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Research article

Enhanced expression of *EsWAX1* improves drought tolerance with increased accumulation of cuticular wax and ascorbic acid in transgenic *Arabidopsis*

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ABSTRACT

Drought can activate several stress responses in plants, such as stomatal closure, accumulation of cuticular wax and ascorbic acid (AsA), which have been correlated with improvement of drought tolerance. In this study, a novel MYB gene, designed as *EsWAX1*, was isolated and characterized from *Eutrema salsugineum*. *EsWAX1* contained a full-length open reading frame (ORF) of 1068 bp, which encoding 355 amino acids. Transcript levels of *EsWAX1* were quickly inducible by drought stress and ABA treatment, indicating that *EsWAX1* may act as a positive regulator in response to drought stress. Ectopic expression of *EsWAX1* increased accumulation of cuticular wax via modulating the expression of several wax-related genes, such as *CER1*, *KCS2* and *KCR1*. Scanning electron microscopy further revealed higher densities of wax crystalline structures on the adaxial surfaces of leaves in transgenic *Arabidopsis* plants. In addition, the expression of several AsA biosynthetic genes (*VTC1*, *GLDH* and *MIOX4*) was significantly up-regulated in *EsWAX1*-overexpressing lines and these transgenic plants have approximately 23–27% more total AsA content than WT plants. However, the high-level expression of *EsWAX1* severely disrupted plant normal growth and development. To reduce negative effects of *EsWAX1* over-expression on plant growth, we generated transgenic *Arabidopsis* plants expressing *EsWAX1* driven by the stress-inducible *RD29A* promoter. Our data indicated the *RD29A::EsWAX1* transgenic plants had greater tolerance to drought stress than wild-type plants. Taken together, the *EsWAX1* gene is a potential regulator that may be utilized to improve plant drought tolerance by genetic manipulation.

1. Introduction

Drought is one of the most serious problems for sustainable agriculture worldwide. For productive and sustainable agriculture, it is important to improve drought stress tolerance by genetic engineering. Previous studies have shown that over-expression of a few MYB transcription factors can improve tolerance to drought stress in plants. MYB transcription factors have a conserved MYB DNA-binding domain (MYB domain), which contain up to three imperfect repeats (named sequentially as R1, R2 and R3, respectively). According to the number of peptide repeats in the MYB domain, the MYB family is categorized into 1R-MYB, 2R-MYB, 3R-MYB and 4R-MYB, respectively (Jin and Martin, 1999). Among the MYB proteins in plants, the MYB family with the two-repeat (R2R3) is the most common one, which have been demonstrated to involve in the regulation of secondary metabolism, control of cellular morphogenesis and plant stress responses (Abe et al., 2003; Ding et al., 2009; Lippold et al., 2009; Seo et al., 2011).

In *Arabidopsis*, *AtMYB2* can be apparently induced by drought stress and functions as a transcriptional activator in abscisic acid (ABA)-mediated gene expression (Abe et al., 2003). The expression of *MYB15* is up-regulated by drought stress, and its over-expression has been shown to improve drought tolerance in transgenic plants by increasing the expression levels of the genes involved in ABA

Abbreviations: EsWAX1-OX, EsWAX1 over-expressing lines; TFs, transcription factors; WT, wild type; ABA, abscisic acid; IAA, indole-3-acetic acid; mJA, methyl jasmonate; KT, kinetin; ACC, 1-aminocyclopropane-1-carboxylic acid; AsA, ascorbate acid; SOD, superoxide dismutase; APX, ascorbate peroxidase; POD, peroxidase; CAT, catalase; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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biosynthesis and signaling (Ding et al., 2009). The *AtMYB41* transcripts can be rapidly increased under various abiotic stresses and *AtMYB44* over-expression confers drought tolerance in *Arabidopsis* by reducing the expression of genes encoding PP2Cs, which have been described as negative regulators of ABA signaling (Lippold et al., 2009). It has recently been shown that over-expression of *AtMYB96* confers drought resistance in transgenic plants via modulating cuticular wax biosynthesis (Seo et al., 2011). In higher plants, cuticular wax forms a hydrophobic layer covering aerial organs, which is deposited either outside of the cuticle (epicuticular wax), or within the cuticular matrix (intracuticular wax). In addition to repelling atmospheric water, epidermal wax provides a protective barrier between the plant and its environment, which functions as a barrier to water loss (Riederer and Schreiber, 2001; Kerstiens, 1996). It has been well documented that drought stress significantly increases cuticular wax accumulation in plants (Kosma et al., 2009). Seo et al. reported that cuticular wax accumulation of plants treated with drought stress showed 3–4 folds higher than that of plants grown under well water conditions (Seo et al., 2011). The similar phenomenon has also been observed in other plant species, such as *Pinus pinaster* and *Eutrema salsugineum*, suggesting that cuticular wax accumulation is closely associated with drought tolerance responses (LeProvost et al., 2013; Xu et al., 2013).

Regulatory mechanisms of drought tolerance have been extensively described (Yamaguchi-Shinozaki and Shinozaki, 2006), including regulation of transcription, functional protection of proteins (such as dehydrins and heat shock proteins), accumulation of osmolytes (proline, glycine betaine, trehalose, mannitol, *myo*-inositol) and induction of ascorbic acid. Among these osmolytes, *myo*-inositol has been shown to be involved in the synthesis of ascorbic acid (AsA) and as a precursor of AsA biosynthesis. It has previously been reported that *myo*-inositol oxygenase (MIOX) is a key monooxygenase which catalyzes the conversion of *myo*-inositol into D-glucuronic acid (D-GlcUA). MIOX proteins have highly conserved across almost all eukaryotes. In animals, MIOX degrades *myo*-inositol into D-GlcUA and then was reduced to L-gulonic acid and ring formation to gulonolactone which is finally oxidized to AsA (Brown et al., 2006). In plants, MIOX has also been shown to catalyze *myo*-inositol to D-GlcUA, which is involved in AsA biosynthesis (Lorence et al., 2004).

Recently, *E. salsugineum* has became an important model plant for studying abiotic stress, which shares similar sequence identity with *Arabidopsis thaliana* (Inan et al., 2004). *E. Salsugineum* is more drought, and cold tolerance than *A. thaliana*. Under normal conditions, there exist significant differences of morphological characteristics between *A. thaliana* and *E. Salsugineum* and the latter exhibits smaller, narrower and waxy leaves. The higher wax level may play a critical role in limiting transpirational water loss across the plant surface. Previous studies have shown that genetic engineering with genes involved in wax biosynthesis can improve tolerance of plants to drought stress (Aharoni et al., 2004; Zhang et al., 2005). A novel R2R3-MYB transcription factor *EsWAX1* showed higher-level expression in expressed sequence tags (ESTs) library generated from abotic stress-treated *E. Salsugineum* plants (Taji et al., 2008), however the researches related to the MYB transcription factors in *E. Salsugineum* were rarely available.

Here, we reported the isolation of the *EsWAX1* gene from *E. Salsugineum*. Ectopic expression of *EsWAX1* significantly increased accumulation of cuticular wax and AsA content in transgenic *Arabidopsis* plants, however high-level expression of *EsWAX1* severely affect plant normal growth and development. Our findings further shown that the *EsWAX1* gene, under control of the *Arabidopsis RD29A* promoter, could be used to generate transgenic plants with improved drought tolerance without impacting plant growth and development.

2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana (Columbia ecotype) and *E. salsugineum* (Shandong ecotype) were used in this study. Plants were grown in greenhouse at 22 °C with 16 h light/8 h dark photoperiod and a relative humidity at approximately 80%. To shorten the flowering time, 7-week-old *E. salsugineum* plants were transferred from 22 °C to a 4 °C cold room for 6 weeks for vernalization.

2.2. Treatments with different exogenous hormones and drought stresses

3-week-old *E. salsugineum* plants grown on MS medium containing 0.8% agar were transferred to MS agar medium supplemented with various exogenous hormones, including 10 μM indole-3-acetic acid (IAA), 10 μM abscisic acid (ABA), 10 μM kinetin (KT), 20 μM methyl jasmonate (mJA), 20 μM 1-aminocyclopropane-1-carboxylic acid (ACC) for the indicated time periods. For analyzing the effects of cold and drought stress on the expression of *EsWAX1*, 3-week-old *E. salsugineum* plants grown on MS agar medium were treated for the indicated time periods according to a previously described method by Seo et al. (2011).

To assess drought tolerance of *RD29A*::*EsWAX1* transgenic *Arabidopsis* plants, the drought stress treatments were performed. 5-week-old wild type (WT) and *RD29A*::*EsWAX1* transgenic plants were firstly grown in soil under well water conditions. These plants were deprived of water for 14 d and then rewatered once. The survival rate recorded 10 d after being rewatered.

2.3. Plasmid construction and plant transformation

Total RNA was isolated from 5-week-old *E. salsugineum* plants using the TRIzol reagent (Invitrogen, USA). Residual genomic DNA was removed by RNase-free DNase I (Invitrogen, USA) treatment. A gene-specific primer pair was designed to amplify the coding region of *EsWAX1* based on the sequence from *E. salsugineum* (GenBank accession no. BAJ34253). The coding region of the *EsWAX1* gene from *E. salsugineum* cDNA was amplified with gene specific primers and was cloned into pGEM-T vector (Promega, USA) for sequencing to verify its integrity. The amplification condition was followed by 94 °C for 5 min, 56 °C for 1 min, 72 °C for 7 min (35 cycles in total) and finally 72 °C for 10 min. The PCR product was cloned into pGEM-T vector (Promega, USA) for sequencing.

For the over-expression of *EsWAX1*, the complete coding sequence of *EsWAX1* was digested with XbaI and SacI, and the digested fragment was inserted into plant binary vector containing a super-promoter consists of a trimer of the octopine synthase (OCS) transcriptional activating element affixed to the mannopine 2 synthase 2′ (mas2′) transcriptional activating element (Lee et al., 2007). To create a construct containing *RD29A*::*EsWAX1* cDNA, the sequence of RD29A promoter from *Arabidopsis* was firstly amplified. The PCR products were cloned into pGEM-T vector (Promega, USA) for sequencing and then digested with HindIII and NcoI. The digested fragment was inserted into plant binary vector pCMBIA1304. Secondly, the *EsWAX1* cDNA was inserted in sense orientation into the NcoI site of the pCMBIA1304-RD29A vector.

The constructed plasmids were transferred into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* plants by the floral dip method (Clough and Bent 1998). These seeds were collected and grown on MS agar medium containing 50 mg/l hygromycinto to screen transgenic plants. Transformants were identified as hygromycin-resistant and verified by PCR. The fragment of