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Cloning of an ADP-ribosylation factor gene from banana (*Musa acuminata*) and its expression patterns in postharvest ripening fruit

Yuan Wang[a,c], Jing Wu[a], Bi-Yu Xu[a], Ju-Hua Liu[a], Jian-Bin Zhang[a], Cai-Hong Jia[a], Zhi-Qiang Jin[a,b,*]

[a] *Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Hainan 571101, China*
[b] *Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Hainan 570101, China*
[c] *College of Agriculture, Hainan University, Hainan 570228, China*

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ABSTRACT

A full-length cDNA encoding an ADP-ribosylation factor (ARF) from banana (*Musa acuminata*) fruit was cloned and named *MaArf*. It contains an open reading frame encoding a 181-amino-acid polypeptide. Sequence analysis showed that MaArf shared high similarity with ARF of other plant species. The genomic sequence of *MaArf* was also obtained using polymerase chain reaction (PCR). Sequence analysis showed that *MaArf* was a split gene containing five exons and four introns in genomic DNA. Reverse-transcriptase PCR was used to analyze the spatial expression of *MaArf*. The results showed that *MaArf* was expressed in all the organs examined: root, rhizome, leaf, flower and fruit. Real-time quantitative PCR was used to explore expression patterns of *MaArf* in postharvest banana. There was differential expression of *MaArf* associated with ethylene biosynthesis. In naturally ripened banana, expression of *MaArf* was in accordance with ethylene biosynthesis. However, in 1-methylcyclopropene-treated banana, the expression of *MaArf* was inhibited and changed little. When treated with ethylene, *MaArf* expression in banana fruit significantly increased in accordance with ethylene biosynthesis; the peak of *MaArf* was 3 d after harvest, 11 d earlier than for naturally ripened banana fruits. These results suggest that *MaArf* is induced by ethylene in regulating postharvest banana ripening. Finally, subcellular localization assays showed the MaArf protein in the cytoplasm.

Introduction

ADP-ribosylation factors (ARF) are 20-kD guanine nucleotide-binding proteins (small GTPases) belonging to the Ras superfamily. Plant ARFs appear to have critical roles in multiple steps of the membrane traffic pathways, including retrograde and anterograde endoplasmic-reticulum–Golgi trafficking, as well as vacuolar transport (Loren et al., 2007). They also act as molecular switches for polar auxin transport in root development (Chong and Zhuang, 2007; Zhuang et al., 2006). A number of genes encoding ARF proteins have been isolated from microorganisms, animals and plants. In higher plants, several cDNAs presumably encoding ARFs have been isolated from rice (Youssefian et al., 1993), potato (Regad et al., 1993) and *Arabidopsis* (Lebas and Axelos, 1994). In particular, there has been detailed structural and functional analysis of the Arf GTPase family in *Arabidopsis*. The *Arabidopsis* genome contains 21 Arf GTPase family members, with isoforms present in both Arf and Arl GTPase subfamilies. In all cases, Arf GTPases act to recruit cytosolic coat proteins to sites of vesicle budding. Arf GTPases recruit COPI and clathrin protein coats to transport vesicles, whereas a specific subset of the Arf GTPase family, the Sar1p GTPases, recruit COP-II coats. However, little is known regarding the function of Arl GTPases. It has been speculated that this gene might play a role in the membrane trafficking steps necessary for proper cell plate deposition during cytokinesis in developing embryos (Vernoud et al., 2003).

Fruits have been classified as climacteric and nonclimacteric on the basis of their patterns of respiration and ethylene production during maturation and ripening (Liu et al., 1999). In climacteric fruits, it has been demonstrated that ethylene triggers ripening, in that massive ethylene biosynthesis begins at the onset of the respiratory climacteric period. In most climacteric fruits, ethylene production begins to increase at the onset of the climacteric period, thereafter increases, and decreases in parallel with the changes in respiratory climacteric toward the full-ripe stage.

Abbreviations: 1-MCP, 1-methylcyclopropene; RACE, rapid amplification of cDNA ends.

* Corresponding author at: Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Yilong W. Rd. 2, Longhua County, Haikou, Hainan 571101, China. Tel.: +86 898 66794563; fax: +86 898 66705617.
E-mail address: zhiqiangjin2001@yahoo.com.cn (Z.-Q. Jin).

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As a typical climacteric fruit, banana is cultivated largely in tropical and subtropical regions of the world. The pattern of ethylene production during ripening in banana fruit differs from other climacteric fruits, and shows a sharp rise and fall in the rate of ethylene production during the early climacteric rise of respiration (Liu et al., 1999). As a result, it has been suggested that the regulatory mechanism of ethylene metabolism in banana fruit may differ from other climacteric fruits. To understand the molecular basis of fruit ripening in banana, we isolated genes that were differentially expressed at the early stage of postharvest banana ripening by using suppression subtractive hybridization (SSH). A cDNA fragment of an *Arf* homolog was obtained that was upregulated at the early stage of postharvest banana ripening (Xu et al., 2007). An intriguing question is whether *Arf* is involved in banana ripening. To address this question, the full-length cDNA encoding Arf was obtained in this study, followed by obtaining the corresponding genomic sequence. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze spatial and temporal expression of the gene in different organs. Real-time RT-PCR was used to analyze the expression level of Arf at different stages of ethylene biosynthesis during fruit ripening to explore the relationship between Arf expression and ethylene biosynthesis.

Materials and methods

Banana fruits and treatments

Banana (*Musa acuminata* L. AAA group, cv. Brazilian) fruits obtained from the banana plantation of the Institute of Tropical Bioscience and Biotechnology (Chengmai, Hainan) were harvested at the mature green stage (100–110 d after flower shooting). Banana hands with similar development stages were selected and three fingers from hands were divided into three groups for different treatments. For natural ripening, the group of bananas was kept at 25 °C and allowed to ripen naturally. For ethylene treatment, the group of bananas was sealed in a closed airtight container and treated with 100 μL/L of ethylene for 24 h. For ethylene inhibition treatment, 1-methylcyclopropene (1-MCP) was used as a potent inhibitor of ethylene responses; bananas were sealed in a closed airtight container and treated on the day of harvest with 1 μL/L of 1-MCP (Biotechnologies for Horticulture Inc., Walterboro, SC, USA) for 24 h at 25 °C. The treated materials were then allowed to ripen at 25 °C, and were subsequently frozen in liquid nitrogen and stored at 80 °C for extraction of total RNA at different stages of ripening.

Measurement of ethylene production

Ethylene production was measured by enclosing fruit samples in an airtight container for 2 h at 25 °C, withdrawing 1 mL of the headspace gas, and injecting this into a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) fitted with a flame ionization detector and an activated alumina column. The ethylene production measurements were obtained as recommended by the manufacturer.

RNA extraction and cDNA synthesis

Total RNA was separately extracted from the roots, rhizomes, flowers and leaves of the plant, from which the fruits were obtained as described previously (Wan and Wilkins, 1994).

For cloning of full-length cDNA, the total RNA from banana fruit tissues (including peel and pulp) was isolated 2 d after harvest, as described by Wan and Wilkins (1994). The cDNA was synthesized using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Isolation of full-length cDNA encoding Arf from banana

Rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNA, using the double-stranded cDNA from banana fruit at 2 d after harvest as a template, based on the fragment cloned by SSH previously (Xu et al., 2007). For 5′ RACE, the forward primer was 5′-tgatgggcgagctttaagtcctc-3′, and the reverse primer was 5′-ccttgcctcaacaacacgatc-3′ (provided in the SMART PCR cDNA Synthesis Kit as the SMARTIIA oligonucleotide). For 3′ RACE, the forward primer was 5′-tggtcaggacaagatcagac-3′, and the reverse primer 5′-ctccgagatctggacgagc-3′ (provided in the SMART PCR cDNA Synthesis Kit as SMART CDS primer IIA). The amplified products of the 5′ and 3′ cDNA ends were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA). Nucleotide sequences of the inserted cDNA fragments were determined on an ABI PRISM310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) using the BigDye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Based on the 5′ and 3′ end sequences of the cDNA, a pair of gene-specific primers was designed (5′-ggctctctgtgttcggcgcgg-3′ and 5′-taacccacacaggtcactc-3′) to allow amplification of the entire open reading frame (ORF). The amplified product was inserted into the pGEM-T easy vector (Promega). The nucleotide sequence was determined on an ABI PRISM310 Genetic Analyzer (Perkin Elmer) using the BigDye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The sequence was analyzed by BLAST (http://ncbi.nlm.nih.gov/blast).

Isolation of genomic DNA sequence of Arf from banana

Genomic DNA was isolated from leaf of banana tissue-cultured plantlets as described by Pua et al. (2000). Based on the full-length cDNA, primers were designed to amplify the Arf genomic sequence. Forward primer was 5′-ggtacaatggggctcacgttc-3′ and reverse primer was 5′-ttaagccttgctggcaatgttg-3′. PCR was carried out as follows: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 50 s, 59 °C for 50 s, 72 °C for 90 s; and an additional cycle at 72 °C for 10 min. The amplified product was inserted into the pGEM-T easy vector (Promega) and sequenced on an ABI PRISM310 Genetic Analyzer using the BigDye Termination Cycle Sequencing Ready Reaction Kit.

RT-PCR

Total RNA was isolated separately from roots, rhizomes, leaves, flowers and fruit tissues (including peel and pulp) as described by Wan and Wilkins (1994). Each total RNA sample was purified using an RNeasy mini spin column (Qiagen Inc., Valencia, CA, USA). Poly $(A)^+$ RNA from each total RNA sample was isolated using OligotexdT30 (TaKaRa, Dalian, China) according to the manufacturer's protocol. The first strand of cDNA was synthesized from 2 μg poly$(A)^+$ RNA from each sample using AMV Reverse Transcriptase (Promega). The following gene-specific primers were designed: forward primer, 5′-ccgtcgactatatcctccg-3′ and reverse primer, 5′-attgcatttggaggatcttg-3′. Each RT-PCR reaction was conducted as follows: 1 cycle at 94 °C for 4 min; 29 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and an additional cycle at 72 °C for 10 min. RT-PCR of actin was used as an internal control. For amplification of actin, the following primer set was designed: forward, 5′-tgtagcaattcaggctgttctt-3′ and reverse, 5′-tcagagatggctggaagagaac-3′.

Real-time RT-PCR analysis of Arf homolog expression

For real-time quantitative RT-PCR, each total RNA was isolated separately from naturally ripened banana fruit at 0, 2, 6, 10, 12, 14 and 16 d after harvest, from ethylene-treated fruit at 0, 1, 2, 3,