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Functional characterization of 1-aminocyclopropane-1-carboxylic acid oxidase gene in *Arabidopsis thaliana* and its potential in providing flood tolerance

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ABSTRACT

Ethylene is a phytohormone that has gained importance through its role in stress tolerance and fruit ripening. In our study we evaluated the functional potential of the enzyme involved in ethylene biosynthesis of plants called ACC (aminocyclopropane-1-carboxylic acid) oxidase which converts precursor ACC to ethylene. Studies on ethylene have proven that it is effective in improving the flood tolerance in plants. Thus our goal was to understand the potential of ACC oxidase gene overexpression in providing flood tolerance in transgenic plants. ACC oxidase gene was PCR amplified and inserted into the pBINmgfp5-er vector, under the control of a constitutive Cauliflower Mosaic Virus promoter. GV101 strain of *Agrobacterium tumefaciens* containing recombinant pBINmgfp5-er vector (referred herein as pBIN-ACC) was used for plant transformation by the 'floral dip' method. The transformants were identified through kanamycin selection and grown till T3 (third transgenic) generation. The flood tolerance was assessed by placing both control and transgenic plants on deep plastic trays filled with tap water that covered the soil surface. Our result shows that wild-type Arabidopsis could not survive more than 20 days under flooding while the transgenic lines survived 35 days, suggesting development of flood tolerance with overexpression of ACC oxidase. Further molecular studies should be done to elucidate the role and pathways of ACC oxidase and other phytohormones involved in the development of flood adaptation.

1. Introduction

Ethylene, one of the five main plant growth regulators, is the first identified gaseous hormone that is of major commercial and biological importance [1,2]. Apart from regulating growth and senescence in plants, ethylene also plays a major role in abiotic stress tolerances, which includes cold, drought and flood tolerance [3–5]. Ethylene is also found to be produced in large amounts when plants are under any pathogen attack or herbivore infestation [6]. It has been suggested that a strict control of ethylene biosynthesis is necessary for plant adaptation and survival during stress [7]. This process has 3 main steps that are catalyzed by various enzymes: (1) conversion of methionine to S-adenosyl-methionine (S − AdoMet) by S-Adomet synthetase; (2) conversion of S-AdoMet to ACC (1-aminocyclopropane-1-carboxylic acid) by ACC synthase and (3) final product ethylene produced from oxidation of ACC by ACC oxidase [7]. The second step also catalyzes the release of MTA (methylthioadenosine) as a by-product. MTA plays a major role in recycling amino acid methionine which helps the plants to sustain a high rate of ethylene synthesis [8].

Ethylene biosynthesis is regulated by different enzymes through different processes in plants. Some studies suggest that ACC synthase regulates the ethylene biosynthesis and certain studies demonstrate that ACC oxidase also plays a critical role in regulation of ethylene production [9]. Moreover, it is also known that ACC oxidase acts as a rate-limiting step for ethylene biosynthetic pathway [10,11]. Studies have also shown that the activity of ACC oxidase increases with increase in ethylene production during stress tolerance [12].

Our aim was to understand the potential of ACC oxidase gene in ethylene production with respect to development of flood tolerance in plants. Flood stress is a severe abiotic stress to which most plants

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List of abbreviations

GM	Genetically Modified
WT	Wild Type
ACC	aminocyclopropane-1-carboxylic acid
SEM	Scanning Electron Microscopy

are sensitive to [13]. The mechanism by which ethylene rescues the plants during flooding is through incorporation of flood adaptive traits such as increased adventitious roots formation, shoot hyponasty, shoot elongation and quiescence [14,15]. These traits help the plant with improved gaseous exchange, as flooding cuts down the oxygen supply to the plants. However, most of the plants are not able to synthesize enough ethylene to develop adaptations after being flooded. This is because the last step of ethylene synthesis from precursor ACC requires oxygen [14,15]. Thus during flooding, plants' cells get filled with ACC that is not converted to the desired product [7,15].

In *Rumex palustris*, it has been reported that the low oxygen concentration caused by floods affects the enzyme activity of ACC oxidase which in turn affects the ethylene production [9]. ACC oxidase enzyme needs more oxygen to get activated [16]. Thus our hypothesis is that overexpression of ACC oxidase gene can compensate the reduction in enzyme activity and create an elevation in the ethylene levels thereby rendering the plant flood tolerant. Transgenic plants over-producing ethylene can reduce the cost, pollution [17], potential risks and labor involved in pretreatment of plants with ethylene [18]. Moreover, these types of transgenic plants can also be cultivated in low-lying lands that are prone to floods [19]. This will improve the arable area for farming and therefore help us to address the situation of food shortage in the near future [19].

2. Material and methods

2.1. Plants

Arabidopsis thaliana seeds of ecotype Columbia (Col-0) were purchased from Lehle Seeds, USA. The seeds were surface sterilized following a standard protocol [20]. Few drops of PPM (Plant Preservative Mixture, Plant Cell technology, USA) was added after sterilization to prevent any fungal infections and shaken thoroughly. The PPM was then washed off with sterile water. Sterile seeds were then vernalized by placing them at +4 °C for 24 h. They were then planted on round plastic pots that contains Pro-Mix HP Mycorrhizae potting soil (Premier Tech Horticulture, USA) and Vermiculite (Good Earth Horticulture,Inc.,USA) at a ratio of 3:1 respectively. Two to three granules of Osmocote (The Scotts Co., USA) dry plant fertilizer was also added to the soil. The pots were transferred to a growth chamber set to room temperature (25 °C) with 16 h of light and 8 h of dark cycle. The plants were watered every other day with tap water. The plants were occasionally clipped to promote growth of axillary buds.

2.2. Recombinant vector construction and cloning

The recombinant vector was constructed through restriction enzyme-based cloning. The vector backbone pBINmgfp5-er [21] was used as the plant expression vector. The ACC Oxidase gene was PCR amplified from PUNI51 plasmid clone (clone name U13773; TAIR Accession 3599683) obtained from The Arabidopsis Information Resource, Ohio (www.arabidopsis.org) (Table 1). The Gene ID for the ACC oxidase gene insert in PUNI-51 is AF446874.1 (www.arabidopsis.org). The gene was inserted into pBINmgfp5-er vector cassette by removing the native jellyfish Green Fluorescent Protein gene (GFP) using restriction enzymes BamH1 and Sac1. The recombinant vector will be referred as pBIN-ACC in this paper. The vector also contains a selectable marker gene that codes neomycin phosphotransferase (nptII), which codes for kanamycin resistance. The vector was then inserted into competent *Escherichia coli* cells through standard heat shock transformation protocol [22]. GeneJet Plasmid Miniprep kit (Thermofisher Scientific,USA) was used to extract the recombinant plasmid from transformed *E. coli* cells. The plasmid was then electroporated into GV101 strain of *Agrobacterium tumefaciens*.

2.3. Transformation of Arabidopsis thaliana

Arabidopsis thaliana was transformed by *Agrobacterium*-mediated floral dip technique [23]. Two to three week old plants (T0-transgenic generation zero) that started to flower were chosen for transformation. Bolts were clipped to promote secondary shoot formation. The transformed plants were identified by growing the seeds on a selective medium that contains kanamycin at the concentration of (37.5 μg/ml). These plants were then transferred to pots filled with the potting soil (as described above). Plants were grown and selected on kanamycin plates till the T_3 generation.

2.4. PCR confirmation of recombinant plasmid

The presence of the recombinant plasmid containing the npt II gene was affirmed by PCR analysis. The PCR consisted of PCR Master Mix (Thermofisher Scientific, USA) (12.5 μl), Plant DNA (300 ng), 10uM NPT II primer pairs (each 1ul) (Table 1) and nuclease free water to a total volume of 25 ul. The amplification was carried out by MultiGene Optimax thermocycler (LabNet International, USA). The PCR conditions were: initial denaturation (95 °C for 3 min), followed by 25 cycles of denaturation (95 °C for 1 min), annealing at 57.6 °C for 30 s and extension (72 °C for 45 s) and a final extension (72 °C for 7 min). After the completion of the cycles, 25 ul of the samples were analyzed on 1% agarose gel containing SYBR Safe DNA staining dye (Thermofisher Scientific, USA) at a concentration of 0.1 ul/ml.

2.5. qPCR confirmation of transgene expression

Total RNA was extracted from the leaves of transgenic and wild

Table 1
Primers used in the study.

Primers	Sequence (5′-3′)	Source
PUNI-51 Forward	GAGAGGATCCCTGTTGGTGTGTCTATT	TAIR.org
PUNI-51 Reverse	GAGAGAGCTCTGGCTGGCAACTA GAA	TAIR.org
NPT II Forward	CACGACGGGCGTTCCTTGC	TaqMan® Plant Transgenic Screening NPTII Detection Kit (Thermofisher Scientific, USA).
NPT II Reverse	GGTGGTCGAATGGGCAGGTAGC	TaqMan® Plant Transgenic Screening NPTII Detection Kit (Thermofisher Scientific, USA).
ACTIN qPCR Forward	TGTACGACCACTGGCATAAAG	This work
ACTIN qPCR Reverse	CCAAGGCCAACAGAGAGAAA	This work
ACC qpcr Forward	TTTTGGGAAGAGGCTTGAGA	This work
ACC qpcr Reverse	GAAGCTGGAGACCACTGACC	This work

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