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Banana *MaEF1A* facilitates plant growth and development

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Abstract

Plant translation elongation factor 1 alpha (EF1A) is both a protein synthesis factor and an important component of plant signal transduction, immune responses, protein trafficking, and apoptosis. However, its role in plant growth and development remains unclear. Herein, a full-length *EF1A* gene was isolated from banana (*Musa acuminata* L.) fruit and termed *MaEF1A*. We found that *MaEF1A* shared a high sequence identify with respective genes in other plants and the deduced amino acid sequence contained conserved regions of GTP-EFTU, GTP-EFTU-02, and GTP-EFTU-03, as well as two tRNA binding domains and six GTP-binding sites which represent functional domains for protein biosynthesis. MaEF1A protein is mainly localized to the nucleus. *MaEF1A* was constitutively expressed in different banana organs including developing fruits, and the highest expression was detected in ovary 4 stage. *Arabidopsis thaliana* L. (ecotype Columbia) was transformed with *MaEF1A* and four transgenic lines were obtained. Three transgenic lines were selected for further phenotypic analyses. Our findings indicate that overexpressed *MaEF1A* could greatly enhance plant height, root length, and both rhachis and silique length by promoting cell expansion and elongation. These experiments suggest an important role for *MaEF1A* in plant growth and development.

Additional key words: *Arabidopsis thaliana,* gene expression, *Musa acuminata*, plant development, transgenic plants.

Introduction

Eukaryotic elongation factors can be divided into transcription elongation factors and translation elongation factors based on their respective roles in biological processes. Transcription elongation factors (TEFs) can facilitate efficient mRNA synthesis and carry out diverse functions during transcription such as modulating the catalytic properties of RNA polymerase II and assisting the progression of that enzyme through repressive chromatin (Saunders *et al*. 2006, Selth *et al*. 2010, Sims *et al*. 2004). In a growing number of studies, TEFs have been shown to play critical roles in plant growth and development. Indeed, plants with deleterious transcription elongation factor-mutations or TEFs that can interact with other proteins, such as the histone H2B mono-ubiquitinase (HUB1) (Van Lijsebettens and Grasser 2010) and the SUPPRESSOR OF Ty4 /5 (SPT4/5), involved in auxin-related gene expression in *Arabidopsis* (Dürr *et al*. 2014), have illustrated the importance of TEFs in maintaining proper gene expression programs (Grasser 2005, Smith and Shilatifard 2013).

Translation elongation factor 1 alpha (EF1A) is an important component for protein biosynthesis (Andersen *et al*. 2003) that can catalyze binding an aminoacyl-tRNA to the ribosome A-site by a GTP-dependent mechanism (Browning 1996). It represents up to 3 - 10 % of total soluble protein and is considered to be one of the most abundant soluble cytoplasmic proteins in a cell (Merrick 1992). Therefore, plant EF1A is not only a protein synthesis factor, but it is also a cytoskeletal protein that plays an important role in plant cells. Indeed, EF1A

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Abbreviations: CaMV - cauliflower mosaic virus; CT - cycle threshold; EF1A - elongation factor 1 alpha; GFP - green fluorescent protein; ORF - open reading frame; RT-PCR - reverse transcriptase-polymerase chain reaction; SEM - scanning electron microscopy; TEF - transcription elongation factor.
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proteins play important roles in signal transduction (Yang *et al*. 1993), viral infections (Yamaji *et al*. 2010, Furukawa *et al*. 2014), and mitochondrial tRNA import (Bouzaidi-Tiali *et al*. 2007). They are also thought to have a role in apoptosis (Ejiri 2002), the regulation of DNA replication and repair networks (Toueille *et al*. 2007), in addition to their molecular chaperone-like activity (Hotokezaka *et al*. 2002, Shin *et al*. 2009). However, little is known about the function of EF1A in plant growth and development.

Among the most important tropical fruits, the banana has the largest biomass. The isolation and mechanistic characterization of key genes that control plant growth and development has a great potential to improve banana yields and quality. Herein, we report the isolation of a full-length cDNA of EF1A, termed *MaEF1A*, from banana and describe our investigation into its role in plant growth and development. Our findings identify a new role for *MaEF1A* in plant growth and development.

Materials and methods

Plants and treatments: Banana (*Musa acuminata* L. AAA group, cv. Brazilian) roots, stems, leaves, flowers, and developing ovaries were obtained from the Institute of Tropical Bioscience and Biotechnology banana plantation (Chengmai, Hainan). Fruits were harvested at the mature green stage (100 - 110 d after flowering). Each sample was frozen separately in liquid N_2 and stored at -70 °C before RNA extraction.

Seeds of wild-type *Arabidopsis thaliana* L. (Columbia ecotype) were purchased from the Arabidopsis Biological Resource Center (the Ohio University, Columbus, Ohio, USA). The seeds were sown on a 1:1:8 mixture (m/m/m) of *Vermiculite*, *Perlite*, and peat moss and irrigated with water every other day unless stated otherwise. *Arabidopsis* plants were grown in a climate chamber at a temperature of 22 °C, an air humidity of 70 %, a 16-h photoperiod, and an irradiance of 50 μmol m^{-2} s^{-1} (*Sylvania GRO-LUX* fluorescent lamps; Utrecht, The Netherlands).

Cloning and sequence analysis of *MaEF1A*: A full-length cDNA sequence of 1 341 bp, termed *MaEF1A*, had been obtained by screening a postharvest banana fruit cDNA library with a pair of λTriplEx2 arm primers, Ptr5 (CTCCGAGATCTGGACGAGC) and Ptr3 (TAATAC GACTCACTCACTATAGGG). The PCR conditions were as follows: 94 °C for 3 min followed by 32 cycles of amplification (94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min), and finally 72 °C for 7 min. The amplified PCR product was inserted into a *pGEM-T easy* vector (*Promega*, Madison, USA) and sequenced on both strands by the *Sangon Company* (Shanghai, China). The resulting sequence was deposited in GenBank and analyzed by *BLAST* (http://ncbi.nlm.nih.gov/blast). Conserved domains were analyzed using *InterProScan*. Motifs were identified using *Scanprosite* (www.mybiosoftware.com/tag/scanprosite). Sequences in the *NCBI* database with the highest similarities to MaEF1A were aligned using the *DNAMAN* software.

RNA extraction, cDNA synthesis, and real-time RT-PCR: The total RNA from all tissues used in this study was extracted using the methods of Asif *et al*. (2000) and Aragón *et al*. (2010). A poly(A)$^+$ mRNA (200 ng) was converted into a cDNA using a *SMART* PCR cDNA synthesis kit (*Clontech*, Palo Alto, USA) in a final volume of 0.02 cm^3, which was subsequently used as real-time PCR template.

Primer sets used in real-time RT-PCR for *MaEF1A* were as follows: *MaEF1A*P1 (GAATGTTGCTGTAAA GGACCTG) and *MaEF1A*P2 (TCATCTTCACCA AACCTGC); *MaActin*P1 (TGTAGCAATTCAGGC TGTTCTT) and *MaActin*P2 (TCAGAGATGGCT GGAAGAGAAC); *AtActin*P1 (GGTAACATTGTGC TCAGTGGTGG) and *AtActin*P2 (AACGACCTTAAT CTTCATGCTGC). A *SYBR® Premix Ex Taq*™ (*TaKaRa*, Dalian China) was used in a 0.025 cm^3 reaction mixture with 0.005 cm^3 of a *ROX* reference dye. Each primer was used at a 100 nM concentration with the equivalent of 100 ng reverse-transcribed RNA template per reaction. In all experiments, appropriate negative controls containing no template RNA were subjected to the same procedure to exclude or detect any possible contamination. Before initiating experiments, a series of template dilutions were performed to determine an optimal template concentration for use in the experiments to maximize target amplification.

Each real-time quantitative PCR was performed using a *Mx3000P* device (*Stratagene*, La Jolla, CA, USA) and *SYBR* chemistry. Thermal cycling conditions were as follows: 94 °C for 3 min followed by 40 cycles of 94 °C for 7 s, 55 °C for 10 s, and 72 °C for 15 s. The samples were run in triplicate, and each experiment was repeated twice. The cycle threshold values (CT) were determined and the relative fold differences were calculated based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001, Hershkovitz *et al*. 2013) using *MaActin* or *AtActin* as endogenous reference genes.

Fusion construct of MaEF1A and its subcellular localization: To generate a MaEF1A construct, the *MaEF1A* cDNA was amplified from a banana fruit cDNA library by PCR using primers *MaEF1A*P3 (CCGCCATGGCAATGG GAAAGGAGAAGGTT) and *MaEF1A* P4 (GGACTAGTTTTTTCTTTTGGGCT GC). The two primers contained *Nco*I and *Spe*I recognition sites, respectively, marked as underline. The resulting PCR fragment, *MaEF1A*, was digested with *Nco*I and *Spe*I and subcloned between the cauliflower mosaic virus (CaMV) 35S promoter and the green fluorescent protein (GFP) reporter gene in a pCAMBIA1302 plant

transformation vector that had also been digested using *Nco*I and *Spe*I. The *MaEF1A* coding sequence in the construct was verified by both restriction analysis and DNA sequencing prior to subcloning. The resulting constructs were introduced into onion epidermal cells using a particle delivery method with a *PDS1000/He* device (*Bio-Rad*, Hercules, CA, USA). The transformed cells were incubated at 26 °C in the dark for 18 h, and green fluorescence was monitored using a fluorescence microscope (*Kawasaki*, Kanagawa, Japan).

***Arabidopsis* transformation:** Transformation of *A. thaliana* was performed using the vacuum infiltration method as previously described (Bechtold *et al*. 1993, Zhu *et al*. 2012) with the *Agrobacterium tumefaciens* L. strain LBA4404. The seeds were harvested and plated on a hygromycin selection medium (50 mg cm^{-3}) to identify T1 transgenic plants. The T1 (line 5) selfed and T3 progenies that were homozygous for hygromycin resistance were used for subsequent studies.

Southern blot analysis: The total genomic DNA was isolated from leaves of both control and putatively transformed plantlets using a *DNeasy* plant mini kit (*Qiagen*, Vanecia, CA, USA). The genomic DNA was digested using *Hind*III under conditions specified in the manufacturer's instructions. Fragments were separated on a 1.2 % (m/v) agarose gel and then blotted onto a nylon membrane (*Amersham*, Chalfont, Buckinghamshire, UK). Crosslinking by UV was performed using a *CL-1000* UV crosslinker at 8 mJ cm^{-2} for 4 min. A hybridization probe was generated using a DNA fragment amplified from the pCMBIA1302-MaEF1A construct as template and a digoxigenin labeling kit (*Roche*, Mannheim, Germany) according to the manufacturer's instructions. Hybridization was performed at 42 °C for 20 h and the membrane was washed twice in a 2× saline sodium citrate (SSC)/ 0.5 % (m/v) sodium dodecyl sulfate (SDS) solution at room temperature for 5 min, then washed twice in a 2× SSC / 0.1 % SDS solution at 68 °C for 15 min, followed by a 2× maleic acid / 0.6 % (v/v) *Tween 20* wash buffer for

Fig. 1. Sequence alignments and analyses of the putative amino acid sequences of EF1A from different plant species. Sequences from *Musa acuminata* (Ma, AAY56337), *Nicotiana paniculata* (Np, BAA34348.1), *Oryza sativa* (Os, AAC15413.1), *Solanum lycopersicum* (Sl, XP_004251154.1), *Arabidopsis thaliana* (At, NP_200847.1), and V*itis vinifera* (Vv, XP_002277159.1) were aligned using *DNAMAN* software. *Boxes* represent GTP-binding sites; sequences *underlined with boxes* indicate tRNA binding domains.

5 min. The membrane was next soaked in a detection buffer with a nitro blue tetrazolium / 5-bromo -4-chloro -3′-indolyl- phosphate *p*-toluidine salt solution for 4 - 12 h to visualize a blue color.

Scanning electron microscopy (SEM): After sowing, six-day-old cotyledons were fixed in stubs using a double-faced tape and coated with a 10 nm-thick platinum layer in a *MEDo020* coating system (*Bal-Tec*, Kettleshulme, UK). The samples were analyzed using a *FEI Quanta 600 FEG* scanning electron microscope (*FEI Company*, Hillsboro, OR, USA). Observations by SEM were performed in the secondary electron mode operating at 15 kV; images were digitally recorded. The samples were assayed in triplicate; each experiment was repeated three times.

Description of statistics: All experiments were repeated three times, and data presented as means ± standard errors. One-way analysis of variance was performed and interactions between means were separated by the least significant difference at $P = 0.05$.

Results

A full-length EF1A gene termed *MaEF1A* was obtained from a banana cDNA library and deposited in GenBank under an accession number of AAY56337. Sequence analysis reveals that *MaEF1A* was 1 344 bp in length, with an open reading frame (ORF) that encoded a single peptide of 447 amino acid residues. Bioinformatics analysis shows that *MaEF1A* shared a 96 % identity with *Nicotiana paniculata* (Np), *Oryza sativa japonica* group (Os), and V*itis vinifera* (Vv); a 95 % identity with *Solanum lycopersicum* (Sl), and a 94 % identity with *Arabidopsis thaliana* (At). The deduced sequence for the *MaEF1A* cDNA contained an integrated ORF and three conserved regions: GTP-EFTU, GTP-EFTU-02, and GTP-EFTU-03. Moreover, MaEF1A contained two tRNA binding domains (P and DKPLRLPLQDVYKIGGIGTVPVGRVE) and six GTP-binding sites (G, GKS, D, PG, NKMD, and RYDE) as determined by *Scanprosite* analysis (Fig. 1).

There were significant differences in the expression of *MaEF1A* between different tissues. The expression of *MaEF1A* could be detected in roots, stems, leaves, flowers, ovary at stage 8, ovary at stage 4, ovary at stage 1, and at 0 day postharvest fruits. Ovary 4, ovary 1, and the root tissues showed the highest expression, whereas the lowest expression could be detected in the stems. The *MaEF1A* expression in ovary 4 were ~8.7-fold higher than in the stems (Fig. 2).

To analyze the subcellular localization of MaEF1A protein, we generated a MaEF1A-GFP fusion construct. The *MaEF1A* gene was fused in-frame to the N-terminus of the *GFP*-reporter gene under control of the CaMV 35S promoter. The plasmids encoding the GFP alone (Fig. 3*A*,*B*) or the MaEF1A-GFP fusion protein (Fig. 3*C*,*D*) were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment. The control

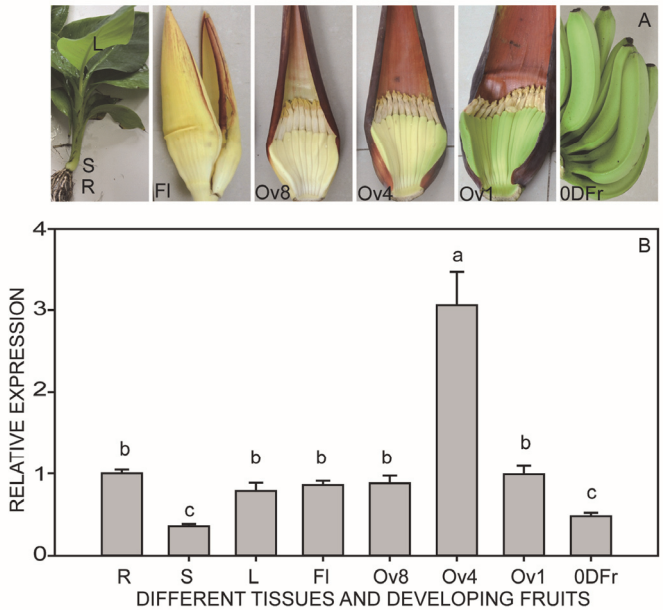

Fig. 2. *A* - Relative expression of *MaEF1A*, as determined by real-time Rt-PCR, in different tissues and stages of fruit development: R - root, S - stem, L - leaf, Fl - flower, Ov8 - ovary at 8 stage, Ov4 - ovaryat 4 stage, Ov1 - ovary at 1 stage, 0DFr - 0 DPH fruits. *B* - The x-axis represents different tissues and stages of fruit development. The y-axis represents relative *MaEF1A* expressions compared to those of *Actin*. Means from three independent measurements and *vertical bars* indicate SE. Significant differences are marked by different letters.

transfected cells showed distribution of GFP throughout the entire cell (Fig. 3*A*,*B*). By contrast, the MaEF1A-GFP fusion protein mostly accumulated in the nucleus (Fig. 3*C*,*D*). The control cells treated with gold particles showed no detectable fluorescence (Fig. 3*E*,*F*).

We selected seven T1 transgenic lines for examination by Southern blot analysis. Our results show that one copy of *MaEF1A* was integrated into the genomes of transgenic lines L3, L6, and L7, whereas two copies could be detected in line L5 (Fig. 4*A*). The expression of *MaEF1A* in transgenic lines L3, L5, L6, and L7 were 3.2-, 3.9-, 3.6-, and 4.9-fold higher than in the wild-type plants (Fig. 4*B*).

We selected three one-copy inserted lines (L3, L6, and L7) for phenotypic analysis. The growth of *Arabidopsis* lines that over-expressed *MaEF1A* was greatly increased. The SEM findings indicate that cotyledon cells of the three transgenic lines were obviously expanded and elongated 6 d after sowing, whereas cotyledon cells of the wild-type plants were packed together and displayed a curly shape (Fig. 5*A,B*). Plant height, root length, and fresh mass per plant in each of the three transgenic lines greatly increased compared to those of the wild-type 15 d after transplanting. Notably for the L7, the plant height, root length, and the fresh mass per plant were 1.5-, 1.7-, and 2.7-fold higher than those of the wild-type (Fig. 5*C*,*D*). We chose the L6 (middle level of gene expression line) to further investigate its flowering and fruiting characteristics. We found that the rhachis of the L6 was longer than that of the wild-type plants (Fig. 5*E*). Moreover, the silique elongated quickly after fruit developing stage 3 (Fig. 5*F,G*).

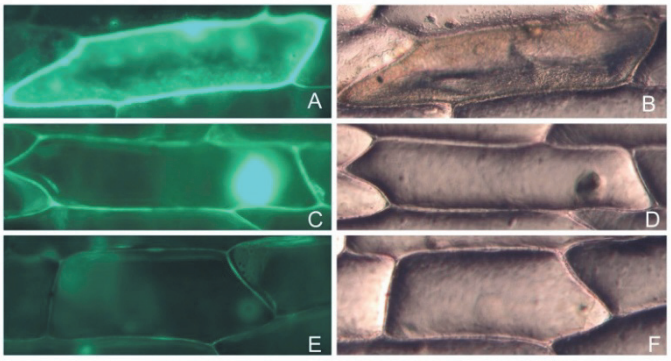

Fig. 3. Subcellular localization of the banana elongation factor 1 alpha - green fluorescent protein fusion protein (MaEF1A-GFP). In onion epidermis cells, pCAMBIA1302-GFP (*A*,*B*) and pCAMBIA1302-MaEF1A-GFP (*C*,*D*) expression vectors were transiently expressed. GFP (*A*,*B*) or MaEF1A-GFP (*C*,*D*) proteins were visualized using a *Nikon* fluorescence microscope 24 h later. *E*,*F* - a negative control, *A*,*C*,*E* - GFP fluorescence, *B*,*D*,*F* - bright-field image.

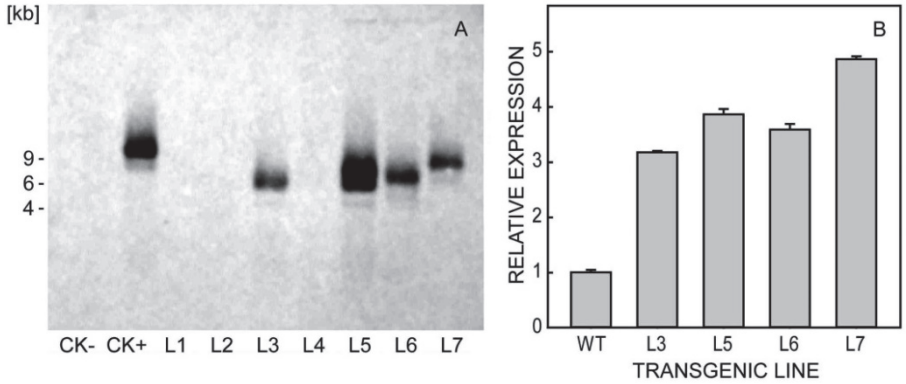

Fig. 4. Analysis of transgenic *Arabidopsis* plants. *A* - Southern blot of transgenic *Arabidopsis*. A total of seven PCR-positive plants were randomly selected for Southern blot analysis. The pCMBIA1302-MaEF1A plasmid was used as a positive control (CK^+), and a non-transformed plant was used as a negative control (CK^-). The blot was hybridized with a probe generated from PCR amplified DNA derived from the pCMBIA1302-MaEF1A construct; line 5 (L5) indicates two insertions, and lines 3, 6, and 7 (L3, L6, and L7) indicate one insertion. *B* - The relative expression of *MaEF1A* in transgenic lines and wild-type (WT), as determined by real-time RT-PCR. Means from three independent measurements and *vertical bars* indicate SE.

Discussion

Plant EF1A participates in protein synthesis and plays important roles in signal transduction (Berthelot *et al*. 2012), immunity (Furukawa *et al*. 2014), regulation (Dürr *et al*. 2014), and apoptosis (Ejiri 2002). However, little is

known about its effects on plant growth and development. In the present study, full-length *MaEF1A* was isolated from the banana genome. Our bioinformatics analysis shows that *MaEF1A* shared a high sequence identity with *N. paniculata*, *O. sativa, V. vinifera*, *S. lycopersicum*, and *A. thaliana*. The deduced amino acid sequence contained the conserved regions GTP-EFTU, GTP-EFTU-02, and GTP-EFTU-03, which suggests it to be a banana EF1A. Moreover, MaEF1A contained two tRNA binding domains and six GTP-binding sites which are functional domains for protein biosynthesis.

Commonly, plant *EF1A* genes are highly expressed in all organs and developing tissues. However, several studies have reported that *EF1A* gene expression can vary during development (Xu *et al*. 2007, Suhandono *et al*. 2014). In the present study, *MaEF1A* was constitutively expressed in various banana organs and developing fruits (Fig. 2). However, the *MaEF1A* expression at the ovary 4 stage, which is a stage when the banana fruit quickly elongates and expands, was especially high (Liu *et al*. 2013) and indicates the need for a high rate of protein synthesis. This finding indicates that *MaEF1A* might play

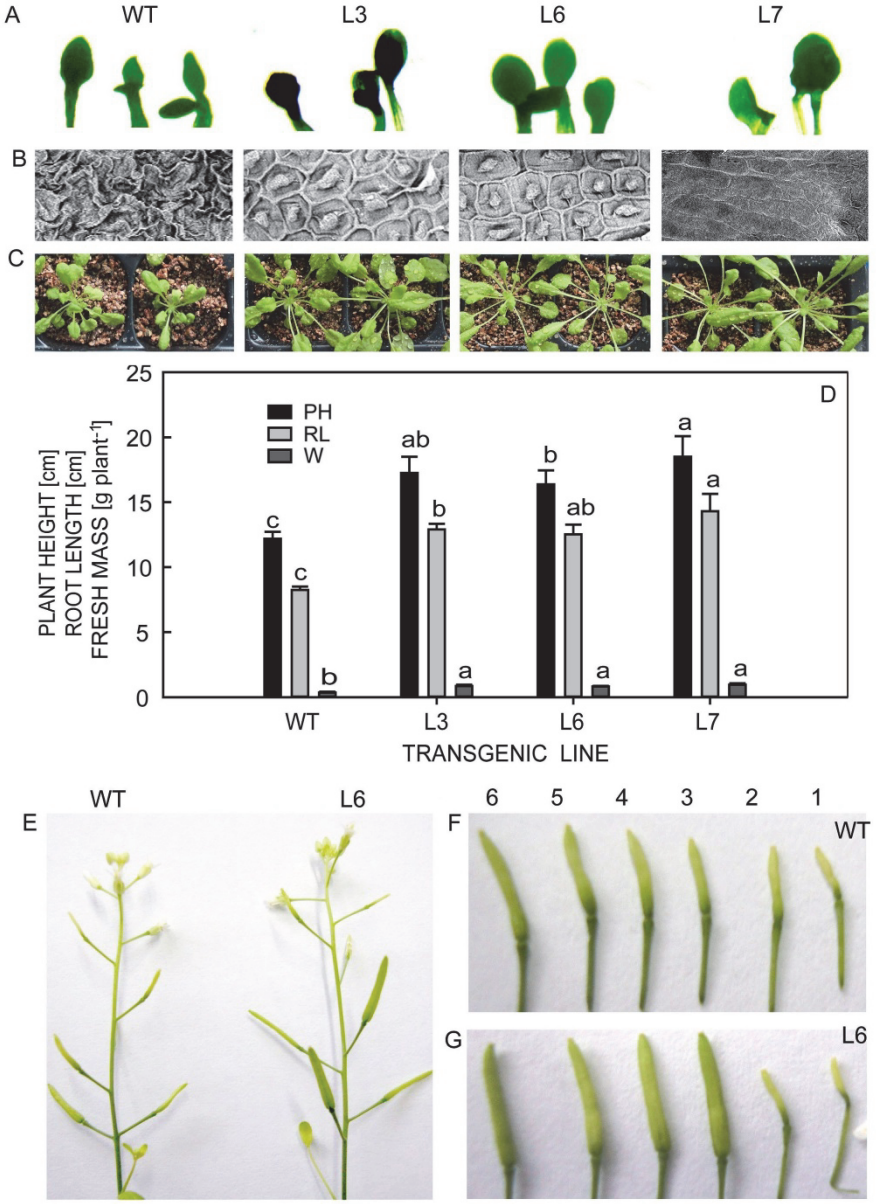

Fig. 5. Phenotypic analysis of transgenic *Arabidopsis*. *A* - Cotyledons of three transgenic lines 6 d after sowing seeds. *B* - Scanning electron microscopy of cotyledons of three transgenic lines 6 d after sowing seeds. *C* - The growth phenotype of three transgenic lines 15 d after transplanting. *D* - Analysis of plant height (PH), root length (RL), and fresh mass (W) per plant of three transgenic lines 15 d after transplanting. Means were generated from three independent measurements and the *vertical bars* indicate standard errors. Significant differences are marked with different letters. Where absent, the *standard error bars* fall within the dimensions of the symbol. *E* - The flower phenotype of the wild type (WT) and L6. *F,G* - Silique analysis of WT and L6.

an important role in banana fruit elongation and expansion. The protein encoded by *MaEF1A* mainly localized to the nucleus suggesting its possible role in regulation of banana growth and development.

To further investigate the *MaEF1A* role in plant growth and development, a plant-expressing vector was constructed and used to transform *Arabidopsis*. Our results indicate that *MaEF1A* was highly expressed in three transgenic lines. Real time RT-PCR analysis detected an additional signal in the wild type plants, which might be a false positive result that occurred because of a high sequence identity. We selected three one-copy insertion lines (L3, L6, and L7) for phenotypic analysis. These analyses reveal that over-expressed *MaEF1A* could greatly improve plant growth and development, including plant height, root length, and rhachis length, by promoting cell elongation and expansion (Fig. 5). Elucidating the mechanism whereby *MaEF1A* promotes cell expansion will require further investigations. Interestingly, the siliques were greatly elongated and expanded, which was consistent with the *MaEF1A* expression pattern in developing banana fruits. Similar results were obtained by Van Lijsebettens and Grasser (2010) and Dürr *et al*. (2014) in their investigations of transcription elongation factor functions. However to date, no reports have been published about the effects of a translation elongation factor on plant growth or development.

In conclusion, this study yielded potential insights into a novel role for *MaEF1A* in plant growth and development by promoting cell elongation and expansion.

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