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AcEBP1, an ErbB3-Binding Protein (EBP1) from halophyte *Atriplex canescens*, negatively regulates cell growth and stress responses in *Arabidopsis*

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ABSTRACT

An ErbB-3-binding protein gene *AcEBP*1, also known as proliferation-associated 2G4 gene (*PA2G4s*) belonging to the M24 superfamily, was obtained from the saltbush *Atriplex canescens*. Subcellular localization imaging showed the fusion protein AcEBP1-eGFP was located in the nucleus of epidermal cells in *Nicotiana benthamiana*. The *AcEBP1* gene expression levels were up-regulated under salt, osmotic stress, and hormones treatment as revealed by qRT-PCR. Overexpression of *AcEBP1* in *Arabidopsis* demonstrated that *AcEBP1* was involved in root cell growth and stress responses (NaCl, osmotic stress, ABA, low temperature, and drought). These phenotypic data were correlated with the expression patterns of stress responsive genes and PR genes. The *AcEBP1* transgenic *Arabidopsis* plants also displayed increased sensitivity under low temperature and evaluated resistance to drought stress. Together, these results demonstrate that AcEBP1 negatively affects cell growth and is a regulator under stress conditions.

1. Introduction

A number of environmental stress factors, such as drought, salinity, extreme temperatures, low or high light intensity, deficiency or toxic levels of nutrients, and diseases, have significant impacts on enzyme activities and gene expression in crop plants, leading to considerable reduction in their growth and productivity [1]. Morphogenesis in plants is largely postembryonic, and along with organ growth is influenced by environmental factors [2]. Plants, as sessile organisms, have evolved exquisite mechanisms to adapt to abiotic stresses, such as drought, high salt, and extreme temperatures [3]. The capacity for growth of plant organs is determined by zones of proliferating cells in meristems. Their growth and proliferation require coordination with other parts of the organism mediated by growth and mitogen factors that may impinge on growth regulation (ribosome biogenesis) or cell cycle control (e.g., DNA replication) [4].

ErbB-3-binding protein 1 (EBP1), also known as proliferation-associated 2G4 proteins (PA2G4s), is an RNA-binding protein belonging to the peptidase M24 family. The protein plays a role in cellular growth and differentiation [5]. EBP1 was originally identified as an ErbB3 receptor-binding protein [6], and exhibits significant homology to the cell cycle-regulated murine protein p38-2G4 [7]. EBP1 is also known to interact with a number of proteins and RNAs involved in either transcriptional regulation or translational control and is a striking example for the adaptation of a conserved enzyme fold to a multi-functional binding platform [8]. Retinoblastoma gene protein Rb, the binding target for EBP1, is an important modulator of cell cycle progression and cellular differentiation in inhibiting E2F1-mediated transcription [9,10]. PKC also plays a role in regulating phosphorylation and function of EBP1 *in vivo* [11] and its two distinctive isoforms (p48 and p42) might regulate cell survival and differentiation [12].

In yeast, the control of cell growth was investigated that attainment of a cell size threshold triggers initiation of cell division, which was probably regulated through nutrition determined translational machinery [13]. In human cells, the HsEBP1 protein was shown to be part of ribonucleoprotein complexes binding to rRNA precursors, small nucleolar RNA species in the nucleoli, and mature ribosomal RNAs. The nucleolar localization of this protein is linked to its ability to inhibit growth [14]. HsEBP1 was also suggested to regulate the production and assembly of translational machinery

Abbreviations: PCR, polymerase chain reaction; eGFP, enhanced green fluorescent protein; cDNA, complementary DNA; MS, Murashige and Skoog; OE, overexpressor; WT, wild-type; VC, vector control; EF1α, elongation factor 1-alpha; FW, fresh weight; DW, dry weight; WC, water contents; ROS, reactive oxygen species; SOD, superoxide dismutase; POP, peroxidase; MeJA, methyl jasmonate; ET, ethylene; Chl, chlorophyll; ABA, abscisic acid; NBT, nitroblue tetrazolium; PEG6000, polyethylene glycol 6000; NME, N-terminal Met excision; PKC, Protein kinase C.

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and to regulate translation in response to cellular stresses [15]. *EBP1* was up-regulated in the sciatic nerve after crush, which was involved in the differentiation and migration of Schwann cells [16]. To date, molecular analyses of cell division and its regulation in plants are sparse. Their mechanisms in plants are more complicated than those in animals, partly because plants are immobile and have to be highly responsive to the ever-changing environments [17]. In plant cells, EBP1 nucleolar localization and the interaction with ribosome biogenesis factors suggest the conserved function for EBP1 in the regulation of protein synthesis and cell growth. In potato and Arabidopsis, EBP1 regulates plant organ growth mainly due to increased cell numbers, affects the expression of different cell cycle genes and influences RBR1 protein level, suggesting that EBP1 functions primarily to promote growth by proliferation, possibly via stimulation of ribosome biogenesis and protein translation [4]. This wide range of activities raises the interesting possibility that plant EBP1 genes are involved in promoting phase I growth (by promoting cell growth), as well as phase II growth (by regulating E2F activity) [18]. However, the direct influence of EBP1 on cell growth and the phase I/II switch are not yet known. In addition, plant EBP1 also associates with mature ribosomes to sustain protein translation during stressful conditions, suggesting that also in plants ribosome biogenesis and translational control regulate growth [19].

Despite its importance in regulating cell growth, many studies on EBP1 mainly focus on animals. Only a handful of genes that have an impact on plant growth or fruit size are known [4]. Studies on EBP1 of woody halophytic plants are sparse. Therefore, identification and characterization of EBP1 genes controlling plant body size, development, growth kinetics, and growth habits are of importance to understand the mechanisms controlling the basic growth in plants. Unveiling mechanisms of how EBP1 in halophytic plants regulates organ growth and plant cell size will advance our knowledge in this field. Moreover, the behavior of EBP1 in response to abiotic stresses is also largely unknown.

The elite halophytes in the genus *Atriplex* (Chenopodiaceae) has been extensively employed in physiological and molecular investigations to explore stress-related novel genes [20]. The fourwing saltbush (*Atriplex canescens*) has been commonly recommended as excellent phytoremediation plant in the lands contaminated by saline-alkali or heavy metals [21]. Tolerance to salinity, drought, heavy metals, and low temperature makes *A. canescens* a source for exploring exclusive genes or new genetic mechanisms that could be applied for genetic manipulation of crops [21]. At the molecular level, identification of stress-responsive genes is an initial step toward understanding plant stress tolerance. Previously, we investigated the mechanisms of salt stress tolerance in *A. canescens*, and we generated the full-length cDNA library of *A. canescens* treated with NaCl (400 mM) [20]. We have now sequenced additional cDNA library colonies and obtained a new stress-responsive gene *AcEBP1* coding for an ErbB-3-binding protein belonging to M24 superfamily. Its expression patterns in response to osmotic stress, high salinity, low temperature, and hormones were determined by qRT-PCR. Transient expression of an AcEBP1-eGFP fusion protein was utilized to investigate the subcellular localization in *Nicotiana benthamiana*. Furthermore, transgenic *Arabidopsis* plants by overexpressing *AcEBP1* were generated to explore its role in controlling plant cell growth and in response to abiotic stresses in *Arabidopsis*.

2. Material and methods

2.1. Plant materials and growth conditions

Nicotiana benthamiana used for transient expression was grown in the greenhouse under following controlled environmental conditions: 21–23 °C, 100 μmol photons m^{-2} s^{-1}, 60% relative humidity, and 16 h light/8 h dark [22]. *Arabidopsis thaliana* ecotype Columbia (Col-0) was used for overexpression experiments in this study. Seeds of wild type and transgenic plants were surface-sterilized with 10% NaClO and washed three times with deionized distilled water (ddH_2O). Sterile seeds were suspended in 0.1% agarose and plated on Murashige and Skoog (MS) medium with 3% sucrose. Plates were stratified in darkness for two days at 4 °C and then transferred to a chamber with the similar environmental conditions as described above for seven days. Seedlings were then potted in soil mix (vermiculite: humus = 1:2) and placed in a tissue culture room with the similar environmental conditions as above.

A. canescens plants used for qRT-PCR analysis were grown in controlled environmental conditions as described above. Seedlings were grown hydroponically in 1/2 strength Hoagland solution with pH 6.0 for 50 days, and the seedlings were treated with various types of abiotic stresses.

2.2. Cloning and sequence analysis of AcEBP1 gene from A. canescens

The full-length cDNA sequence of *AcEBP1* was cloned from the cDNA library of *A. canescens* based on EST sequencing and analysis [20]. The server of the BLASTX program at NCBI (http://www.ncbi.nlm.nih.gov/) was employed to search for the homologs of the sequence of *AcEBP1*. Sequence alignments of AcEBP1 with EBP1 proteins from other plants and animals were completed by using DNAMAN 6.0 [23]. Prediction of physical and chemical parameters was done using the ProtParam tool (http://web.expasy.org/protparam/). The phylogenetic tree was generated using neighbor-joining method in MEGA5 [24]. Prediction of protein localization was performed by PSORT Prediction (http://psort.hgc.jp/form.html).

2.3. AcEBP1-eGFP subcellular localization

To investigate subcellular localization of AcEBP1, its coding sequence with no stop codon was amplified using two oligonucleotide primers (AcEBP1-pCHF3300-F and AcEBP1-pCG-3300-R, Supplementary Appendix Table A.1). The PCR product was ligated into the pCG3300 vector (GenBank No. KF206145) and resulted in pCG3300-*AcEBP1* construct with an *AcEBP1-GFP* fusion fragment under the control of cauliflower mosaic virus (CaMV) 35S promoter [25]. The construct was further confirmed by sequencing. To perform transient expression, leaves from four-week-old *N. benthamiana* and were infiltrated with *Agrobacterium tumefaciens* strain EHA105 harboring pCG3300-*AcEBP1-eGFP*. The localization of AcEBP1-eGFP fusion protein in leaves was observed at 488 nm using a confocal laser scanning microscope (Olympus Fluoview FV1000) as previously reported [22]. The construct with eGFP alone (pCG3300) was used as the control.

2.4. Generation and identification of AcEBP1 in Arabidopsis

For overexpression of *AcEBP1*, PCR fragment was inserted into pCHF3300 (GenBank No. KF206142) and was driven by CaMV 35S promoter. The resultant construct was characterized by sequencing and then introduced into *A. tumefaciens* strain EHA105 to transform wild-type *Arabidopsis* (Col-0) by flower dipping. Seeds were germinated on MS medium supplemented with 3% sucrose, and for the selection of transgenic plants 8 mg/L basta was added to the medium. One-week-old plants were transferred to pots under conditions as described above until seeds were generated. Transgenic *Arabidopsis* plants were characterized by PCR with primer pairs AcEBP1-pCHF3300-F and AcEBP1-pCG-3300-R (Supplementary Appendix Table A.1). To select homozygous lines, seeds from