The Translation Elongation Factor eEF-1Bβ1 Is Involved in Cell Wall Biosynthesis and Plant Development in Arabidopsis thaliana

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Abstract

The eukaryotic translation elongation factor eEF-1Bβ1 (EF1Bβ1) is a guanine nucleotide exchange factor that plays an important role in translation elongation. In this study, we show that the EF1Bβ protein is localized in the plasma membrane and cytoplasm, and that the transcripts should be expressed in most tissue types in seedlings. Sectioning of the inflorescence stem revealed that EF1Bβ predominantly localizes to the xylem vessels and in the interfascicular cambium. EF1Bβ gene silencing in eff1β caused a dwarf phenotype with 38% and 20% reduction in total lignin and crystalline cellulose, respectively. This loss-of-function mutant also had a lower S/G lignin monomer ratio relative to wild type plants, but no changes were detected in a gain-of-function mutant transformed with the EF1Bβ gene. Histochemical analysis showed a reduced vascular apparatus, including smaller xylem vessels in the inflorescence stem of the loss-of-function mutant. Over-expression of EF1Bβ in an eff1 mutant background restored a WT phenotype and abolished ectopic lignin deposition as well as cell expansion defects in the mutant. Taken together, these data strongly suggest a role for EF1Bβ in plant development and cell wall formation in Arabidopsis.

Introduction

Translation is one of the vital processes involved in the fine regulation of gene expression through ensuring direct, rapid, reversible and spatial control of protein concentration [1], and thereby affects developmental processes in both prokaryotes and eukaryotes. Translation elongation in eukaryotes requires a set of soluble non-ribosomal proteins known as eukaryotic elongation factors (eEFs) [2]. They include eEF1A and eEF1B factors, which are involved in the recruitment of aminoacyl-tRNAs onto the ribosome, and eEF2 factor, which mediates ribosomal translocation. eEF1B is essential for growth [3] and plays a role in oxidative stress resistance in yeast [4]. eEF1B is also involved in distributing eEF1A between polypeptide chain elongation and actin-binding activities [5], and in cell cycle regulation [6].

The plant eEF1B is a trimer composed of the structural protein (eEF1Bβ) plus two nucleotide exchange subunits (eEF1Bδ and eEF1Bβ) [6] and is intermediate in complexity between yeast and metazoans. The yeast eEF1B is made up of two subunits, a guanine nucleotide exchange protein (eEF1Bγ) and a structural protein (eEF1Bγ), whereas the metazoan complex is a heteromer of at least four subunits: the structural protein (eEF1Bγ), two nucleotide exchange factors (eEF1Bδ and eEF1Bβ), plus the unique valine-tRNA synthetase (Val-RS) [6]. The nucleotide exchange function is achieved primarily by the eEF1Bδ isoform, and the exact physiological functions of eEF1Bβ and eEF1Bδ are not yet known.

The plant cell wall is a complex and dynamic structure composed of polysaccharides (cellulose and hemicellulose), proteins, and phenolic compounds (primarily lignin, but also other phenolic acid linkages) [7]. The cell wall not only strengthens the plant body, but also plays key roles in plant growth, cellul differentiation, intercellular communication, water movement and defense [8]. Disruption of either cellulose or lignin biosynthetic and regulatory genes leads to stunted phenotypes, irregular xylem development and weak stem formation [9,10], but the link between disruption of monolignol biosynthesis and dwarfism is not clearly established [10]. Recently, a direct relationship between cell wall biosynthesis and cytoskeleton was reported [11,12]. This was significant in light of the physical interaction established earlier between eEF1β and actin in the cytoskeleton of Dictyostelium discoideum [13].

In this study, we investigated the role of eEF-1Bβ1 (locus At1g30230, referred to hereafter as EF1Bβ1) in plant development...
by studying changes in cell wall structure and composition within a dwarfed T-DNA insertion line, SALK_046102C [14]; referred to hereafter as eff, as a result of the mis-expression of the EF1β gene. We also investigated the impact of EF1β over-expression in the Arabidopsis etopic lignification 1 (allelic eli1-1, hereafter el1) mutant. This plant line has a mutation within cellulose synthase CES3 and exhibits ectopic lignification in cells normally free of lignin [15]. Using these genetic tools, we show that EF1β not only affects plant growth and cell elongation, but also plays a role in the biosynthesis of cellulose and lignin and points to EF1β as a novel regulator of plant development and cell wall biosynthesis.

Materials and Methods

Plant materials and growth conditions
SALK_046102C seeds were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, USA [14]. Plants from this accession were mutated in the EF1β gene (At1g30230) and did not show any phenotype until the following generation, when we observed 11 plants (eff) with strong dwarf phenotypes out of 40 plants, indicating that the original SALK_046102C line was heterozygous. The el1 mutant line was obtained from Dr. D. Bonetta at the University of Ontario Institute of Technology, Oshawa, Canada. Arabidopsis thaliana, ecotype Columbia (Col 0), and mutant lines eli1 [15] and eff were grown in Promix BX (www.premierhort.com) in a growth room (16-h light/8-h dark) under fluorescent white light (150 μmol m⁻² s⁻¹) at 22°C after stratification at 4°C for 48 hr to synchronize germination. For plate-grown seedlings, sterilized seeds were sown on 0.5x MS medium with 1% sucrose in the light, or without sucrose in the dark. For growth in the dark, seeds were exposed to fluorescent white light (150 μmol m⁻² s⁻¹) at 22°C for 6 hr to induce germination, after which the plates were wrapped individually with aluminum foil. The age of the seedlings was defined starting at the end of the cold treatment.

Mutant genotyping
Segregating plants from SALK_046102C seeds were used for T-DNA analysis and to develop the homozygous line, eff. The homozygous dwarf plants were used in all subsequent analysis. The T-DNA insertion into the EF1β gene was confirmed in eff plants by PCR with T-DNA border and gene-specific primers (LBB1, 3, LP and RP; Table S1) designed by SIGnAL T-DNA Verification Primer Design Tool (Salk Institute Genomic Analysis Laboratory, CA, USA). To determine the nature of the mutation and T-DNA copy number, the mutant was backcrossed to the WT (Col 0) and the presence of T-DNA in BC₁-F₁ plants was confirmed by PCR. BC₁-F₂ seeds were obtained from BC₁-F₁ plants through selfing. BC₁-F₂ seeds from four individual plants were used for characterization of the eff mutant. Genotyping of the el1 mutant was carried out previously by others [15,16].

Plasmid construction and transformation
The promoter and 5′-UTR of EF1β (1931-bp fragment including the start codon ATG) was amplified from Arabidopsis genomic DNA by PCR (primers P1+P2, Table S1) and cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen). The promoter was recombined into the Gateway destination vector pMDC163 containingUidA gene [17] through an LR clonal reaction (Invitrogen). The coding region of EF1β was amplified by PCR (primers P3+P4, Table S1) from Arabidopsis cDNA and cloned into pGEM-T Easy vector (Promega), then transferred into the binary vector pBINPLUS-35S as a BamHI and SacI fragment to generate the over-expression construct EFBOX. To generate a translational fusion, EF1β cDNA without the stop codon was amplified by PCR (primers P5+P6, Table S1) from Arabidopsis leaf cDNA, cloned into pENTR/D-TOPO, then the insert was transferred into the pEarleyGate 101 vector upstream of the yellow fluorescence protein (YFP) [18] using the Gateway recombination system (Invitrogen) to create the 35S-EF1β-YFP construct. All clones were confirmed by sequencing.

The constructed vectors were electroproporated into Agrobacterium tumefaciens strain LBA4404 except for 35S-EF1β-YFP, which was introduced into GV3101. Arabidopsis plants were transformed by the floral-dip method [19]. For the complementation study, the EFBOX construct was introduced into eff and el1 mutant lines by the same method. Transgenic Arabidopsis seedlings were selected on growth medium containing 0.5x Murashige and Skoog salt mixture (PhytoTechnology Laboratories, KS, USA), 1% (w/v) sucrose (pH 5.8), and 0.8% (w/v) plant agar (Sigma) supplemented with 25 mg/l hygromycin or 50 mg/l kanamycin. Plants positive for the T-DNA were further confirmed by PCR.

Confocal microscopy
Roots of four-day-old Arabidopsis seedlings that were transformed with the EF1β-YFP fusion construct were examined on a DM IRE2 inverted microscope equipped with an HCX PL APO 1.20 63x water-immersion objective. Images were collected in a 512×512 format on a TCS SP2 confocal system (Leica Microsystems) using a scanning speed of 400 Hz. YFP was visualized by exciting the samples with the 514 nm argon laser line and collecting fluorescence with an emission window set at 520-580 nm. To plasmolyze the cells, seedlings were incubated in ½ MS containing 0.75 M sorbitol for 15 minutes, and then incubated in 0 μM SynaptosRed™ Reagent (SR; Calbiochem) for 5 minutes to stain the plasma membrane.

β-glucuronidase (GUS) histochemical assay
For the GUS staining assay, 12-day-old seedlings grown on MS media and tissue collected from the base of the inflorescence stem from 6-week-old plants grown on soil were tested with 5-bromo-4-chloro-3-indolyl glucuronic acid according to Hematy et al. at 37 C for 4 h [20]. Stem pieces were mounted in 3% agarose, and 30 μm sections were prepared using a Leica VT1000S vibratome (www.leica.com). Seedlings and sections were visualized with Nikon SMZ 1500 and Zeiss Axioskop 2 plus microscopes, and images were captured using a NIKON DXM 1200 digital camera.

Histochemical staining for lignin
For phloroglucinol staining, 5-day-old dark green seedlings or 30 μm sections from the base of the inflorescence stems were stained with 2% phloroglucinol in 95% ethanol and concentrated HCl (v/v, 2:1) for 5 min. For Maule staining, 100 μm sections were treated for 10 min with 1% KMnO₄ and then rinsed with water. Sections were then treated for 3 min with 10% HCl, rinsed in water, and mounted in concentrated NH₄OH. For anatomical analysis of el1 and el1-EFBOX plants, 30 μm sections from the base of 6-week-old inflorescence stem were treated with 0.02% aqueous solution of Toluidine Blue. All samples were observed under a light microscope and photographed using a NIKON DXM 1200 digital camera.

Lignin content and monomeric composition analysis
Total lignin from the inflorescence stem was determined using the thioylglicolic acid (TGA) method according to Brinkmann et al. [21] with slight modifications. Briefly, cell wall residue (CWR) was prepared by extracting the ground stem with toluene/ethanol (2:1,
v/v), 95% ethanol, and water (three times each). Extractive-free CWR was dried at 70°C overnight. Aliquots of 10 mg dried CWR (3 replicates per individual sample) were weighed into 2 ml screw cap tubes (Sarstedt) and mixed with 1.5 ml of 2 N HCl and 0.3 ml thioglycolic acid (TGA). Subsequent analysis was carried out as per Brinkmann et al. [21], and the relative amount of lignin was measured considering WT absorbance at 280 nm as 100%.

Lignin monomer composition was determined using thioacidolysis as described by Foster et al. [22], except that 10 mg of CWR was used as the starting material and all reagents were scaled up accordingly. We used an Agilent 7890 GC/5975 MSD with an HP-5MS column (Agilent, 30 m × 0.25 mm i.d., 0.25 μm film thickness) for monolignol analysis. Total ion chromatogram peaks were identified by relative retention times using tetracosane as an internal standard, as well as by determining characteristic mass spectrum ions of 299 m/z and 269 m/z for syringyl (S) and guaiacyl (G) monomers, respectively. The relative composition of the lignin components was quantified by setting the total peak area of the lignin peaks to 100%.

Cellulose content analysis

Cellulose content was determined using a colorimetric method [23] and expressed as μg cellulose mg⁻¹ DW.

Quantitative real-time RT-PCR

For quantitative real time reverse transcription PCR (qRT-PCR) experiments, total RNA was extracted from 6-week-old inflorescence stems using TRIzol reagent (Invitrogen). RNA was treated with Turbo DNAse (www.ambion.com) to eliminate trace amounts of genomic DNA. Reverse transcription reactions were performed with Superscript™ III Reverse Transcriptase (Invitrogen) using 2.0 μg of RNA per reaction; then the cDNA was diluted 25-fold with nuclease-free water. Polymerase chain reactions were carried out in a 96-well plate in a LightCycler® 480 II (http://www.roche-applied-science.com/lightcycler) using SYBR® Green Master Mix (Roche) in a reaction volume of 20 μl. Five reference genes [adenine phosphorybosyl transferase 1 (At1g27450), elongation factor EF1a (At5g60390), eukaryotic initiation factor eIF4A1 (At3g13920), UBC21 (At5g25760), and UBQ10 (At4g05320)] were tested in the experiment, and the two most stable genes (EF1α and eIF4A1) were selected for data normalization using geNorm software [24]. PCR efficiency was determined from amplification plots using the program LinRegPCR [25].

Statistical analysis

The t-tests were performed using the STATISTIX for Windows 2.2 program (Analytical Software, Tallahassee, FL, USA).

Results

Sub-cellular localization of EF1Bβ protein to the plasma membrane

To investigate the subcellular localization of EF1Bβ, we transformed Arabidopsis plants with a translational fusion of EF1Bβ and the yellow fluorescent protein (EF1Bβ::YFP) under the control of the 35S promoter. YFP fluorescence was observed in the cytosol and periphery of epidermal cells of the root tips of stably transformed seedlings (Figure 1A–C). Following plasmolysis with sorbitol, EF1Bβ::YFP localization remained clearly visible in the plasma membrane and cytosol (Figure 1D–F). These results are consistent with the results of proteomics studies that predicted EF1Bβ to be a plasma membrane [26,27] as well as a cytosolic [28] protein. However, in some transformed lines, bright yellow dot like aggregates were observed in the cytoplasm after plasmolysis (Figure S1). It is possible that EF1Bβ::YFP protein partially coagulated at high expression levels because of plasmolysis although this phenomenon remains to be elucidated.

Expression of EF1Bβ promoter in seedlings and inflorescence stem

To investigate the expression pattern of EF1Bβ, 1931 bp upstream sequence containing the putative promoter region and 5’-UTR of EF1Bβ was fused to the UidA reporter gene expressing GUS, and the construct was introduced into Arabidopsis plants.

Figure 1. Confocal images showing localization of EF1Bβ-YFP (A and E) in Arabidopsis root tip cells plasmolyzed with 0.75 M sorbitol. SynaptoRed (SR) was used as a plasma membrane marker (B and F). Panels C and G are the merged images of the YFP and SR channels and D and H show the DIC images. Lower panels (E-H) show a close-up of root tip cells. Bars = 10 μm. Arrowheads (A and E) highlight the YFP localization in the cell periphery.

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Twelve-day-old seedlings from 10 independent lines showed ubiquitous GUS staining activity throughout the plant (Figure 2A,B,C,D,E) although the expression was relatively low in hypocotyls. GUS activity was most intense in the elongating zones, such as just above the root tips (Figure 2C). Sections from the basal part of the inflorescence stem of 6-week-old plants were used to analyze promoter expression in the vascular elements. GUS activity was mainly concentrated in the vascular bundles, particularly in the xylem and phloem, and in the interfascicular cambium region (Figure 2F). Flower and silique epidermis layers showed fairly strong expression, with the strongest expression in the pistil and stamens (Figure 2G, H), but almost no expression was observed in the seeds (Figure 2I). In summary, GUS expression was highest in elongating cells and in vascular tissues, indicating that EF1β might be associated with cell wall biosynthesis in interfascicular and xylary fibers in addition to its role in translation elongation.

We also investigated the expression pattern of the EF1β gene in different tissues of WT Arabidopsis by qRT-PCR (primers listed in Table S1). EF1β transcripts were detected in all of the tissues under study (Figure 3), but transcript abundance varied among the tissues. Relatively higher levels of EF1β transcript were detected in root samples from seedlings and 6-week-old plants than in other tissues (P≤0.01) (Figure 3). This expression pattern is in accordance with data obtained using the EF1β promoter::GUS fusion (Figure 2).

Genetic analysis and complementation of the efβ mutant (SALK_046102C)

To dissect the role of EF1β in cell wall formation, we initially confirmed the genetic basis and phenotype of efβ, a T-DNA insertion mutant of EF1β (SALK_046102C). At the seedling stage, all the efβ plants grew normally and no visible phenotype differing from the WT phenotype was observed. However, a
strong dwarf phenotype (Figure 4) developed later during rosette development and reproductive growth in some plants. To determine the nature of the mutation and T-DNA copy number, homozygous dwarf efβ was backcrossed (BC) to the WT (Col 0). All 35 BC F1 plants showed WT phenotype and the presence of T-DNA. Of 266 BC F2 plants, 200 plants showed a WT phenotype, whereas 66 plants showed a mutant phenotype. This result conforms to a theoretical segregation ratio of 3:1, indicating a monogenic recessive mutation ($P \leq 0.01$). We randomly selected 32 plants with WT phenotypes from the BC F2 population and used genomic DNA from segregating plants as template for PCR to determine the presence or absence of T-DNA. Of 32 plants, 21 plants had T-DNA insertion, whereas 11 plants did not contain T-DNA. This result supports a theoretical ratio of 2:1 and also reflects a monogenic recessive mutation caused by T-DNA insertion at a single locus ($P \leq 0.01$).

To support the above evidence that disruption of EF1βB function by T-DNA insertion is responsible for its dwarf phenotype, we performed a complementation experiment, in which mutant effβ plants were transformed with an EF1βB over-expression construct EFBOX. We obtained five independent transformants (T1) with a restored (WT) phenotype (Figure 4). In addition, the expression of EF1βB in WT and effβ lines was measured in inflorescence stems from 6-week-old plants by qRT-PCR (primers listed in Table S1). In effβ, no expression of EF1βB was detected, whereas significant expression was detected in the WT (Figure 5A). These results confirm that effβ and its stunted phenotype are a direct result of T-DNA insertion into the EF1βB gene.

Effect of EF1βB on cell wall structure

To investigate the consequences of altered EF1βB expression on cell wall structure, we over-expressed EF1βB in WT to develop an over-expression line (transformed with 35S::EF1βB, hereafter referred to as EFBBOX) and used it in subsequent analysis along with effβ. The expression level of EF1βB in inflorescence stem as determined by qRT-PCR was 10-fold higher in EFBBOX compared to WT (Figure 5B). For structural analysis, sections from the basal part of the inflorescence stem of 6-week-old plants of WT, EFBBOX and effβ were histochemically stained with phloroglucinol, Maule and Toluidine Blue reagents, and analyzed by light microscopy. Phloroglucinol stain reacts with coniferaldehyde groups in lignin, and the color intensity grossly reflects the total lignin content [29]. With phloroglucinol, effβ plants exhibited significantly shrunken interfascicular fibers, and smaller sized and a reduced number of xylem vessels, which suggested that overall lignin content was reduced in effβ compared to the WT and EFBBOX lines (Figure 6A–C). Yellow-brown coloration from Maule staining indicated that the xylary elements of all three types of lines were predominantly composed of G-lignin (Figure 6D–F). Red coloration (from Maule staining) indicated that S-enriched lignin [29] was predominant in the interfascicular region of all three types of lines, and was also present in a patchy pattern within the vascular region of EFBBOX (Figure 6F). However, the extent of the red coloration was somewhat lower in effβ due to the reduced interfascicular fibers (Figure 6D–F). In Toluidine Blue staining, some cells in between the cortex and phloem of the effβ stem showed unusual expanded cell shapes in addition to smaller and reduced xylem elements, and these unusual features were not found in either WT or EFBBOX stems (Figure 6G–I). These histochemical observations indicated that

Figure 4. Phenotypes of Arabidopsis wild type, effβ and EFBBOX-complemented plants. Complemented mutant plant was transformed with a 35S::EF1βB cDNA.
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Figure 5. EF1βB expression in inflorescence stem of WT and effβ (A), and of WT and EFBBOX (B). Data represent mean transcript abundance ± SD relative to EF1α and elF4A1 from three independent experiments each replicated three times. ** indicates significant difference at $P \leq 0.01$.
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disruption of EF1β expression caused a reduction in total lignin content (mainly S-lignin) concurrently with growth reduction and a reduction in vascular elements and interfascicular fibers. They also showed that over-expression of EF1β increases these elements and fibers in Arabidopsis (Figure 6C, F, I).

**Effect of EF1β on lignin content and composition**
Changes in plant phenotype as well as structural and histochemical changes in the inflorescence stem of efβ prompted us to investigate the total lignin content in the inflorescence stems of WT, EFβOX and efβ plants by the thioglycolic acid method [21]. Total lignin content was reduced by 38% in inflorescence stems of efβ plants compared to the WT, whereas the lignin level was almost unaffected in EFβOX plants (Figure 7A). This result is consistent with lignin histochemical analysis (Figure 6).

Angiosperm lignin predominantly contains G and S subunits [10,22]. Therefore, we determined the relative amounts of G and S monomers in the inflorescence stems by thioacidolysis [30]. This analysis showed that the S-lignin rather than G-lignin (and hence the S/G ratio) was significantly lower in efβ plants compared to WT, but no significant differences in S/G ratios could be detected between EFβOX and WT plants (Table 1).

**Effect of EF1β on cellulose content**
Cellulose is the main component of cell wall and is indispensable for growth and development. Since disruption of EF1β function resulted in a dwarf phenotype and affected lignin, modulation of EF1β expression might also affect cellulose content. To investigate this possibility, we measured the cellulose fraction of cell wall from the inflorescence stem using a quantitative colorimetric assay [31]. We found no significant change in cellulose content in EFβOX, whereas efβ showed a 20% reduction in cellulose level relative to WT (Figure 7B). A significant reduction (20%) in cellulose level may reflect a general decrease in secondary wall thickening in efβ plants.

**EF1β over-expression affected cellulose biosynthesis genes**
Because of the phenotypic and anatomical changes caused by altered EF1β expression, we set out to evaluate the effect of
EF1Bβ on the expression of select cell wall-related genes in inflorescence stems of EFβOX and efβ plants.

The transcript levels of 10 genes involved in the lignin biosynthetic pathway (listed in [32]) and LACCASE4 (LAC4) did not change significantly between the lines (Figure 8A, B). We also tested the expression pattern of one primary cell wall cellulose synthase gene CESA3 [33], three secondary cell wall genes CESA4, CESA7 and CESA8 [34], and a membrane-bound endoglucanase KORRIGAN1 (IRX2). Of all cellulose genes tested, only CESA3 and CESA7 showed significant differences in expression, but only in the EFBOX plants, where CESA3 and CESA7 expression was increased in relation to expression in efβ (Figure 8B).

Table 1. Lignin composition of EF1ββ gain- and loss-of-function mutants.

| Genotype | Monomer composition | S/G |
|----------|---------------------|-----|
|          | S lignin (%)        | G lignin (%) |
| WT       | 24.56±0.38          | 75.44±0.38   | 0.32   |
| EFβOX    | 22.01±2.51          | 77.99±2.51   | 0.28   |
| efβ      | 15.08±2.06*         | 84.92±2.06*  | 0.17*  |

Lignin monomer composition in the inflorescence stem was determined by thioacidolysis method. Data presented as means ± SD of three independent experiments with three technical replicates for each experiment. * indicates significant difference of S/G ratio in efβ relative to the WT and EFβOX at P≤0.05. doi:10.1371/journal.pone.0030425.t001

EF1β1 rescues the eli1 phenotype and abolishes ectopic lignification

The eli1 mutant exhibits ectopic lignification and developmental abnormalities, including a stunted phenotype and disorganized xylem [15], due to a mutation in CESA3 [16]. In addition to cellulose biosynthesis, this gene plays a role in normal cell expansion [15]. By evaluating eli1 and several other dwarf mutants, these authors found a linkage between cell expansion, the initiation of secondary cell wall formation, and subsequent lignification. As our results suggested a role for EF1ββ in plant growth and development, we investigated whether EF1ββ had any role in the eli1 phenotype. When we expressed 35S::EF1ββ in the eli1 background (hereafter referred to as eli1-EFβOX), transformants showed a restored growth phenotype similar to that of the WT (Figure 9A) indicating a role for EF1ββ in rectifying the growth defects of eli1. We also determined the transcript levels of...
**Figure 9.** Phenotypes of Arabidopsis wild type, eli1 and EF1-FOX-complemented plants (A). Expression of EF1ββ in WT and eli1-EF1OX inflorescence stems (B). Data presented as mean transcript abundance ± SD relative to EF1β and eEF4A1 of three independent experiments and each replicated three times. ** indicates significant differences relative to WT transcript levels at P ≤ 0.01. Phloroglucinol staining of roots (C) and hypocotyls (D) from 5-day-old dark grown seedlings showing ectopic lignification in eli1 but not in eli1-EF1BOX or in WT background. Cross sections of the base of the inflorescence stem of 6-week-old plants; WT (E,H), eli1 (F,I), and eli1-EF1OX (G,J) stained with phloroglucinol (E-G) and Toluidine Blue (H-J).

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EF1ββ in eli1-EF1BOX and WT inflorescence stems to establish a link between eli1 phenotype rescue and EF1ββ transcript level. The rescued plant showed an 11-fold higher expression of EF1ββ relative to WT (Figure 9B). The higher expression of EF1ββ in the restored plants supports the notion that EF1ββ over-expression was responsible for the phenotypic complementation of eli1.

Five-day-old seedlings of WT, eli1 and eli1-EF1BOX were used to compare lignin accumulation using phloroglucinol-HCl. Seedlings were grown in the dark without sucrose so they remained photosynthetically inactive and avoided any influence of external sugar on lignin [35]. This way the genetic makeup would predominantly contribute to lignin deposition. Under these conditions, phloroglucinol staining in the eli1 mutant showed patches of cells which accumulated ectopic lignin in both the root and hypocotyl, but were more concentrated in the root (Figure 9C, D). This result is consistent with previous studies on eli1 [15,35], which showed strong ectopic lignin accumulation in the roots of seedlings grown in darkness. In contrast, no ectopic lignification was observed in the root or hypocotyl of either WT or eli1-EF1BOX seedlings grown under the same conditions (Figure 9). These observations indicate that EF1ββ over-expression abolished ectopic lignification in eli1 and restored the WT phenotype. Basal stem sections of eli1 showed moderately stained ectopic lignification in the pith parenchyma cells, while the intensity of staining in the xylem elements and interfascicular fibers of eli1 was relatively low (Figure 9F). Again, this lignin abnormality was absent from the eli1-EF1BOX plants, showing a restored WT phenotype (Figure 9G).

Sections from the basal part of inflorescence stems were also stained with Toluidine Blue to observe cell structure. Sections of eli1 exhibited smaller cells with disrupted xylem vessel formation and abnormal development of cortex cells, tracheids, and some pith parenchyma cells (Figure 9I) as reported previously [15]. In contrast, sections from the rescued plant showed normal cell size and vascular development similar to that of the WT (Figure 9J). These observations established that EF1ββ can complement the cell expansion defect of eli1 and ultimately abolish ectopic lignin accumulation in the eli1 mutant.

Expression profile of cell wall genes in eli1-EF1BOX

In the above experiments we found that EF1ββ over-expression in eli1 background restored the WT phenotype and abolished ectopic lignification in eli1 (Figure 9). To extend this analysis to the transcriptional level, we compared the expression pattern of phenylpropanoid and cellulose biosynthesis genes, namely PAL1, C4H, CCaOMT1, F3H1, OMT1, CES13, CES14, CES17 and CES18, in the WT and eli1-EF1BOX plants using 2-week-old seedlings grown on plates. Except for CES13, no other gene including CES17 showed statistically significant differences in expression in WT and eli1-EF1BOX lines (Figure S2). CES13 expression was almost two-fold higher in eli1-EF1BOX relative to WT.

Discussion

Accumulating evidence indicates that components of the translational apparatus have functions in cells beyond their conventional role in protein synthesis [36]. In plants, cytoskeleton and cell wall biosynthetic activities now appear to be closely linked [11,12,37]. Due to this close relationship and the fact that EF1B is an actin-binding protein [13], we hypothesized that EF1ββ may play a role in plant development and cell wall biosynthesis. To investigate this hypothesis, we used gain- and loss-of-function mutants of EF1ββ in a detailed study using molecular, biochemical and histological approaches.

Through a localization study, we found that EF1ββ is likely localized to the plasma membrane and cytosol. The plasma membrane localization is compatible with a role for EF1ββ in the synthesis of cell wall components, such as cellulose. Cellulose synthesizing machineries are located in the plasma membrane [9], and two other plasma membrane bound proteins, KORRIGAN1 [38] and KOBITO1 [39], are involved in cellulose synthesis although they are not components of the cellulose synthase proteins. Recently, Gu et al. showed that cellulose synthase-interactive protein 1 (CSI1), a plasma membrane localized non-CESA protein is directly involved in cellulose synthesis in the primary cell wall through interaction with CESA isoforms [40]. These findings are in agreement with a possible role for EF1ββ in cellulose biosynthesis.

EF1ββ promoter::reporter gene expression analysis revealed EF1ββ to be preferentially expressed in developing organs, and in developing fibers and vessels that undergo secondary wall synthesis. Relatively moderate-to-high levels of EF1ββ transcript were detected in all tested organs, which indicate the ubiquitous expression of this gene and underscores the importance of EF1ββ in plant growth and development. This expression pattern was corroborated when a dwarf phenotype was generated by the disruption of EF1ββ expression (particularly in later stages of development), and is supported by the role EF1β plays in yeast growth [3]. Recently Vain et al. reported that a homozygous T-DNA insertion mutant of the gene encoding eukaryotic translation initiation factor 4A (eIF4A) in Brachypodium distachyon exhibited a dwarf phenotype (43-46% of the height of the plant without T-DNA insertion) due to a decrease in both cell number and cell size, and the plants were completely sterile [41]. Both eIF4A and EF1β are involved in translation, and silencing of the two genes resulted in dwarf phenotypes though eIF4A plants were fully fertile. A stunted growth phenotype was observed in plants with disrupted expression of different genes involved in cellulose and lignin biosynthesis [9,10,16]. Arabidopsis irregular xylem 1, 3, and 5 mutants which correspond to mutations in CES14, CES17, and CES19 are characterized by collapsed xylem vessels and stems with ~ 70% lower levels of cellulose compared to wild-type plants [42-44]. Our results revealed that disruption of EF1ββ also caused significant reductions in lignin and cellulose levels in cell walls and a change in vascular morphology and structure of the inflorescence stem of the eIF4A mutant. As with the Aspen PhCt09A1 homolog of KORRIGAN1 [45], the gain-of-function EF1BOX plants showed cellulose and lignin contents similar to WT. Reduction of cell size and changes in cell shape due to disruption of cellulose gene was also observed in the eli1 mutant and was restored to the WT phenotype in the eli1-EF1BOX plants.

The reduced cellulose and stunted phenotype with changed cell shape in eIF4A are consistent with reduced growth and misshapen cells found in mutants affecting other plasma...
membrane-associated proteins, including cellulose biosynthesis and related genes such as CESA3 [15], KORRIGAN1 [39] and KOBITO1 [39], and suggest that EF1ββ may be involved in cellulose biosynthesis. Vascular tissues, either primary or secondary, of higher plants play essential roles in the transport of water, nutrients, and signaling molecules and in physical support [46]. So, the alteration in the size and shape of the vessel elements in elfβ could impact the transport of nutrients and water to the stem, which could contribute to the reduced stem size of the mutant plants. Disruption of lignin regulatory genes, such as MYB58 and MYB63 [47], and lignin structural genes, such as hydroxycinnamyl alcohol dehydrogenase (CAD) [48,49] and hydroxycinnamoyl CoA reductase (CCR) also resulted in a wide range of developmental defects, including dwarfism, reduction of cell wall thickness, deformed cell shape, and sterility. EF1ββ is a plasma membrane and cytosolic protein whereas phenylpropanoid enzymes tend to be cytoplasmic or ER localized [10]. Considering the involvement of this EF1ββ in translational elongation, a direct role for this protein in lignin biosynthesis is unlikely, but rather its role in lignin biosynthesis may be through maintaining normal plant development.

Lignin monomer composition, when expressed as the syringyl/guaiacyl (S/G) ratio, was altered due to down-regulation of the EF1ββ gene in elfβ plants relative to WT and EF1BOX plants. S-rich lignins are predominantly deposited in the interfascicular fibers of Arabidopsis, whereas cell walls of xylem vessels are rich in guaiacyl lignin [35]. The reduced interfascicular fiber region of elfβ may have resulted in/from lower S-lignin and a lower S/G ratio in the inflorescence stem of elfβ relative to WT and EF1BOX plants. Recently, Berthet et al. demonstrated a strong reduction in lignin content with a substantial increase in S/G ratio in an Arabidopsis laccase double mutant (lac4-2Las17) with higher saccharification efficiency [29]. In contrast, Sonbol et al. reported a positive relationship between lower S/G ratio and increased availability of cell wall polysaccharides in Arabidopsis [50], while Srinivasa Reddy et al. demonstrated in alfalfa that the S/G ratio is not necessarily related to cell wall digestibility [51]. These apparent contradictions underline the importance of additional research with EF1ββ to elucidate the relation between lignin composition and cell wall digestibility and to determine its potential use as a tool to engineer plant cell walls for higher digestibility.

Stunted growth and reductions in cellulose and lignin contents in elfβ plants led us to investigate the effects of modulating EF1ββ transcript levels on the expression of select lignin and cellulose biosynthesis genes. However, no significant differences were observed in levels of transcripts of lignin biosynthesis or cellulose synthase genes except for CESA3 and CESA7 in either EF1BOX or elfβ relative to the WT. Histochemical analysis revealed increased vascular development in the EF1BOX plants which might be related to the upregulation of CESA3 or CESA7 transcript levels. Recently Hématy et al. reported that a functional THESEUS1 (THE1), a plasma-membrane-bound receptor-like kinase gene, was required for the dwarf phenotype and ectopic-lignin accumulation in greenhouse-grown cellulose-deficient mutants el1-1, rsw1-10, and pom1-2, but this gene also did not up-regulate any of the monolignol biosynthetic genes [20]. Since EF1ββ is involved in translation elongation, it is likely that this gene predominantly affects cellulose or lignin biosynthesis at the posttranscriptional level or regulates both of these processes in some indirect fashion.

The inhibition of cellulose synthesis triggers a set of characteristic cellular changes and altered transcript levels for hundreds of genes [20]. The ectopic lignification mutant el1 has been extensively studied to elucidate the role of the CESA3 gene in cellulose biosynthesis, cell expansion, and plant morphology and lignin deposition [16]. We over-expressed EF1ββ in the el1 background, which successfully abolished ectopic lignifications and restored the WT phenotype. CESA3 is a cell expansion protein and is important for plant development [15,16]. Phenotypic and functional rescue of el1 by EF1ββ is consistent with a role for this protein in cell expansion. Gene expression patterns of selected cell wall-related genes in WT and el1-EF1BOX were unaltered except for CESA3 which also reflected the reversal of el1 characteristics by EF1ββ. However, upregulation of the CESA3 transcript level in el1-EF1BOX might be related to the elevation of EF1ββ transcript levels, although the mechanism is unknown. Both genes are involved in cell elongation and it is possible that this common role is responsible for this elevation. Varying degrees of ectopic lignification occur in el1 [15], rsw1 [52], kornigan1 [53], and dot3 [54] and the degree of ectopic lignification was correlated with the degree of cell expansion. However, we did not observe any ectopic lignification in elfβ (data not shown). As with the Arabidopsis mutants, pom-pom and cobra, this implies that reduced cell expansion does not necessarily lead to ectopic lignin accumulation [15,55] and strongly suggests the involvement of other (or indirect) mechanism(s) or feedback affecting the synthesis of other cell wall components. For example, fluorescent live-cell imaging of CESA6 [56] and CESA3 [57] identified significant intracellular Golgi reserves of CESA proteins which did not exclusively coincide with cellulose synthase complex (CSC) assembly. Golgi bodies are known to “pause” on microtubules and affect the excretion of CSCs in Arabidopsis [37]. Hence, intracellular trafficking of CESAs could play a role in the developmental and environmental regulation of cell wall composition. In addition, Caño-Delgado et al. showed that reduced cellulose synthesis rather than lignification was responsible for reduced growth of and ectopic lignification in the el1 mutant [16]. Indirect effects would explain the role of EF1ββ in lignin biosynthesis as a consequence of the disruption of cellulose biosynthesis.

In conclusion, EF1ββ is a novel regulator of plant development and plays an important role in cell wall formation. Disruption of its expression negatively impacts plant growth and development as well as vascular tissue development. Over-expression of this gene rescued the cell expansion defects of el1, which further confirms an important role for EF1ββ in plant development. However, EF1ββ should be studied in more detail to further unravel the mechanism of cell wall biosynthesis and to confirm whether elfβ is hypostatic or epistatic relative to el1.

Supporting Information

Figure S1 Confocal images showing localization of EF1ββ-YFP (A and D) in Arabidopsis root tip cells plasmolyzed with 0.75 M sorbitol. SynaptoRed (SR) was used as a plasma membrane marker (B and E). Panels C and F are the merged images of the YFP and SR channels. Panels A and C are showing bright yellow circular bodies, possibly the accumulation of EF1ββ-YFP. Lower panels (D-F) show a close-up of root tip cells. Bars = 10 μm.

Figure S2 Expression of select cell wall-related genes in WT and el1-EF1BOX. Data presented as mean transcript abundance ± SD relative to EF1α and elf4a1 of three independent experiments and each replicated three times. * indicates significant differences relative to WT transcript levels at P < 0.05.

Table S1 List of primers used in this study.

[DOC]
Author Contributions

Conceived and designed the experiments: AH MG ZH. Performed the experiments: ZH LA BM. [J]. Analyzed the data: AH MG ZH LA BM. [J]. Contributed reagents/materials/analysis tools: BM LA JJ. Wrote the paper: ZH LA AH MG.

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