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Gene expression of pathogenesis-related protein during banana ripening and after treatment with 1-MCP

Ravi Kesari, Prabodh Kumar Trivedi*, Pravendra Nath

Plant Gene Expression Laboratory, National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow 226 001, India

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ABSTRACT

Pathogenesis-related (PR) proteins are generally considered as plant defense proteins associated with preventing or limiting pathogen and insect attack. Identification and characterization of a fruit PR1, the *MaPR1a* gene, is reported here for the first time from banana. The *MaPR1a* cDNA is 606 bp long and contains an open reading frame of 489 nucleotides encoding 162 amino acid residues. The deduced amino acid sequence of *MaPR1a* has a high level of identity with PR1 proteins from other plants. Southern blot as well as nucleotide sequence analyses using the Global Musa Genomics Consortium database suggests that *MaPR1a* is encoded by a multigene family in banana. Ethylene exposure of unripe mature banana fruit induced *MaPR1a* expression, which increased with ripening, and 1-methylcyclopropene (1-MCP) treatment prior to ethylene exposure inhibited expression. No expression was detected in any other tissue which suggests that *MaPR1a* gene family members are fruit-specific and ripening related. The 1506 bp proximal promoter of the gene shows the presence of *cis*-acting elements which could bind to sets of transcription factors as well as regulate jasmonic acid (JA) and salicylic acid (SA) signalling. Our expression analysis suggests that in addition to ethylene, JA and SA also induce expression of *MaPR1a* in fruit tissue.

1. Introduction

Plants defend themselves by triggering cascades of signaling events that eventually lead to the synthesis of low molecular weight secondary metabolites, generation of reactive oxygen species (ROS), lignification of cell walls, induction of the hypersensitive response (HR) and activation of a number of pathogenesis-related (PR) genes. The PR genes have been characterized from many plant species and expression is shown to be inducible upon infection with pathogen or insect attack. These PR proteins have been classified into 17 different families (Van Loon and Van Strien, 1999) and include β-1,3-glucanases (PR-2), chitinases (PR-3, -4, -8 and -11), osmotin with thaumatin-like proteins (PR-5), defensins (PR-12), thionins (PR-13), lipid-transfer proteins (PR-14), etc. The regulation of PR expression is known to be controlled by signaling compounds such as abscisic acid (ABA), ethylene, jasmonic acid (JA), salicylic acid (SA), mechanical wounding, exposure to UV-light, osmotic stress and microbial infection (Kitajima and Sato, 1999; Santamaria et al., 2001; van Loon et al., 2006; Loake and Grant, 2007; Zhang and Shih, 2007; Sels et al., 2008).

Although most PR proteins have been found to be inducible after pathogen infection, the expression of various PRs has also been reported to be activated during plant growth and development. *Arabidopsis* and rice contain 22 and 39 PR-1-type genes, but only 1 and 2, respectively, have been demonstrated to be inducible by pathogens or insect attack. A large number of other PR-1 genes are expressed constitutively in roots or floral tissues of these plants which suggests a role during plant growth and development (van Loon et al., 2006). Some of the PRs have also been reported to be accumulated under specific physiological conditions such as pollen development (Worrall et al., 1992), seed germination (Wu et al., 2001), leaf senescence, and fruit development and ripening (Quirino et al., 2000; Buchanan-Wollaston et al., 2003; Monteiro et al., 2007; Zhang and Shih, 2007; Charles et al., 2008).

Banana is one of the important staple foods in the world and has been extensively studied for *in vitro* propagation, pathogen defense and fruit ripening. Through two-dimensional SDS-PAGE of *in vitro* translation products (Medina-Suarez et al., 1997), differential screening of cDNA libraries (Clendennen and May, 1997), mRNA differential display (Gupta et al., 2006) and PCR-based suppression subtractive hybridization (Kesari et al., 2007), various differentially regulated ESTs during banana fruit ripening have been identified. Out of a large set of differentially expressing ripening-related ESTs, several have been shown to be defense and stress related. The objective of this study was to characterize one of the PR genes, *MaPR1a*, which shows fruit-specific and ripening-related expression. The proximal promoter of the *MaPR1a* contains *cis*-acting elements which have been shown in other systems to interact with

* Corresponding author. Tel.: +91 522 2297958; fax: +91 522 2205836/2205839.
E-mail address: prabodht@hotmail.com (P.K. Trivedi).

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various transcription factors as well as transcriptional regulation of genes by SA and JA signaling.

2. Materials and methods

2.1. Plant material and treatments

Hands of mature unripe banana (*Musa acuminata*; dwarf cavandish, genome AAA, var. Robusta Harichhal) were obtained from a local farm. This variety of banana does not ripen naturally and requires exposure of ethylene or other hydrocarbons to initiate ripening for commercial purposes. Fruit from the same whorl of the hand representing similar developmental stages were treated with 100 μL/L ethylene for 24 h at 22 °C in an air-tight container as described previously (Trivedi and Nath, 2004). Containers were opened every 6 h, flushed with air to remove CO_2 accumulation and maintain O_2 concentrations and replaced with 100 μL/L ethylene fresh each time. Fruit were allowed to ripen in air for 7 d under the same conditions and referred as Day 0 (prior to ethylene treatment) to Day 7. For 1-MCP treatment, fruit were pre-exposed to 10 μL/L of 1-methyl cyclopropene (Ethyl Bloc™ from Biotechnologies for Horticulture Inc., Walterboro, SC, USA) for 12 h followed by ethylene treatment as described above. Fruit pulp from bananas was separated every 24 h for a period of 7 d after ethylene treatment, frozen in liquid nitrogen and stored at −70 °C. Pulp tissue was also collected at different stages (stages 1–4) of development. Stages 1–4 of the fruit were defined in order of increasing size viz. 6, 10, 14 and 18 cm respectively, of which stage 4 represented mature green fruit. Samples of different tissues such as stem, pseudostem, leaves, bract and flowers were collected from 8 months old field grown banana plants and frozen in liquid nitrogen and stored at −70 °C until used. For the wounding experiment, banana leaves were mechanically perforated using a metal forceps and sampled at various time points. Jasmonic acid treatment of banana leaf and fruit was by applying to the outer surface a solution of 100 mM methyl jasmonate (Aldrich, Steinheim, Germany) in water with 0.1% Tween 20, using a cotton pad to keep the surface wet for 5 min before allowing the liquid to dry. For the treatment of banana fruit with salicylic acid, banana fingers were dipped in 2 L of distilled water containing salicylic acid (1000 μM) and teepol (0.2%) at room temperature for 6 h with occasional shaking. After 6 h, fingers were removed from the solution and dried with tissue paper and kept in air at room temperature. Samples were taken at various time points and frozen in liquid nitrogen and stored at −70 °C.

2.2. Determination of fruit firmness

Five fruit from each treatment were used to measure fruit firmness. To measure the firmness, peel from one side of the banana finger was removed and an measurements were carried out at three different places using a Penetrometer (Model FT327 and FT011, McCormick Fruit Tech, USA) and recorded in newtons (N). The average of three readings was taken as a measure of firmness for individual fruit. Data are expressed as mean +/− standard deviation (S.D.) of all replicates.

2.3. Cloning of full-length cDNA

Total RNA from banana pulp as well as other vegetative tissues was isolated according to Asif et al. (2006). RNA isolated from the pulp of Day 4 ethylene treated banana fruit samples was used to prepare a smart cDNA library. For isolation of mRNA from the total RNA, the PolyATtract mRNA isolation system was used (Promega Corporation, Madison, WI, USA) as per the manufacturer's recommendations. Two micrograms of mRNA was used as the template to synthesize cDNA using a SMART™ PCR cDNA synthesis kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Rapid amplification of cDNA ends (RACE) was performed using the SMART cDNA library as the template. For the 5′ RACE, two oligonucleotides (GSP1: 5′ CGC TGG GTT GTA GTT GCA GAT GAT G 3′ and GSP2: 5′ GGG TTG TAG TTG CAG ATG ATG AAG 3′) were designed as gene specific primers from the partial sequence of PR1 obtained from our previous study (Gene Bank Accession No. DQ663582, Kesari et al., 2007). The PCR product was then cloned into the pTZ57R TA cloning vector and three independent clones were sequenced using M13 universal and reverse primers for both plus and minus strands. Sequencing was carried out on an automated DNA sequencing system (ABI 373A, Applied Biosystems Inc., USA) using the dye terminator cycle sequencing kit.

2.4. RNA gel-blot analysis

Total RNA obtained from various treatments and tissues was resolved on a denaturing 1.2% formaldehyde agarose gel as described by Sambrook et al. (1989). RNA was transferred to Hybond N+ nylon filters (Amersham Biosciences, Buckinghamshire, NA, USA) by vacuum transfer using the vacugene apparatus from Amersham-Pharmacia. A 189 bp fragment containing 3′ *MaPR1a* cDNA was labeled for the use in northern blot hybridization by random priming using α^{32}PdCTP as the radiolabel. Prehybridization and hybridizations were performed at 42 °C in a formamide based hybridization buffer as described by Sambrook et al. (1989). Washings were performed in 2× SSC (saline sodium citrate), 0.1% SDS (sodium dodecyl sulphate) for 2× 15 min, 0.5× SSC, 0.1%SDS for 2× 15 min and 0.1× SSC, 0.1% SDS for 15 min, all at room temperature and finally a high stringency wash was given to the blot at 50 °C with 0.1× SSC, 0.1% SDS for 15 min. Blots were exposed to Kodak XOMAT X-ray film (KODAK, India Ltd., Vakola, Santacruz, Mumbai) and stored at −80 °C for 1–5 d depending on signal intensity.

2.5. Southern blot analysis

Genomic DNA was isolated from young green leaves as described by Dellaporta et al. (1983). Five micrograms of genomic DNA were digested with BamHI, EcoRI, HindIII, SacI, XbaI and XhoI restriction enzymes (Fermentas, USA), separated on 0.8% agarose gel and blotted onto Hybond N+ nylon filters. The DNA gel-blot was pre-hybridized, hybridized, washed and exposed to X-ray film according to method given in the RNA gel-blot analysis section.

2.6. In silico sequence analysis

Sequence similarity searches and signal peptide predictions were performed using the basic local alignment search tool (BLAST, National Center for Biotechnology Information, Bethesda, MD) and SIGNALP version 1.1 WorldWide Web Prediction Server (Center for Biological Sequence Analysis, Technical University of Denmark), respectively. Homologous genes in banana were identified by searching the Global Musa Genomics Consortium database (http://www.musagenomics.org). Deduced polypeptide alignment was carried out using the clustal method of DNASTAR Megalign (DNASTAR Inc., Madison, WI). A phylogenetic tree of full-length deduced amino acid sequences of PR was constructed using the Phylip 3.5c package employing parsimony and bootstrap analysis (100 replicates).

2.7. Cloning of MaPR1a proximal promoter

Total DNA from young banana leaf was isolated according to Dellaporta et al. (1983) and a genome walker library was con-