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Isolation and Expression Analysis of the Ethylene Receptor Gene *MiETR1b* in Mango (*Mangifera indica*)

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Abstract

A mango *ETHYLENE RESPONSE1* (*ETR1*) gene, designated *MiETR1b*, was isolated from the cotyledon of mango (*Mangifera indica* L. 'Zihua') using RT-PCR, and the 5′ and 3′ rapid amplification of cDNA ends. The full-length cDNA was 2 530 bp, with an open reading frame of 2 220 bp, and it encoded a putative protein of 739 amino acids. The genomic DNA sequence of *MiETR1b* was 4 116 bp in length, having a 3 305 bp sequence from the start to terminator codon, containing six exons and five introns. The deduced amino acids possessed conserved domains of the GAF and HATPase_c superfamilies. A phylogenetic tree analysis indicated that MiETR1b had the highest similarity to MiETR1 from *M. indica* and a high similarity to CsERS1, DlETR1, TcERS1 and PtETR1. Quantitative real-time PCR showed that *MiETR1b* was expressed in the proximal and distal cut surfaces throughout the adventitious root formation period. Meanwhile, the expression of *MiETR1b* in the distal cut surface was significantly up-regulated within 6–48 h. Pre-treatments with indole-3-butyric acid and 2,3,5-triiodobenzoic acid significantly down-regulated *MiETR1b* expression at 1 d and 6 h, respectively. However, more ethylene was produced from 12 to 24 h, while ethylene production decreased after 4 days of culturing. In conclusion, *MiETR1b* might play an important role during the adventitious root formation of mango cotyledon segments, which is related to ethylene production.

Keywords: *Mangifera indica*; *MiETR1b*; expression analysis; adventitious root

1. Introduction

Over 70% of propagation systems used in the horticultural industry depends on the successful rooting of cuttings (Davies et al., 1994; Rout et al., 2006); therefore, adventitious rooting is a prerequisite for the successful production of viable plant clones. However, many horticultural plants, especially woody plants, lack the physiological ability to produce adventitious roots, which presents a challenge for the horticultural industry (Pijut et al., 2011; Verstraeten et al., 2013). Thus, it is important to understand the formation mechanism of adventitious roots during the development of factory seedlings.

AUX and PIN proteins play important roles in the lateral root formation of plants (De Smet et al., 2006; Peret et al., 2009; Bellini et al., 2014), but the molecular mechanism of adventitious root formation remains unclear (Verstraeten et al., 2013; Bellini et al., 2014). Adventitious roots always develop from cells neighboring vascular tissues; however, the lack of early molecular markers makes the identification of the original cell(s) challenging (Bellini et al., 2014). Adventitious roots may form at any location in the plant other than the roots, and the formation is controlled by endogenous plant hormones and environmental stimulation. Auxins promote adventitious root formation (Pacurar et al., 2014b), while cytokinin inhibits their formation. Exogenous cytokinin suppresses adventitious root formation in segments, while mutants defective in cytokinin biosynthesis or perception display an increased production of adventitious roots (Cano et al., 2014). However, the role of ethylene in adventitious rooting remains controversial, with some reports indicating that ethylene promotes adventitious root formation (De Klerk et al., 1999; Bellini et al., 2014) and others indicating that ethylene inhibits adventitious root formation (Muday et al., 2015). Ethylene may interact

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with auxin, and a complex auxin-ethylene crosstalk involved in the control of adventitious root formation was also reported recently (Pacurar et al., 2014a). Thus, determining the molecular mechanism behind ethylene's role in adventitious rooting would be helpful for understanding rooting regulation.

In a previous study, we found that adventitious roots formed only at the proximal cut surface (PCS) of mango (*Mangifera indica* L. 'Zihua') cotyledon segments, whereas no roots were formed on the opposite, distal cut surface (DCS) or the longitudinal cut surface. During this process, no exogenous plant growth regulators were added. Therefore, it was a good system for studying adventitious root formation (Li et al., 2008). Ethylene was released when the plants were wounded (León et al., 2001). In the rooting system of our study, a mango cotyledon was cut into 2 cm long segments, creating two cut surfaces where ethylene might be released. Thus, we could determine whether ethylene is involved in adventitious root formation in mango cotyledon segments.

The first step of ethylene signaling is the binding of ethylene to ethylene receptors. In *Arabidopsis thaliana,* five ethylene receptor genes have been identified, including *ETHYLENE RESPONSE1* (*ETR1*), *ETR2*, *ETHYLENE RESPONSE SENSOR1* (*ERS1*), *ERS2* and *ETHYLENE INSENSITIVE4* (*EIN4*) (Chen et al., 2005; Kendrick and Chang, 2008). Based on a phylogenetic analysis, the ethylene receptors can be divided into two subfamilies that share structural features. Subfamily 1 contains ETR1 and ERS1, and subfamily 2 contains ETR2, ERS2 and EIN4 (Chen et al., 2005). Subfamily 1 receptors play a more important role during ethylene signaling (Hua and Meyerowitz, 1998; Wang et al., 2003; Qu et al., 2007).

In this study, we measured the release of ethylene during adventitious root formation in mango cotyledon segments and cloned the *MiETR1b* gene, which encodes ETR1. In addition, we studied the *MiETR1b* transcript levels at different time points, as determined by quantitative real-time PCR.

2. Materials and methods

2.1. Materials and the induction of adventitious roots

Mature mango (*M. indica* L. 'Zihua') fruits were collected at the South Subtropical Crop Research Institute (Zhanjiang, China), and adventitious root formation was induced using the method described by Li et al. (2008). In June–August 2013, embryos of similar size were selected after being dissected from mango fruits (a matured mango embryo was approximately 6–7 cm in length and 2–3 cm in width), rinsed with tap water for 1 h and surface-sterilized with 10% (v/v) commercial bleach for 10–15 min. After 3–5 washes in sterile distilled water, embryos were placed on sterile filter paper, and the cotyledons were cross-cut into 2-cm segments (the embryo axis was also excised and discarded), which were placed horizontally in a 150 mL Erlenmeyer flask containing 20 mL of 0.7% (w/v) autoclaved agar medium at pH 5.8 without plant growth regulators or sucrose (AM). The explants were cultured at (28 ± 2) °C in the dark for the indicated period.

Previous results showed that 492.1 μmol·L^{-1} indole-3-butyric acid (IBA) significantly increased adventitious root formation at the PCS of mango cotyledon segments, whereas 200 μmol·L^{-1} 2,3,5-triiodobenzoic acid (TIBA) completely inhibited root formation (Li et al., 2008). To investigate the influences of IBA and TIBA on *MiETR1b* expression, selected embryos were pre-treated with 492.1 μmol·L^{-1} IBA or 200 μmol·L^{-1} TIBA. Pre-treatments were performed on a shaker (60 r · min^{-1}) at (28 ± 2) °C in the dark.

After culturing on AM for 0 h, 6 h, 1, 2, 4, 7 or 10 days, samples (2 mm) were collected from the PCS and DCS of cotyledon segments and immediately frozen in liquid nitrogen. The samples were stored at – 80 °C until RNA extraction.

2.2. Cloning of the MiETR1b gene

LA *Taq*, TaKaRa Agarose Gel DNA Purification Kit Ver.4.0, PrimeScript II 1st Strand cDNA Synthesis Kit, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), dNTP mixture, competent *Escherichia coli* DH5α cells and the pMD18-T vector were all purchased from Takara Bio Inc., Dalian, China.

Total RNA was extracted from the PCS of 4-day-old mango cotyledon segments using the method of Xiao et al. (2003). The cDNA was synthesized from 2 μg of RNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Dalian, China) with Oligo dT as a primer, according to the manufacturer's instructions. This cDNA was used as a template to isolate DNA fragments by PCR, using a pair of degenerate primers (MiETRSP1 and MiETRSP2; Table 1) based on regions that were found to be highly conserved in 10 other ETR1s using

Table 1 Primer applications and sequences

Application	Primer name	Primer sequence (5′ – 3′)
DNA fragment amplification of *MiETR1b*	MiETRSP1	GCCCTGATGCTGGTGCAYATHATHCC
	MiETRSP2	GGTTCATCACGGCCARRAARTCRTT
First round PCR of 3′ RACE	MiETRSP3	TCGTGTTCCACTCCTGCATCTCTCAA
Second round PCR of 3′ RACE	MiETRSP4	AAACAGCCATTCATGCTCGCAAC
First round PCR of 5′ RACE	MiETRSP5	AGGAGTGGAACACGAACTGCAACCA
Second round PCR of 5′ RACE	MiETRSP6	ATGTCGCCCAGTTTCTTCCTGTGT
Amplification of full-length cDNA and genomic DNA of *MiETR1b*	MiETRSP7	TGGCAGCTGGCTCATCTGTGCTTAT
	MiETRSP8	GGGTCTCATGAATTGTTCACGGGCTTA
Primers for quantitative real-time PCR	MiETRSP9	TCAGGTGGCTGTTGCTCTTT
	MiETRSP10	ATCGTTGCGAGCATGAATGG
Actin for quantitative real-time PCR	MiETRSP11	CCACTGCTGAACGGGAAAT
	MiETRSP12	GTGATGGCTGGAAGAGGAC