

## Cloning and expression analysis of rubredoxin from cold-treated banana leaves

### Clonación y análisis de la expresión del gen de rubredoxina de hojas de banano sometidas a tratamiento de frío

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**Abstract.** A banana (*Musa AAA*, Cavendish subgroup cv. Brazil) cDNA encoding a putative rubredoxin-like protein (*MaRd1*) was obtained from total RNA isolated from cold-treated banana leaves using rapid amplification of cDNA ends (RACE) technique. *MaRd1* cDNA contained 597 nucleotides encoding 198 amino acids in the open reading frame. *MaRd1* protein showed 56% amino acid identity with that of *Pyrococcus furiosus* rubredoxin (P24297). A chloroplast transit peptide and a transmembrane region were detected at the N-terminus and the C-terminus, respectively, of the deduced amino acid sequence of *MaRd1* gene. Southern blotting revealed the occurrence of at least two copies of *MaRd1* in the banana genome. Real time quantitative RT-PCR analysis revealed that the expression of *MaRd1* gene was mainly in leaves, pseudo-stems and immature fruits, while it was barely detectable in roots and flowers. Cold and salt stresses induced higher levels of *MaRd1* transcript accumulation in leaves. This finding indicated a role of *MaRd1* in the response to these abiotic stresses.

**Keywords:** Rubredoxin; Expression; Banana; Stress response.

**Resumen.** Un ADNc de banano (*Musa AAA*, Cavendish subgroup cv. Brazil) que codifica para una rubredoxina putativa (*MaRd1*) fue obtenido a partir de ARN total aislado de hojas de banano tratadas con frío por la técnica de amplificación rápida de los extremos del ADNc (RACE). El ADNc de *MaRd1* constaba de 597 nucleótidos que codifican para 198 aminoácidos. Estos aminoácidos de la putativa *MaRd1* de banano mostraron un 56% de identidad con los correspondientes a la rubredoxina de *Pyrococcus furiosus* (P24297). Un péptido de tránsito de cloroplasto y una región de transmembrana fueron detectados en los extremos N-terminal y C-terminal, respectivamente, de la secuencia deducida del gen de la *MaRd1*. El análisis utilizando la técnica de Southern reveló la presencia de al menos dos copias del gen en el genoma de banano. El análisis por RT-PCR cuantitativa en tiempo real reveló que el gen *MaRd1* se expresa principalmente en las hojas, pseudo-tallos y frutos inmaduros, mientras que su expresión fue débilmente detectable en raíces y flores.

**Palabras clave:** Rubredoxina; Expresión, Banana; Respuesta al estrés.

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Recibido / Received 2.VII.2010. Aceptado / Accepted 3.VIII.2010.

## INTRODUCTION

Temperature might drop to levels lower than usual, causing damages to many species of plants. Crops of tropical origin, such as banana and rubber, are especially vulnerable because they are not normally exposed to such stress. Prolonged cold exposure usually causes wilting, chlorosis or necrosis, and stunting growth and development in susceptible plants (Lyons, 1973; Wang, 1990). Exposure of cold-sensitive plants to cold results in significant and rapid physiological changes, such as shifts in photosynthesis and respiration rates, ion leakage from cell membranes, and the production and accumulation of toxic compounds including reactive oxygen species (ROS) in plant cells (Lyons, 1973; Huner & Williams, 1988; Xin & Browse, 2000; Suzuki & Mittler, 2006). Low temperatures generally stimulate acclimation or adaptation responses from plants. These responses enhance cold tolerance on the affected plants (Levitt, 1980; Provart et al., 2003). Cold acclimation is a complex and global process involving, but not limited to, accumulation of proline and other cryoprotectants, changes in membrane lipid composition, alterations in photosynthetic carbon metabolism and detoxification of ROS (Iba, 2002; Stitt & Hurry, 2002; Provart, 2003).

Banana varieties (*Musa* spp.) initially originated in tropical regions of Southeast Asia. They are sensitive to cold, which can result in serious losses in commercial banana production. Conventional breeding for cold tolerance in banana has proven more difficult than in many other crops because most banana cultivars are triploid. Therefore, modern biotechnology may offer the best hope for genetic improvement in banana. We studied cold inducible genes in banana with the goal of genetically modifying banana for cold tolerance. In this study, we identified an expressed sequence tag (EST) encoding for a rubredoxin (Rd) protein.

Rd proteins play an important role in prokaryotes and eukaryotes, and have been reported as electron transfer molecules branching electrons in several biochemical pathways (Chen & Mortenson, 1992; Yoon et al., 1999; Wastl et al., 2000). Rd is involved in reactions for scavenging ROS in *Archaeoglobus fulgidus* (Rodrigues et al., 2005). Enhancer1 (ENH1), an Rd-like protein, enhances salt stress tolerance in *Arabidopsis* by ROS detoxification (Zhu et al., 2007). This study explores possible connections of *MaRd1* gene to cold response in banana.

## MATERIALS AND METHODS

**Plant materials.** Banana (*Musa AAA*, Cavendish subgroup cv. Brazil) plants were obtained from a commercial nursery (Hainan Wanzhong Co., Ltd) in Hainan province, China. They were cultivated in an experimental plot of loamy soil under natural daylight conditions at the Institute of Tropical Bioscience and Biotechnology, Haikou, China. They were irrigated twice a week with tap water and supplied with a local organic fertilizer

at a rate of 1 kg per tree once monthly. The Real-Time quantitative RT-PCR samples of roots, pseudo-stems, leaves, flowers, and immature fruits were harvested during summer from adult plants (about 250 cm tall) 70 days after flowering.

Banana plantlets were generated and propagated *in vitro* to avoid pathogen contaminations. These plantlets were then grown in 9 cm ceramic pots (Jiajiayi Porcelain Industry Co., Ltd, Dehua, China) in a mixture of loam and coconut husk (60:40 v/v, pH 6.0). They were watered twice a week with tap water and once a week with a plant nutrient solution containing: 0.7 g/L  $\text{KNO}_3$ ; 0.7 g/L  $\text{Ca}(\text{NO}_3)_2$ ; 0.8 g/L  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ ; 0.28 g/L  $\text{MgSO}_4$ ; 0.12 g/L  $\text{Fe}_2(\text{SO}_4)_3$ , and 0.6 mg/L  $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_3\text{BO}_3$ ;  $\text{MnSO}_4$ ;  $\text{ZnSO}_4$  and  $\text{CuSO}_4$ , respectively. Greenhouse conditions were 16 hours light (300  $\mu\text{mol}/\text{m}^2/\text{s}$ , fluorescent lamp) at 28 °C, and 8 hours dark at 21 °C. The relative humidity was maintained at 85%.

Banana plantlets with 4 well-developed leaves were placed at 5 °C, 28 °C or 39 °C (temperature treatments) in climate cabinets (SPX-250/300IC, Shanghai Boxun Industry & Commerce Co., Ltd.) They were under a 16 h light/ 8 h dark photoperiod at 300  $\mu\text{mol}/\text{m}^2/\text{s}$  (fluorescent lamps). Nine plantlets were used in each temperature treatment. The third and fourth well-developed leaves from the top were harvested after 3 days.

Salt treatments were also applied to plantlets with 4 well-developed leaves. These plantlets were irrigated using the plant nutrient solution described above plus sodium chloride (Guangzhou Chemical Reagent Factory). The salt was added 4 times at a rate of 25 mM or 50 mM per addition. Interval between additions was 2 days. Plantlets were kept at either 100 mM or 200 mM for 2 days. Control plantlets were watered with the nutrient solution without sodium chloride.

The third and fourth mature leaves from 9 plantlets were harvested for each of the above temperature and salt treatments, frozen in liquid nitrogen and stored at -80 °C until needed.

**Total RNA extraction and cDNA synthesis.** Total RNA from each banana tissue was isolated using Concert™ Plant RNA Reagent (Cat. 12322-012, Invitrogen, USA) and quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Starlab, USA) at 260 nm. Leaf total RNA from 5 °C-treated plantlets was used to synthesize 5'- and 3'-RACE-Ready cDNA according to the manual of BD SMART™ RACE cDNA Amplification Kit (Cat. 634914, Clontech, USA). First-strand cDNA used for Real-Time quantitative RT-PCR was synthesized using M-MLV reverse transcriptase (Cat. M1701, Promega, USA) in 25  $\mu\text{l}$  reactions according to the manufacturer's instructions.

**Rapid-amplification of cDNA ends.** The 5' and 3' flanking regions of *MaRd1* gene were obtained by 5'- and 3'- RACE using the BD SMART™ RACE cDNA Amplification Kit according to the manufacturer's instructions. Primers were designed based on an EST sequence from the suppression subtractive hybridization (SSH) library using cDNA of 5 °C treated banana leaves as tester,

and those of 28 °C as driver (unpublished). 5'-RACE PCR was performed using the cDNA as template and the *MaRd1*-L<sub>1</sub> primer (5'-TGCCTCCTAGCCCCGAAGTCTGGTT-3') and *MaRd1*-L<sub>2</sub> primer (5'-CCGGCGATCTCCACGCTCTTGCTCT-3') of *MaRd1* gene, the universal primer A mix (UPM), and the nested universal primer A (NUP) of the RACE kit. 3'-RACE PCR was performed using the *MaRd1*-R<sub>1</sub> primer (5'-GAGGAGCGCTTCGCGGTCTCAACA-3') and *MaRd1*-R<sub>2</sub> primer (5'-CCGGCATCTACGAGTGCCGCTTTG-3') of *MaRd1* gene, UPM and NUP. PCR products were purified with the QIAquick Gel Extraction Kit (Cat. 28704, Qiagen, USA), cloned into the pGEM®-T Easy Vector System I (Cat. A1360, Promega, USA), and transformed into *E. coli* strain DH5 $\alpha$  competent cells (Cat. CB101-01, TIANGEN, China) by heat shock. White colonies were picked, and plasmid DNA was isolated using QIAprep Spin Miniprep kit (Cat. 27106, Qiagen, USA). The presence of cDNA insert was confirmed by *EcoRI* (Cat. FD0274, Fermentas, Lithuania) digestion. Sequencing was performed on both strands by Sangon (Shanghai, China).

**Sequence analysis.** Prediction of chloroplast transit peptides (cTP) of *MaRd1* protein was performed using ChloroP 1.1 at <http://genome.cbs.dtu.dk/services/ChloroP/>. Transmembrane helices prediction was made using TMHMM at <http://www.cbs.dtu.dk/services/TMHMM/>. Multiple sequence alignment was carried out by the DNAMAN program (V5.2, LynnonBiosoft, Quebec, Canada).

**Real-Time quantitative RT-PCR (RQ-PCR).** For expression profiling of *MaRd1* gene, gene-specific primers (*MaRd1*-RF, ACTCCTCCAAGCCCACCAATAC; *MaRd1*-RR, ACTCGTAGATGCCGGTGTGAG; *Mb-Act1*-F, TGTACGTTGCTATCCAGGC-T; *Mb-Act1*-R, TGATGGCTGGAAGAGAACCT) were designed, and RQ-PCR was performed using cDNAs from roots, pseudostems, leaves, flowers, and immature fruits. The RQ-PCR was performed with an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Inc.) using SYBR Green I as dye. A banana actin gene, *Mb-Act1* (GenBank accession AF246288), was used as an internal control in each reaction. Each sample in triplicate was quantified by the comparative cycle threshold method (Wittwer, 1997). The same methods were employed to detect the transcription levels of *MaRd1* gene in the temperature and salt stress experiments.

**Southern blot hybridization.** Genomic DNA from young leaves of banana plants was isolated by the cetyl-trimethyl-ammonium bromide method (Rogers and Bendich, 1985). Twenty micrograms genomic DNA was digested with the restriction enzymes *EcoRI* and *HindIII* (Cat. FD0274 and FD0504, Fermentas, Lithuania), separated on 1% (w/v) agarose gel, and blotted to a Hybond-N<sup>+</sup> nylon membranes (Cat. RPN1510B, Amersham Pharmacia Biotech) according to the manufacturer's instructions. DNA probe was made using *MaRd1* cDNA as template and

with a DIG High Prime DNA Labeling and Detection Starter Kit I (Cat. 11745832910, Roche, Penzberg, Germany) in accordance with the manufacturer's instructions. The membrane was pre-hybridized and hybridized at 48 °C, washed at 68 °C and stained according to the manufacturer's instructions.

## RESULTS

**Identification and cloning of *MaRd1* gene.** A previous study in our lab identified a 237 bp banana EST that showed homology to a rubredoxin in other species. We designed PCR primers based on the sequence of this EST and isolated a sequence from banana leaves of plants treated at 5 °C using 5'- and 3'-RACE strategy. This sequence, deposited in GenBank (accession numbers DQ875443), is designated as *MaRd1*. It contains a 597 bp open reading frame (ORF) that encodes 198 amino acid residues with a theoretical molecular mass of 21.58 kDa.

Multiple sequence alignment using the DNAMAN program revealed that the amino acid sequence of the *MaRd1* protein was highly similar to the 8 orthologous Rd proteins from the GenBank (Fig. 1). A putative cTP with 53 amino acid residues was detected in the N-terminus of the deduced polypeptide of the *MaRd1* gene ([www.cbs.dtu.dk/services/ChloroP/](http://www.cbs.dtu.dk/services/ChloroP/)). A hydrophobicity profile of the deduced polypeptide of the *MaRd1* gene at the C-terminus (amino acid residues 177-197) (<http://www.cbs.dtu.dk/services/TMHMM/>) was predicted as membrane-spanning regions. The deduced polypeptide of the *MaRd1* gene has 56% identity with the Rd protein of *Pyrococcus furiosus* (P24297) (Blake, 1991). Rd is a small nonheme iron protein, and the iron atom is coordinated by four cysteine residues (Fe(S-Cys)<sub>4</sub>) which were arranged in two cysteine boxes (CXXC) with conserved spaces between them (Fig. 1). These cysteine residues participate in the formation of active sites with iron (Cheng & Markley, 1995).

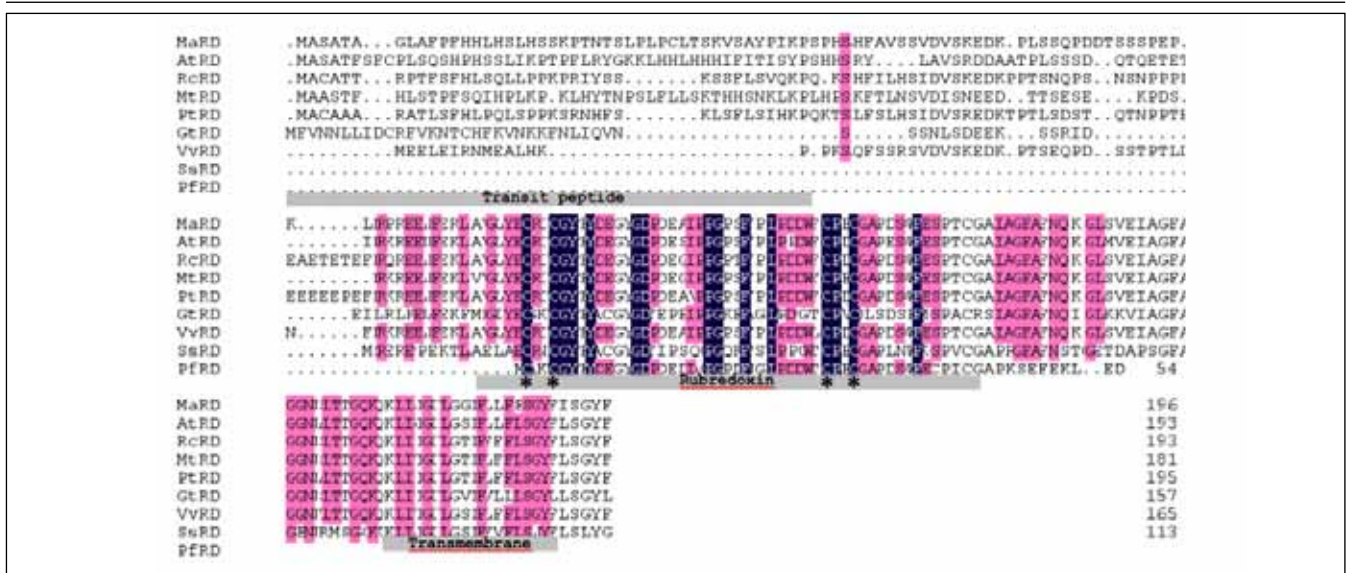
Genomic Southern blot was carried out with the *MaRd1* cDNA as probe (Fig. 2). Several bands were detected from *EcoRI* and *HindIII* digestion, suggesting that the banana genome has multiple copies of the *MaRd1* gene.

**Transcript profile of *MaRd1* gene assessed by RQ-PCR.** Expression of the *MaRd1* gene in different tissues of the banana plants was analyzed by RQ-PCR using gene specific primers (Fig. 3). The highest expression was detected in leaves, followed by pseudo-stems and immature fruits, all being green. In comparison, the transcript levels of the *MaRd1* gene were barely detectable in roots and flowers.

**Induced transcript levels of *MaRd1* gene in response to temperature and salt stress.** To determine whether *MaRd1* gene transcript levels change in response to different temperature treatments, RQ-PCR was performed with gene specific primers on leaf total RNA from banana plantlets grown at 5 °C, 28 °C or 39 °C for 3 days (Fig. 4A). The *MaRd1* gene transcript was

**Fig. 1.** Alignment of several Rd proteins. *Musa* spp. (this work, *MaRd1*, DQ875443), *Arabidopsis* (*AtRd*, AAD25628), *Vitis vinifera* (*VvRd*, CAN65205), *Ricinus communis* (*RcRd*, EEF49703), *Medicago truncatula* (*MtRd*, ACJ83960), *Populus trichocarpa* (*PtRd*, XP\_002319619), *Guillardia theta* (*GtRd*, CAB40406), *Synechocystis* sp. PCC6803 (*SsRd*, NP\_440410), *Pyrococcus furiosus* (*PfRd*, P24297). Sequences were aligned by DNAMAN program using default parameters. Numbers in parentheses refer to GenBank accession numbers. Identical amino acids (100% similarity) are shaded black, and similar amino acids (less than 100% similarity) in at least 75% of the compared sequences are shaded pink. The cysteine ligands of the iron ion are marked with \*.

**Fig. 1.** Alineación de varias proteínas Rd. *Musa* spp. (este trabajo, *MaRd1*, DQ875443), *Arabidopsis* (*AtRd*, AAD25628), *Vitis vinifera* (*VvRd*, CAN65205), *Ricinus communis* (*RcRd*, EEF49703), *Medicago truncatula* (*MtRd*, ACJ83960), *Populus trichocarpa* (*PtRd*, XP\_002319619), *Guillardia theta* (*GtRd*, CAB40406), *Synechocystis* sp. PCC6803 (*SsRd*, NP\_440410), *Pyrococcus furiosus* (*PfRd*, P24297). Las secuencias se alinearon utilizando el programa DNAMAN usando parámetros establecidos. Los números en paréntesis se refieren a los números de las cepas en el Banco de Genes. Los aminoácidos idénticos (100% de similitud) están coloreados de negro, y los aminoácidos similares (similitud menor del 100%) en al menos 75% de las secuencias comparadas están coloreados de rosa. Los ligandos de cisteína del ion hierro están marcados con una \*.



at a lower level on leaves from the control (28 °C) plantlets. The transcript levels increased significantly on plants treated at 5 °C. However, there was no noticeable difference between the control and the heat (39 °C) treatment.

When banana plantlets were irrigated with the nutrient solution, *MaRd1* expression levels were relatively low (Fig. 4B). Treatment with 100 mM NaCl greatly increased *MaRd1* transcript levels on leaves. NaCl at 200 mM resulted in appreciably higher levels of detectable *MaRd1* transcript.

## DISCUSSION

Rds are known as nonheme iron proteins, where one iron is covalently attached to four cysteines (Meyer et al., 1995). They have been found in a wide range of prokaryotes and eukaryotes (Lovenberg & Sobel, 1965; Peterson & Coon, 1968; Aurich et al., 1976; Ragsdale & Ljungdahl, 1984; Seki et al., 1988; Sieker et al., 1994; Beinert et al., 1997; Wastl et al., 2000). The prokaryotic Rds are short proteins with a cysteine ligands core. Based on the amino acid spacing between the cysteine ligands to iron, Rd can be classified as type I Rd or type II Rd. Type I Rd, the most common, has a CX2C...CX2C motif binding the iron, and type II Rd has two extra amino acid residues between the first two cysteines, CX4C...CX2C (Rodrigues et al., 2005). According to this classification, the *MaRd1* protein belongs to

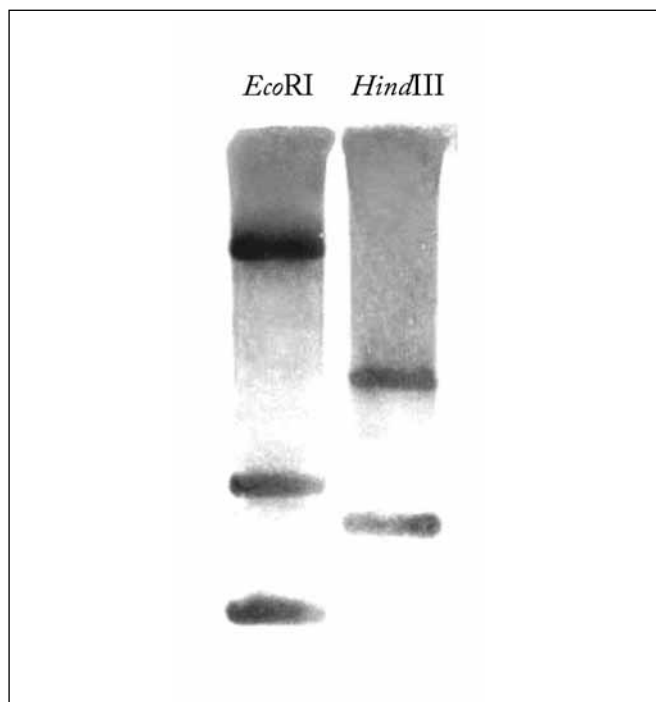
type I Rd. The eukaryotic Rd proteins usually contain, in addition to the cysteine ligands core, a cell signal peptide at the N-terminus, and a membrane anchoring domain at the C-terminus (Wastl et al., 2000). *MaRd1* is similar to the cryptomonad *Guillardia theta* Rd protein (Fig. 1). It has a N-terminus cTP that directs the preprotein into the stroma of the chloroplast, and a C-terminus that includes a transmembrane region which presumably acts as a membrane anchor (Wastl et al., 2000).

Rd proteins in prokaryotes are a cofactor in several biochemical pathways (Chen & Mortenson, 1992), an electron acceptor for pyruvate ferredoxin oxidoreductase (Yoon et al., 1999), and an electron donor for neelaredoxin in reactions for removing harmful superoxide (Rodrigues et al., 2005). Eukaryotic Rd in *Guillardia theta* [nucleomorph-encoded rubredoxin (nmRub)] is associated with the thylakoid membrane, and co-localized with PSII membrane particles and PSII core complexes (Wastl et al., 2000). It was suggested that nmRub could act as an electron transfer molecule, branching electrons from PSII to plastid membrane-located pathways. It could also replace the cytochrome b<sub>6</sub>/f complex or plastoquinone from the photosynthesis machinery under some circumstances. Because nmRub is the closest homolog to *MaRd1*, functions of *MaRd1* could be the same as suggested for nmRub. Our results on *MaRd1* transcripts, showing a predominant expression in green tissues, agree with this hypothesis.

To our knowledge, there is no published data on the expression pattern of eukaryotic Rd. The closest *Arabidopsis* homolog

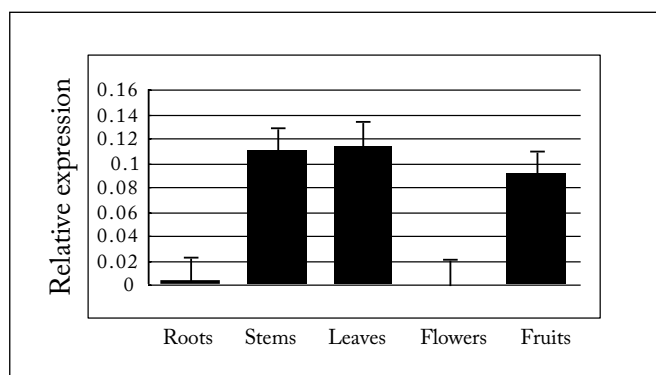
**Fig. 2.** Southern blot of *MaRd1* gene. The genomic DNA was isolated from immature leaves of banana plants followed by digestion with restriction enzymes [*EcoRI* (left lane), and *HindIII* (right lane)]. The digoxigenin-labelled *MaRd1* cDNA was used as probe.

**Fig. 2.** Mancha más al sur del gen *MaRd1*. El ADN genómico fue aislado de hojas inmaduras de plantas de banana seguido por digestión con enzimas de restricción [*EcoRI* (banda a la izquierda), y *HindIII* (banda a la derecha)]. El cADN *MaRd1* marcado con digoxigenina fue usado como prueba.



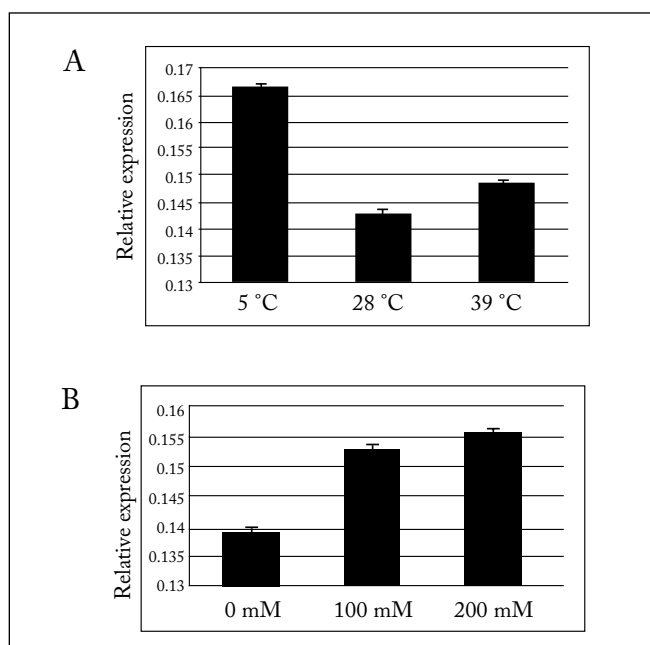
**Fig. 3.** The transcript profile of *MaRd1* gene in various tissues of banana plants. Analyses were performed by RQ-PCR using SYBR Green I on ABI PRISM® 7000 Sequence Detection System with total RNA extracted from various tissues of banana plants. Transcript levels were calculated from triplicate data, after normalization against banana Mh-Act1 gene using the standard curve method.

**Fig. 3.** Perfil de la transcripción del gen *MaRd1* en varios tejidos de plantas de banana. Los análisis se desarrollaron por RQ-PCR usando SYBR Green I en un Sistema de Detección de Secuencias ABI PRISM® 7000; el ARN total fue obtenido de varios tejidos de plantas de banana. Los niveles de transcripción se calcularon por triplicado, después de la normalización respecto al gen Mh-Act1 de la banana usando el método de la curva estándar.



**Fig. 4.** The expression analyses of *MaRd1* gene in response to temperature and salt stress by RQ-PCR. (A) banana plantlets were treated at 5 °C, 28 °C or 39 °C for 3 days. (B) plantlets irrigated with 0 mM, 100 mM or 200 mM NaCl for a week. Total RNA was obtained from the leaves of treated plantlets.

**Fig. 4.** Análisis de la expresión del gen *MaRd1* en respuesta al estrés por temperatura y sal por RQ-PCR. (A) juveniles de banana tratados a 5 °C, 28 °C ó 39 °C por 3 días. (B) juveniles regados con 0 mM, 100 mM ó 200 mM NaCl durante una semana. El ARN total se obtuvo de las hojas de los juveniles tratados.



of *MaRd1* is At1g54500 and, according to the website <http://www.bar.utoronto.ca/>, At1g54500 is expressed mostly in green tissues, which is similar to what we found. However, according to the same website, the expression of At1g54500 is either reduced or unchanged in *Arabidopsis* by salt or cold treatments for up to 24 hours. In contrast, these 2 treatments increased the transcript levels of *MaRd1* in our experiment. This discrepancy could be caused by the difference in treatment length (3 to 7 days in our experiments), plant species or study sequences.

Abiotic stresses, such as cold and salt, induce production and accumulation of toxic compounds that include ROS in plant cells. These compounds disrupt cellular homeostasis and uncouple major physiological processes (Xin & Browse, 2000; Suzuki & Mittler, 2006; Kotak et al., 2007; Zhu et al., 2007). Plant chloroplast thylakoids are the main generation site of ROS (Asada, 2006). The continuous production of ROS can disrupt photosynthetic electron transport (Niyogi, 1999). Rd is a thylakoid membrane protein in organisms with oxygenic photosynthesis (Wastl et al., 2000; Peltier et al., 2004). ENH1 that is an Rd-like chloroplast protein enhances salt stress tolerance in *Arabidopsis* by ROS detoxification (Zhu et al., 2007). The likely cellular localization of *MaRd1* suggests a role of this gene in photosynthesis. Enhanced expression of *MaRd1* gene in response to cold and salt stresses, as shown in this study, implies that *MaRd1* could be involved in banana cold and salt responses and participate in eliminat-

ing ROS. This hypothesis is supported by the report that Rd in *Archaeoglobus fulgidus* acted as an electron donor for neelaredoxin in reactions for removing a harmful superoxide species (Rodrigues et al., 2005). It would be interesting to investigate the role of *MaRd1* in cold and salt stress in banana by genetically modifying the expression of this gene in this plant species.

In conclusion, a *MaRd1* gene was isolated from cold-treated leaves of banana plants. Higher transcript of the *MaRd1* gene in immature fruit, pseudo-stems and leaves indicate that the gene was associated with chloroplast. Induction of *MaRd1* by cold and salt suggests a role of this gene in response to these stresses.

## ACKNOWLEDGMENTS

We thank Dr. Nanfei Xu (Bayer CropScience LP) and Ms. Amy McCaskill (BASF Plant Science LLC) for their careful reading and editing of this manuscript. This work was supported by the National Nonprofit Institute Research Grant of CATAS-ITBB (ITBBZD0931).

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