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Identification of a PTC-containing *DlRan* transcript and its differential expression during somatic embryogenesis in *Dimocarpus longan*

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ABSTRACT

RAN (Ras-related nuclear protein) plays crucial roles in multiple cellular processes in yeast, animals and plants. Here we present a *DlRan* gene and its alternative splicing transcripts containing premature terminator codons (PTCs), identified from embryogenic cultures in longan. Multiple alignment and splicing pattern analyses indicated that *DlRan-1* transcript harboring PTC was the consequence of alternative splicing. The accumulation of *DlRan* PTC-containing transcripts increased significantly when the embryogenic calli were treated with the translation inhibitor, cycloheximide, indicating that *DlRan-1* may be targeted by NMD. The analysis of expression profiles of *DlRan* transcripts revealed that differential expression levels of the alternative spliced *DlRan* transcripts occurred during the development of embryogenic callus, globular-shaped embryos, and cotyledon-shaped embryos, respectively, in the longan somatic embryogenesis, and were in consistent with the embryo development in corresponding wild-type transcripts. The present work offers evidence to speculate that the alternatively spliced PTC-containing transcripts can be functional and may shed light on expression regulation of *DlRan* during development of the longan somatic embryos.

1. Introduction

Longan (*Dimocarpus longan* Lour.) originated in the south of China, is a member of the Sapindaceae, a family also including the fruit lychee (Anupunt and Sukhvibul, 2005; Lai et al., 2000). Longan is an evergreen drupaceous fruit crop, which is widely grown in many subtropical and tropical countries, mainly in China, Thailand, India, Australia and the United States (Matsumoto, 2006). It is an important commercial fruit crop, particularly, in the south-east of China, in Fujian province which is considered as one of the largest longan fruit production regions in the world, with an estimated growing areas of over 15,000 ha and production yield of 200,000 tons per annum (Huang et al., 2005; Lai et al., 2000). Longan fruit are very sweet and tasty and are one of the most favored and popular tropical fruits in China. In recent years, the production of longan fruit has increased steadily and contributed greater benefits to both growers and local economy.

An extraordinary phenomenon in fruit development occurs in selected longan cultivars, in which the development of embryo or seed is halted halfway and an abortive seed arises. As a trade-off, the abortive seeds usually develop much thicker arils than those of normal seeds. The character of abortive seeds is considered a valuable and important index for premium cultivars in longan breeding due to their high ratio of fresh aril and seed. In recent years the mechanisms involved in the formation of aborted seeds in longan fruit development have been studied extensively (Xu et al., 2012). Somatic embryogenesis (SE) resembles zygotic embryos and undergoes almost the same developmental stages (Dodeman et al., 1997) and can serve as an ideal model system for the study of morphological, physiological, molecular and biochemical events occurring during the onset of embryogenesis (Lai et al., 2000; Sharma et al., 2008; Yang and Zhang, 2010; Zimmerman, 1993). Lai et al. (2000) established a highly efficient SE system from immature longan zygotic embryos (40 to 50 days after fertilization) and by using the SE model system, considerable work including controlling synchronization of embryogenesis (Chen and Lai, 2002) has been done to understand the developmental pathways of the longan SE.

SE consists of reprogramming differentiated somatic cells toward the embryogenic pathway. Characterization of genes taking part in signal transduction and transcription factor regulation pathways, such as *SE receptor-like kinases* (*SERKs*) (Schmidt et al., 1997) and *LEAFY COTYLEDON* (*LEC*) (Curaba et al., 2004) has triggered a great interest in revealing cellular and molecular mechanisms of SE in higher plants (Yang and Zhang, 2010). In our previous work, we found that RAN protein was expressed differentially at different embryo developmental stages in longan SE (Fang et al., 2011). Based on this finding, we anticipated that RAN might be involved in some regulatory functions in the process of embryogenesis.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; AS-NMD, alternative splicing coupled with NMD; CE, cotyledon-shaped embryos; CHX, cycloheximide; EC, embryogenic callus; GE, globular-shaped embryos; LEC, LEAFY COTYLEDON; NMD, nonsense-mediated mRNA decay; PTC, premature terminator codons; RACE, Rapid amplification of cDNA ends; RAN, Ras-related nuclear protein; RANGAP1, RAN GTPase-activating protein; RCC1, regulator of chromosome condensation 1; RT-PCR, reverse transcription polymerase chain reaction; SE, somatic embryogenesis; SERK, SE receptor-like kinases; SMG, Suppressor with Morphogenetic effects on Genitalia; UPF, Up-stream frameshift.

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RAN (Ras-related nuclear protein) is one of five small GTPase superfamilies and is an abundant Ras-like GTPase, which was initially characterized for its role in modulating the nucleo-cytoplasmic transport of macromolecules across the nuclear envelope (Bischoff et al., 1999; Joseph, 2006). Like other GTPases, RAN switches between a GTP-bound and a GDP-bound form and these transitions are regulated by a guanine nucleotide exchange factor, regulator of chromosome condensation 1 (RCC1) and the GTPase-activating protein (RANGAP1). RCC1 is mainly located in the nucleus, while RanGAP1 is largely cytosolic. This asymmetrical distribution is important in determining the directionality of nuclear transport (Gorlich and Kutay, 1999). From animal cell studies, there is considerable evidence that RAN controls several key cellular processes, from mediating the nuclear transport of RNA and proteins, to guiding spindle assembly at the onset of mitosis and nuclear envelope reassembly at the end of mitosis (Görlich, 1998; Hetzer et al., 2000; Zheng, 2004). Although functions of *Ran* family genes in nucleo-cytoplasmic transport have been extensively studied in animals and yeasts (Abe et al., 2008; Cimica et al., 2011; Gorlich and Kutay, 1999; Yudin and Fainzilber, 2009), it remains poorly understood in plants (Lü et al., 2011; Wang et al., 2006). In plants, RAN proteins from tomato, *Arabidopsis*, wheat, rice and tall fescue have been characterized and shown to perform functions in primordial meristem development, regulation of cell division and sensitivity to auxin (Ach and Gruissem, 1994; Chen et al., 2011; Kim et al., 2001; Lü et al., 2011; Vernoud et al., 2003; Wang et al., 2006). Wang et al. (2006) reported that overexpression of *TaRAN1* in transgenic *Arabidopsis* and rice increased the proportion of cells in the G2 phase of the cell cycle and resulted in elevation of the mitotic index and prolongation of the life cycle. Overexpression also led to an increased quantity of primordial meristem and decreased number of lateral roots. More recently, it was found that expression of *OsRAN2* was essential for mitosis, with overexpression of *OsRAN2* enhancing cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division (Chen et al., 2011). Evidence from yeast, animals and plants elucidated that RAN homologs are essential for the normal functioning of eukaryotic cells and have been well conserved (Chen et al., 2011).

Post-transcriptional regulation has received focus as an important mechanism to control gene expression in higher plants, and alternative splicing is one of the key components in modulating gene function by expanding the diversity of expressed mRNA transcripts (Cheng and Chen, 2004; Lewis et al., 2003; Reddy, 2007). Alternative splicing can regulate mRNA levels through the production of transcripts containing premature termination codons (PTCs) that link with nonsense-mediated decay (NMD) system (Syed et al., 2012). Eukaryotic cells exhibit quality-control mechanisms that recognize and degrade mRNAs that have not completed nuclear pre-mRNA processing and NMD is one of such quality-control systems. NMD would degrade mRNA possessing premature termination codons (PTCs) that can arise by mutation and gene rearrangements, transcription errors or alternative splicing (Riehs-Kearnan et al., 2012). In mammalian, worm and yeast studies the NMD machinery conferring embryonic lethality such as the core NMD components up-stream frameshift (UPF) and suppressor with morphogenetic effects on genitalia (SMG) protein families have been well documented (Avery et al., 2011; Metzstein and Krasnow, 2006; Wittkopp et al., 2009). Recently, it has been found that NMD is also conserved in plants and several of its components have been functionally characterized in *Arabidopsis* (Arciga-Reyes et al., 2006; Hori and Watanabe, 2007; Riehs-Kearnan et al., 2012). Evidence from these studies demonstrated that NMD is essential for plant viability as null mutations in SMG7 and UPF1 cause embryo and seedling lethality, respectively. To our knowledge, little work has been done to date to explore RAN molecular mechanisms of the regulation of gene expression in plant embryo development or SE.

In the present study, we identified the longan *Ran* candidate gene and expression profiles of *DlRan* were obtained in embryogenic calli, globular and cotyledon-shaped longan SE. We also isolated novel splice variants of *Ran* that harbor premature termination codons (PTCs) from the longan embryogenic cultures, and multiple alignment and splicing analyses indicated that these PTC-containing transcripts were derived from alternative splicing. Furthermore, increasing the accumulation of *DlRan* PTC-containing transcripts as the result of the treatment of cycloheximide, a protein biosynthesis inhibitor, indicated that there might have been the potential regulation by the NMD system in longan SE.

2. Materials and methods

2.1. Plant materials

The establishment of embryogenic callus line "Honghezi" of longan was referred from Lai et al. (2000) and these embryogenic calli were maintained on medium M1 (M0: MS basal salts (Murashige and Skoog, 1962), 2% sucrose and 6 g/L agar, pH 5.8) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and medium M2 (M0 supplemented with 1 mg/L 2,4-D, 0.5 mg/L kinetin, 5 mg/L $AgNO_3$), alternatively, with subculture at an interval of 20 ds. To obtain synchronized somatic embryos at globular-shaped and cotyledonary stages, embryogenic calli cultured on the medium M1 was transferred to the medium M0 supplemented with 0.1 mg/L 2,4-D and M0 (2,4-D free medium), respectively, as described previously (Chen and Lai, 2002; Lai et al., 1997, 2000). All cultures were kept in dark conditions and the temperature maintained at 25 ± 1 °C.

2.2. DNA extraction and isolation of genomic DNA encoding DlRan

Genomic DNA extraction from embryogenic callus was performed using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Isolation of genomic DNA encoding *DlRan* was performed using Takara LA Taq (Takara biotechnology, China) with two specific primer pairs (Supplementary Table S1) following the manufacturer's instructions.

2.3. RNA extraction and quantitative real-time PCR analysis

Total RNAs were extracted from embryogenic callus, globular-shaped and cotyledonary-shaped embryos, respectively, using the TriPure Isolation Reagent (Roche) according to the manufacturer's instructions and treated with DNase I (Takara biotechnology, China) to remove genomic DNA.

The cDNAs were synthesized with random primers and Oligo dT Primer using the SYBR ExScript™ kit (Takara biotechnology, China). Real-time PCR reactions were performed on the Lightcycler 480 system (Roche Applied Science, Switzerland) in a total volume of 20 μL in each well containing 10 μL of 2× SYBR Premix Ex Taq™ II (TaKaRa), 1 μL of cDNA (in 1:10 dilution), and 0.4 μL primers (0.2 μmol each). The PCR conditions were: denaturation 95 °C for 30 s, then 35 cycles of 95 °C at 5 s, 60 °C at 30 s and 72 °C at 30 s. Reactions were run in triplicates and EF-1a and Fe-SOD as endogenous control (Lin and Lai, 2010) was included in each run. Expression data were analyzed with geNORM (version 3.5) (Vandesompele et al., 2002). The specificity of primer annealing was investigated by melting curve with a temperature gradient from 65 °C to 94 °C, then, confirmed by 3% agarose gel electrophoresis followed by sequencing. Primer pairs used for Q-PCR analyses are listed in Supplementary Table S2.

2.4. Rapid amplification of cDNA ends (RACE)

5′ RACE was performed using 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and a gene specific primer: 5′-CCTGTGGA ATGTAACCTGCT-3′. 3′ RACE was performed using a First-Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. 5′ RACE and 3′ RACE reactions were carried out using two specific primers