Identification of alternatively spliced MsRan transcripts involved in low temperature response in Musa spp.

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Abstract

Ran is involved in response to external stimuli. In this study, six MsRan gene cDNA sequences were isolated from wild banana (Musa spp. AB group) from Sanming City, China. Sequence analysis reveals that MsRan3A, MsRan3A-1a, and MsRan3C contained Ran protein domains including a GTP hydrolysis domain, a RanGAP-binding domain, and an acidic tail, whereas two G boxes (G4 and G5) were absent in MsRan3A-6a. The physicochemical property of MsRan3A, MsRan3A-1a, MsRan3A-6a, and MsRan3C appeared to differ significantly. Real time quantitative PCR (qPCR) analysis indicates that MsRan3A-1, MsRan3A-5, MsRan3A-6, MsRan3A-6a, and MsRan3C-1 were expressed in roots, leaves, peduncles, bracts, flowers, peels, and pulp of the wild banana. MsRan3A-1a was expressed at extremely low levels in these tissues and was undetectable by qPCR. The MsRan genes were found to be involved in responses to a low temperature stress but with different response patterns. Furthermore, salicylic acid significantly enhanced MsRan gene expressions suggesting the involvement of these genes in salicylic acid signal transduction.

Additional keywords: gene expression, salicylic acid, wild banana.

Introduction

Banana (Musa spp.), an important food staple and income-generating fruit crop in tropical and subtropical regions, is sensitive to biotic and abiotic stresses such as pests, drought, and low temperatures. In particular, a low temperature has a serious impact on banana yield and fruit quality (Yang et al. 2012). In a previous study, we identified several wild banana populations with cold resistance, including one from Sanming, Fujian Province, China (Lai et al. 2006, 2007). The identification of cold resistance related genes from cold resistant wild banana and the investigation of the underlying mechanisms are useful for genetic improvement and production of banana.

abnormalities in rice, and Chen et al. (2011) have shown that development in OsRAN2-knockdown rice plants is delayed, and spindle organization is aberrant. These results indicate that Ran has a fundamental role in plant development.

According to several studies, Ran is also involved in responses to an external stimulus. For example, Ran protein expression is enhanced under heat stress (Ferreira et al. 2006, Xu and Huang 2008, 2010), and low temperatures stimulate Ran transcript accumulation as well (Chen et al. 2011, Paul and Kumar 2011). Miché et al. (2006) revealed that jasmonic acid, a plant hormone that participates in signaling multiple stresses, induces Ran protein expression in rice roots. Lee et al. (2008) determined that various light sources regulate expression of the Ran gene through phytochrome-mediated signaling pathways. An enhanced accumulation of the Ran protein contains cell division by promoting the normal export of intranuclear tubulin at the end of mitosis and regulates cold resistance in rice. The results of these studies imply that Ran plays a crucial role in plant stress response.

Fifteen DNA sequences and six cDNA sequences of Ran genes have been isolated from wild banana previously (Zhang et al. 2014, 2015a). In this study, we isolated MsRan genes and alternative spliced transcripts from the wild banana. We then assessed their tissue-specific expression profiles as well as their expressions under low temperature stresses and salicylic acid (SA) treatment.

Materials and methods

Plants and treatments: Wild banana (Musa spp. AB group) from Sanming city was grown in our germplasm repository (the Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, China). Suckers were collected, rinsed, and treated as described by Zhang et al. (2006). The explants were inoculated into a half-strength Murashige and Skoog (MS) medium (pH 5.8) supplemented with 30 g dm⁻³ sucrose, 6.0 g dm⁻³ agar, 3.0 mg dm⁻³ 6-benzyladenine (BA), 0.1 mg dm⁻³ naphthaleneacetic acid (NAA), and 0.5 g dm⁻³ active carbon and cultivated at 28 ± 1 °C under the dark. After budding, the cultures were transferred and maintained at a temperature of 28 ± 1 °C, a 12-h photoperiod, and an irradiance of 200 μmol m⁻² s⁻¹. For propagation, the established cultures were transferred to an MS medium supplemented with 30 g dm⁻³ sucrose, 6.0 g dm⁻³ agar, 1.0 mg dm⁻³ 6-BA, and 0.1 mg dm⁻³ NAA (pH 5.8). For cold and SA treatments, shoot cultures subcultured on the propagation medium for 20 d were used. For tissue-specific expression analysis, we collected roots, leaves, peduncles, bracts, flowers, peels, and pulp from adult plants with fruits at 60 % maturity (Fig. 1 Suppl.). To examine the responses of wild banana to low temperature stresses, shoot cultures were exposed to various temperatures (0, 4, 8, 13, 20, and 28 °C) for 36 h and to 8 °C for various time periods (0, 1, 4, 8, 12, and 24 h). Salicylic acid treatment was carried out by spraying shoot cultures with distilled water containing 0 (control) or 0.5 mM SA. Both control and SA-treated shoot cultures were subsequently incubated under 8 °C. Samples were taken at 1, 4, 8, 12, and 24 h. The tissues and treated shoot cultures were harvested and immediately frozen in liquid nitrogen and stored at -80 °C until further use.

Cloning MsRan genes: The total RNA was extracted from the frozen samples using an E.Z.N.A. plant RNA kit (Omega Bio-Tek, Norcross, GA, USA) followed by treatment with DNase I (Takara Biotechnology, Dalian, China) to remove genomic DNA. The cDNA was synthesized with a RevertAid™ first-strand cDNA synthesis kit (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). MsRan gene fragments were amplified using three primer pairs (RanF-4/RanR-4, RanF-5/RanR-5, and RanF-8/RanR-8) designed according to Ran gene sequences from the Musa acuminata genome (http://banana-genome.cirad.fr/) and from the National Center for Biotechnology Information (NCBI) database. To clone the full-length cDNA sequence of MsRan genes, we carried out a rapid amplification of cDNA ends (RACE) using primers designed according to the obtained MsRan gene fragments. The 3′-RACE was performed using a first-strand cDNA synthesis kit (Fermentas) with an oligo-dT adapter primer (AP). The cDNA was then subjected to nested PCR using gene-specific primers (Ran3′RACE-1, Ran3′RACE-2, and Ran3′RACE-3) and a universal amplification primer (UAAP). In the case of 5′-RACE, the first-strand cDNA was synthesized using an oligo-dT primer and SuperScript™ III (Invitrogen, Carlsbad, CA, USA), and the product was purified using a MiniBEST DNA fragment purification kit (Takara). A homopolymeric tail was then added to the cDNA 3′-end using phytochrome-mediated signaling pathways. Wang et al. (2006) demonstrated that overexpression of TaRAN1 renders Arabidopsis hypersensitive to auxin. Zang et al. (2010) has reported similar results in rice with transgenic plants overexpressing OsRAN2 exhibiting an enhanced sensitivity to salinity, osmotic stress, and abscisic acid. Chen et al. (2011) further indicated that OsRAN2-overexpressing transgenic rice displays an enhanced cold tolerance. These authors suggested that OsRAN2 maintains cell division by promoting the normal export of intranuclear tubulin at the end of mitosis and regulates cold resistance in rice. The results of these studies imply that Ran genes play a crucial role in plant stress response.
terminal deoxynucleotidyl transferase (Takara) and dCTP. Nested PCR was carried out with two upstream primers (AP3 and AUAP) and three gene-specific downstream primers (Ran5’RACE-1, Ran5’RACE-2, and Ran5’RACE-3) to amplify the 5’-end of MsRan genes. The full-length MsRan was amplified by RT-PCR using primers designed according to 5’- and 3’-untranslated region (UTR) sequences. After resolving the PCR products on a 1 % (m/v) agarose gel, the bands were excised, purified, subcloned into a pMD18-T vector (Takara), and sequenced. All primers used for isolating MsRan genes are listed in Table 1 Suppl.

Bioinformatics analysis: Nucleotide and deduced amino acid sequences and amino acid sequence alignments were analyzed by the DNAMAN software. The open reading frames (ORFs) of MsRan cDNAs were determined using ORF Finder (www.ncbi.nlm.nih.gov/orf/) and translated into the corresponding amino acid sequence. Homology comparisons were conducted using the BLAST program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). The theoretical isoelectric point (pI) and mass values of mature peptides were calculated using the ProtParam tool (http://web.expasy.org/protparam). A phylogenetic tree was constructed by the neighbor-joining method with 1 000 bootstrap replicates in MEGA 5.

Real-time quantitative PCR: The total RNA (500 ng) was transcribed into cDNA with random primers and an oligo(dT) primer using a PrimeScript RT reagent kit (Takara). Real time quantitative PCRs (qPCRs) were performed on a Lightcycler 480 system (Roche Applied Science, Basel, Switzerland) as described by Fang et al. (2016). Reactions were run in three biological replicates and three technical replicates using 18S rRNA as an internal control. Analyses of expression data were performed with GeNorm v. 3.5 (Vandesompele et al. 2002). The specificity of the products from the real time qPCR experiments were confirmed by melting curve analysis and sequencing. All primers are listed in Table 2 Suppl.

Results

Six MsRan cDNAs were isolated from wild banana by real time PCR coupled with 5’- and 3’-RACE using primers listed in Table 1 Suppl. Sequence analysis indicates that MsRan3A-1, MsRan3A-5, MsRan3A-6, and MsRan3C-1 contained a 663-bp ORF, whereas MsRan3A-1a and MsRan3A-6a contained ORFs of 585 and 483 bp, respectively. The comparison of nucleotide sequences demonstrates that they were highly homologous. The sequences of MsRan3A-1 and MsRan3A-1a were identical and the sequences of MsRan3A-5, MsRan3A-6, and MsRan3A-6a were similar to one another. The genome sequence encoding MsRan3A-1 and MsRan3A-1a (GenBank accession No. KC898288) and the ones encoding MsRan3A-5, MsRan3A-6, and MsRan3A-6a (GenBank acc. No. KC898291) have been previously isolated (Zhang et al. 2014). The comparative structural analysis of MsRan3A-1 and MsRan3A-1a reveal that the coding region of MsRan3A-1a lacked a 78-bp exon (Fig. 1). The comparison of the structures of MsRan3A-5, MsRan3A-6, and MsRan3A-6a indicates that the coding region of MsRan3A-6a lacked a 180-bp exon and that the 3’-UTR of MsRan3A-5 lacked a 75-bp sequence (Fig. 1).

Fig. 1. Schematic representations indicate splicing patterns of MsRan gene transcripts. Bold lines indicate introns, black boxes indicate exons, ATGs indicate start codons, TAGs indicate termination codons, arrows indicate locations where sequence deletions exist.
Fig. 2. Multiple alignments of Ran sequences from banana and Arabidopsis. A-H - predicted Ran proteins from the banana genome encoded by: A: Achr1T05300, B: Achr1T11790, C: Achr1T14580, D: Achr4T00780, E: Achr5T06130, F: Achr5T09850, G: Achr6T01500, H: AchrUn_randomT07030, I: GSMUA AchrUn randomP07030. Conserved GTP binding and hydrolysis domains (G1-G5) are indicated by bold lines. An effector-binding domain (RanGAP-binding) and an acidic C-terminal region (acidic tail) are indicated with asterisks and triangles, respectively.

MsRan3A-1, MsRan3A-5, and MsRan3A-6 were found to encode a protein of 221 amino acids with a predicted molecular mass of 25.1 kDa and a pI of 6.38. A protein encoded by MsRan3C-1 was also found to comprise 221 amino acids with a predicted molecular mass of 25.0 kDa and a pI of 6.38. Sequence deletions in MsRan3A-1a and MsRan3A-6a were responsible for the absence of 26 and 60 amino acids in their deduced proteins, respectively. A predicted molecular mass and pl of MsRan3A-1a were 22.3 kDa and 8.15, respectively, whereas corresponding values in MsRan3A-6a were 18.1 kDa and 4.88. Analysis by BLAST reveals that predicted MsRan proteins shared a high similarity with Ran homologs from other plants. Multiple alignments with Ran homologs from Arabidopsis and M. acuminata indicate that MsRan3A, MsRan3A-1a, and MsRan3C...
contained several conserved motifs including G boxes (G1 - G5), a RanGAP-binding domain and an acidic C-terminal region (Fig. 2). In MsRan3A-6a, however, G4 and G5 domains, known to be involved in GTP-binding and hydrolysis, were lacking. To study the relationships of predicted MsRan proteins and their orthologs in other species, we generated a phylogenetic tree of deduced amino acid sequences of the MsRans and 19 other Ran homologs. In the phylogenetic tree, the Ran sequences from banana, except for MsRan3A-1a and Achr5T06130, fell into the same clade and were closely related to AtRan3. This placement suggests that MsRan3A, MsRan3A-6a, MsRan3B, MsRan3C, and MsRan3D share a conserved role with Ran3 (Fig. 3).

Real time qPCR analysis was performed to assess the expression pattern of the MsRan genes in different wild banana tissues (Fig. 4). MsRan3A-1, MsRan3A-5, MsRan3A-6, MsRan3A-6a, and MsRan3C-1 were expressed in all tested tissues. MsRan3A-1, MsRan3A-5, and MsRan3C-1 were weakly expressed in roots, peduncles, bracts, and peels with the lowest expression in roots, and were highly expressed in leaves, flowers, and pulp. MsRan3A-6 and MsRan3A-6a had the highest expressions in leaves followed by peduncles and showed the lowest expression in bracts. Interestingly, only a small amount of MsRan3A-1a was detected in any tested tissue (Ct values were greater than 34).

To investigate the effects of low temperatures on MsRan gene expressions, we subjected in vitro seedlings of wild banana to various temperatures (Fig. 5).
Expression of *MsRan3A-1* decreased slightly under 20 °C treatment and increased to a higher level under 13 °C treatment. When exposed to a temperature of 8 °C, *MsRan3A-1* expression was reduced to the lowest level, at even lower temperatures (4 and 0 °C), however, its expression was enhanced. As temperatures were lowered from 28 to 13 °C, expression of *MsRan3C-1* increased. Similar to *MsRan3A-1*, *MsRan3C-1* showed the lowest expression under 8 °C with even lower temperatures enhancing its expression (Fig. 5). The lowest expressions of *MsRan3A-5*, *MsRan3A-6*, and *MsRan3A-6a* were detected during 20 °C treatment. Expressions of these genes gradually increased as temperature was lowered from 20 to 8 °C, and the highest expressions were detected at 0 °C.

We also performed time-course experiments involving 8 °C treatment. Expressions of *MsRan3A-1*, *MsRan3A-5*, *MsRan3A-6*, and *MsRan3A-6a* increased slightly from 0 to 4 h, and after 8 h of treatment, their expressions rapidly decreased to their lowest levels and remained there (Fig. 6). Conversely, expression of *MsRan3C-1* was reduced after 1 h of treatment and then rose after 4 h to pre-treatment levels. Similar to other transcripts, *MsRan3C-1* was expressed at the lowest level after 8 h of treatment and remained at a low level thereafter (Fig. 6). These data suggest that *MsRan* genes are involved in a response to low temperature stress with various transcripts responding to low temperatures according to different patterns.

We also examined the effects of SA on expressions of *MsRan* genes under low temperature stress (Fig. 7). *MsRan* gene expressions were significantly enhanced after 1, 8, 12, and 24 h of SA treatment at 8 °C. After 4 h of SA treatment, however, *MsRan3A-1*, *MsRan3A-6*, *MsRan3A-6a*, and *MsRan3C-1* expressions were only slightly repressed, whereas *MsRan3A-5* expression was unaffected. These results imply that *MsRan* genes participate in SA signalling.

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**Fig. 4.** Expression analysis of *MsRan* genes in various tissues of adult wild banana. The total RNA was extracted from various tissues and subjected to real time quantitative PCR analysis. All data were normalized to the expression of 18S rRNA. Means ± SEs, *n* = 3.
Fig. 5. Expression analysis of MsRan genes under low temperature stresses. The total RNA was extracted from shoot cultures treated by different temperatures and subjected to real time qPCR analysis. All data were normalized to the expression of 18S rRNA. Means ± SEs, n = 3. Significant differences were determined by one-way ANOVA (P < 0.05) and are indicated by different letters.

Discussion

Plant Ran proteins are abundantly expressed in meristematic tissue (Haizel et al. 1997, Lü et al. 2011). Fang et al. (2014) have demonstrated that DlRan transcripts are highly expressed during developmental stages when cells are actively proliferating. Wang et al. (2006) reported that TaRAN1-overexpressing Arabidopsis plants exhibit increased primordial meristems, tillering, apical dominance, and the number of rosette leaves and of cells in the root meristem zone. These studies suggest that Ran is also involved in regulation of cell division. Our results indicate that predicted MsRan proteins are highly similar to Ran proteins from other plants. MsRan transcripts were weakly expressed in old tissues and highly expressed in young ones. Furthermore, their expressions were significantly reduced after treatment at 8 °C for 8 h. It was reported that banana growth is arrested and injury occurs under this temperature (Yang et al. 2012). However, different transcripts showed varied expression patterns. The various MsRan genes may thus have divergent functions. Further investigation is needed to determine their exact function.

Alternative splicing is a post-transcriptional regulatory mechanism that plays important roles in regulation of gene expression (Lareau et al. 2004, Stamm et al. 2005). This is a powerful mechanism for regulation of plant stress response (Mastrangelo et al. 2012). Alternatively spliced Ran transcripts have rarely been identified. Fang et al. (2016) isolated an alternatively spliced DlRan transcript containing a premature terminator codon from somatic embryos of longan and
proposed its involvement in regulation of the DlRan gene expression. In the present study, three alternatively spliced transcripts of MsRan were isolated from wild banana. Exon deletions were detected in the coding regions of MsRan3A-1a and MsRan3A-6a. The predicted protein encoded by MsRan3A-1a contained all of the conserved motifs, whereas that of MsRan3A-6a lacked the G4 and G5 boxes, which are involved in GTPase hydrolysis. MsRan3A-1a was not detected in any tested sample, whereas MsRan3A-1 was differentially regulated in various tissues as well as under the low temperature and SA treatments. In contrast to MsRan3A-6, MsRan3A-6a was found to be more abundant in flowers than in peels (Fig. 3). We propose that MsRan3A-1a and MsRan3A-6a may have different functions than their wild-type counterparts. The exon deletion in MsRan3A-5 was found in the 3’-UTR. The 3’-UTR contains various regulatory elements and plays roles in post-transcriptional regulation of gene expression (Garneau et al. 2007, Keene 2007). Unlike MsRan3A-6, the highest expression of MsRan3A-5 was detected in flowers and pulp. These results suggest that MsRan3A-5 and MsRan3A-6 might function differently. Further study is needed, however, to elucidate whether the exon-deletion in the 3’-UTR of MsRan3A-5 has altered its function.

Plants respond to stresses by reprogramming gene expression, thereby giving rise to metabolic alterations. To activate gene expression, environmental signals must be transduced into a nucleus through a series of signal transduction pathways. Nucleo-cytoplasmic partitioning of proteins is proposed as a vital mechanism involved in regulation of plant response to environmental signalling (Merkle 2003, Meier and Somers 2011). These results confirm the importance of Ran, which plays a role in nucleo-cytoplasmic trafficking proteins.

Fig. 6. The effects of a low temperature (8 °C) on expression of MsRan genes in wild banana. The total RNA was extracted from 8 °C treated shoot cultures at different time points and subjected to real time qPCR analysis. All data were normalized to the expression of 18S rRNA. Means ± SEs, n = 3. Significant differences were determined by one-way ANOVA (P < 0.05) and are indicated by different letters.
Ran was shown to participate in response to stresses in several plants (Ferreira et al. 2006, Jiang et al. 2007, Li et al. 2007, Xu and Huang 2008, 2010, Yoshimura et al. 2008, Chen et al. 2011, Paul and Kumar 2011). Chen et al. (2011) reported that 4 °C treatment of rice significantly enhances expression of OsRAN2. However, we found that expressions of MsRan genes increased slightly after 1 and 4 h, then decreased and remained at a low level after the seedlings were treated at 8 °C for 8 h. When the seedlings were exposed to different temperatures, the abundance of MsRan genes varied. We thus propose that the differences of temperature are responsible for the discrepancy between our results and the previous study. The low expression of MsRan transcripts when treated by 8 °C for more than 8 h may be related to growth arrest and injury, which may occur under this temperature (Yang et al. 2012). Our results also indicate that MsRan genes respond to low temperature in various patterns. When the temperature was lower than 8 °C, expressions of MsRan3A-1 and MsRan3C-1 were re-enhanced. These could be due to that the banana seedlings adapted their metabolic processes to cope with the cold stress. These results suggest that MsRan genes are involved in a response to low temperature stress.


Salicylic acid treatment increases chilling tolerance of many plants (Mutlu et al. 2013a). Kang et al. (2003a,b)

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Fig. 7. Expressions of MsRan genes under salicylic acid (SA) treatment in wild banana. Shoot cultures sprayed with 0.5 mM SA or water (control) and incubated under 8 °C. The total RNA was extracted from treated and control plantlets at different time points and subjected to real time quantitative PCR analysis. All data were normalized to the expression of 18S rRNA. Means ± SEs, n = 3. Significant differences were determined by one-way ANOVA (P < 0.05) and indicated by asterisks.
demonstrated that SA treatment enhances chilling tolerance of banana seedlings. In the present study, MsRan gene expressions in seedlings treated with 0.5 mM SA were significantly enhanced. Especially at the end of the treatment, the MsRan genes were still expressed at a high level. We propose that SA modulates MsRan expression, and a high expression of MsRan genes induced by SA is responsible for an enhanced chilling tolerance of banana.

In conclusion, six MsRan transcripts including three alternatively spliced transcripts were isolated from wild banana and bioinformatically characterized. The high similarity of these sequences to other plant Ran sequences suggests the former are orthologs. The MsRan genes were differentially regulated in various tissues, and they were involved in responses to the low temperature stress. Also, SA significantly enhanced expression of the MsRan genes.

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