to those used in our previous study (Wang et al., 2020a). In brief, total RNA was isolated from the samples using the RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany) and was used to synthesize cDNA using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). qPCR was performed using the SYBR Premix Ex Taq™ Kit (TaKaRa) with a Light Cycler 480 (Roche Applied Science, Penzberg, Germany). Relative gene expression was calculated using the 2^{- $\Delta\Delta$ CT} method (Livak and Schmittgen, 2001) with the reference gene *PagActin* as an internal standard. Three biological replicates with four technical replicates were performed for each sample. All primers used for RT-qPCR are listed in Table S1.

GUS staining and activity analyses

To detect salt-induced expression, 4-week-old *Pro_{PagWOX11/12a}::GUS* plants were subjected to salt treatment (200 mM NaCl) for 0, 6 and 9 h. At least 20 roots were harvested from three biological replicates (more than 10 individuals each) for histochemical

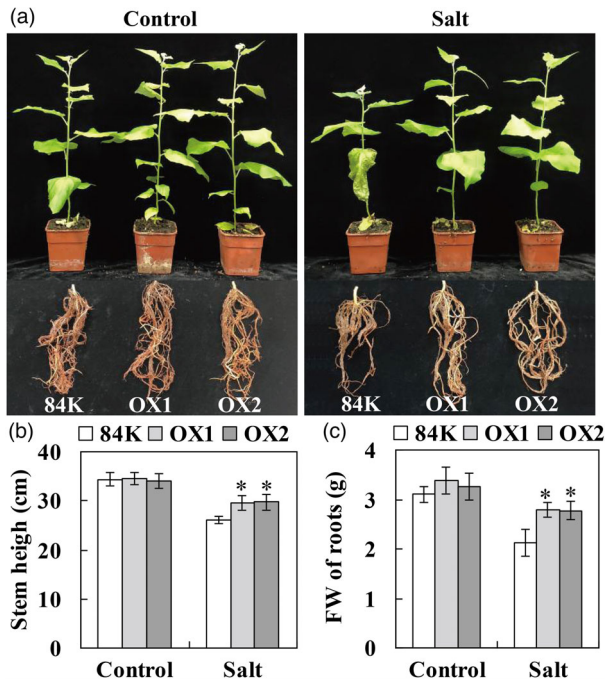


Figure 8 Enhanced salt tolerance in transgenic poplars overexpressing *PagCYP736A12*. (a) Shoot and root phenotypes of 84K and transgenic (OX) plants. (b, c) Plant height and root fresh weights (FW) of 84K and OX line plants. Four-week-old non-transgenic 84K and transgenic (OX1 and OX2) poplar plants were cultivated and salinity (200 mM NaCl) for 10 days as described in Figure 3. The plant height and root FW of individual plant were measured. The experiments were performed three times and the similar results were obtained. The bars represent means \pm SD ($n = 10$) and the asterisks indicate significant differences between non-transgenic 84K and transgenic plants (* $P < 0.05$).

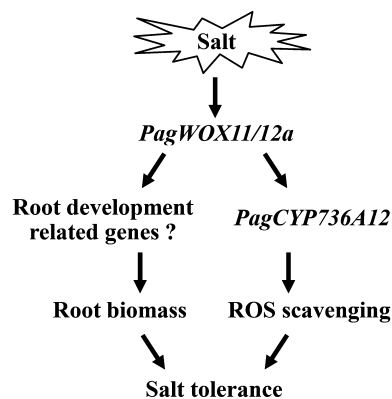


Figure 9 Proposed model of *PagWOX11/12a* gene involved in tolerance to salt stress. *PagWOX11/12a* is induced by salt stress then activates both the rooting-related genes to promote root biomass and *PagCYP736A12* to increase ROS scavenging capacity, which results in salt tolerance of poplar plants.

staining and GUS activity assay. GUS staining was performed as previously described by Liu *et al.* (2014a) GUS activity was quantitatively measured using spectrophotometry (Jefferson *et al.*, 1987). The experiments were repeated three times.

Genetic transformation of poplar

The full-length *PagCYP736A12* cDNA was cloned into the pMDC32 binary vector driven by the CaMV 35S promoter, introduced into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19) according to Wang *et al.* (2020a) and then transformed into 84K poplar using the *Agrobacterium*-mediated leaf disc method following the protocol described by Liu *et al.* (2014a) Putative transgenic plants were selected on 1/2 MS medium supplemented with hygromycin (3 mg/L) and verified by RT-qPCR. The details of the primers used are shown in Table S1.

Analysis of salt stress tolerance

To examine the rooting ability under salt stress, shoot segments of transgenic lines (OE and DR) and non-transgenic 84K poplar plants (approximately 3 cm in length with 2–3 young leaves) were cut from sterilized plants and cultivated on 1/2 MS solid medium supplemented with 0, 50, 75 and 100 mM NaCl for 20 days. The phenotypes of the root systems were photographed and root biomass was quantified. To evaluate salt stress tolerance, 4-week-old non-transgenic 84K and transgenic (OE and DR) plants grown on 1/2 MS solid medium were transplanted to plastic pots with a mixture of soil and perlite, grown in a greenhouse for 20 days and subsequently irrigated with 0 (control) or 200 mM NaCl (salt treatment) solution every 2 days for 20 days. Plant height was measured periodically after the beginning of the salt treatment. After 20 days of culture, the growth rate was calculated, the roots and aerial parts were collected, the FW was measured, and the DW was determined after incubation in an oven at 80 °C for 72 h. The experiments *in vitro* and soil conditions were performed three times, and at least 10 plants were used for each genotype per experiment.

Histochemical staining, physiological indices and ion analysis

To detect ROS levels, DCFH-DA (Sigma-Aldrich, St Louis) was used for H_2O_2 detection in the root tips of non-transgenic 84K and transgenic poplar plants cultivated on 1/2 MS solid medium supplemented with 0 and 75 mM NaCl for 20 days. The confocal images were visualized by a fluorescence microscope (Olympus Bx51, Japan) as previously described (Shi *et al.*, 2019). At least 20 roots were harvested from three biological replicates (more than 10 individuals each) for DCFH-DA staining.

The H_2O_2 and MDA contents in the fresh roots were measured using customized kits (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) following the manufacturer's instructions. The content of AsA and GSH, and the antioxidant enzyme activity levels of APX, GR, CAT and SOD were evaluated using the corresponding assay kits (Suzhou Keming Biotechnology Co. Ltd., Suzhou, China). The Na^+ and K^+ content was measured using atomic absorption spectroscopy (TAS990, Persee, Beijing, China). These experiments were performed three times.

RNA-seq analysis

Transgenic OE (OE1 and OE3), DR (DR7 and DR9) and non-transgenic 84K poplar plants were cut from sterilized plants and cultivated on 1/2 MS solid medium supplemented with 0 (control) and 75 mM NaCl (salt treatment) for 20 days. Root tissue samples were collected from 10 individuals for each of the three biological replicates and used for RNA-seq analysis. Total RNA isolation, quality examination, library construction, sequencing, gene annotation and DEG analysis were performed by BioMarker

Technologies. Gene function annotation, including Gene Ontology, eggNOG, Kyoto Encyclopedia of Genes and Genomes and Swiss-Prot, was carried out using the 84K poplar genome database (<https://doi.org/10.6084/m9.figshare.12369209>) as previously described (Huang *et al.*, 2021). DEGs were defined by a fold change ≥ 2 with a false discovery rate (P -value) < 0.05 . Finally, to verify candidate genes from the RNA-seq analysis, RT-qPCR was performed on roots from transgenic and 84K plants, prepared separately from the RNA-seq sample. All primer sequences used are described in Table S1.

Y1H assay

The full-length coding sequence (CDS) of *PagWOX11/12a* was inserted into the pGADT7-Rec2 vector (Clontech) to generate the effector construct pGAD-PagWOX11/12a. Three tandem copies of the WOX11-binding motif 'TTAATGC' and the *PagCYP736A12* promoter fragment upstream of the transcription starting sites (spanned from -1 to -1000) were PCR amplified and inserted into the pHIS2 vector (Clontech) to generate recombinant reporter constructs. The effector and reporter constructs were co-transformed into yeast Y187 strain cells using a Matchmaker™ Yeast One-Hybrid System (Clontech) and then placed on SD/–Leu/–Trp (DDO) and SD–Trp/–Leu/–His (TDO) media containing 30 mM 3-AT. The Y1H assay was conducted as previously described (Wang *et al.*, 2020a). The pGAD-PagWOX11/12a and p53HIS2 were used as negative control. The pGAD-Rec2-53 and p53HIS2 were used as positive control. The experiments were performed in triplicates. All primers used are shown in Table S1.

ChIP assay

The procedures for vector construction, plant transient transformation and ChIP-qPCR were identical to those used in previous studies (Ji *et al.*, 2014; Li *et al.*, 2014; Wang *et al.*, 2020a). In brief, the CDS of *PagWOX11/12a* was inserted into the pBI121 vector containing the *GFP* reporter gene to generate a recombinant construct (*35S::PagWOX11/12a-GFP*) and introduced into *A. tumefaciens* strain GV3101 for 84K poplar transient transformation. Transiently transgenic poplars were grown on 1/2 MS solid medium under normal conditions for 48 h or cultured on 1/2 MS medium for 36 h, and then subjected to 200 mM NaCl for 12 h. The roots of the transgenic plants were used for the ChIP assay. The sonicated chromatin was immunoprecipitated with an anti-GFP antibody (ChIP+) or immunoprecipitated without an anti-GFP antibody (Mock). qPCR was used to study the fold enrichment of the studied promoter fragments and was performed with the promoter fragment of the *PagActin* gene as an internal control. The PCR products were detected by 1.2% agarose gel electrophoresis. The experiments were performed three times. All primers used are shown in Table S1.

Dual-luciferase reporter assay

The full-length open reading frame of *PagWOX11/12a* was cloned into the pGreenII 62-SK effector vector, and the promoter of *PagCYP736A12* (1000 bp) used in the Y1H assay was cloned into the pGreenII 0800-LUC reporter vector. The recombinant plasmids and negative control vectors were introduced into *A. tumefaciens* GV3101 (pSoup-p19). The effector and reporter vectors were co-transformed into *N. benthamiana* leaves, as previously described (Yao *et al.*, 2020). D-Luciferin (10 μ M) was sprayed onto tobacco leaves and then photographed using an LB985 NightSHADE fluorescence imaging system (Berthold Technologies, Bad Wildbad, Germany). Dual-luciferase (LUC) activity

was determined using a GloMax 20/20 luminometer (Promega, Madison, WI) and a Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions. The experiments were performed at least three times with six technical repeats. The primers used are listed in Table S1.

Statistical analysis

The experimental data were presented as the mean \pm standard deviation (SD) and were subjected to inferential statistical analysis using Student's *t*-test. Significant differences between the two groups of data were evaluated for comparisons ($*P < 0.05$).

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Screening *PagCYP736A12* gene from transcriptome data

Figure S2 Promoter sequence of the *PagCYP736A12* gene

Figure S3 Sequence alignment and phylogenetic analysis of *PagCYP736A12* and other plant CYP736A12 proteins

Figure S4 Na⁺ and K⁺ content in the roots of 84K and transgenic poplar plants

Figure S5 RT-qPCR analysis of the expression of *PagWOX11/12a* and *PagCYP736A12* in 84K and transgenic poplars

Figure S6 RT-qPCR analysis of *PagCYP736A12*-transformed poplars

Table S1 Primer sequences used in this study.

Table S2 Up-regulated DEGs ($n = 236$) in comparisons between 'OE vs 84K' under control growth conditions and 'OE vs 84K' under salt conditions

Table S3 Down-regulated DEGs ($n = 223$) in comparisons between 'DR vs 84K' under control growth conditions and 'DR vs 84K' under salt conditions.

Table S4 DEGs ($n = 17$) in both sets of data listed in Table S2 and S3.