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Ectopic Expression of *E2FB*, a Cell Cycle Transcription Factor, Accelerates Flowering and Increases Fruit Yield in Tomato

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Abstract Cell division and plant development are two interconnected and dependent processes. Nowadays, the sequencing of plant genomes has allowed the identification of the majority of cell cycle regulators. However, the function of many of these cell cycle genes remains unclear. At present, the majority of cell cycle studies have been focused on the model plant *Arabidopsis thaliana*. In this model plant, the cell cycle transcription factor E2FB acts as positive regulator of cell proliferation. In this work we analyzed the effect of expressing *E2FB* in tomato. E2FB-expressing plants show reduced levels of cell cycle genes in mature leaves and grow and develop faster than wild type (wt). E2FB^{OE} plants flower significantly earlier than wt and produce more and bigger fruits, with the total fruit yield significantly higher in E2FB^{OE} than in wt plants. Taken together, our data indicate that the rate of cell proliferation and differentiation is important for tomato development and that cell cycle genes are good candidates to manipulate to improve crop productivity.

Keywords E2F · Cell cycle · Tomato · Fruit yield

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Introduction

Vegetables and fruits are essential components of the human diet as sources of vitamins, minerals, and fiber, and they provide antioxidants that might prevent chronic diseases. Tomato (*Lycopersicum lycopersicum* L., formerly *Lycopersicum esculentum* Miller) is an economically important crop worldwide and is one of the foremost model plants in agricultural research (Barone and others 2008). Genetic modifications of crop plants to introduce desirable traits such as nutritional enhancement, disease and pest resistance, and enhanced crop productivity have revolutionized agriculture, becoming a promising technology to boost food production in the world (Chrispeels 2002).

The growth and development of multicellular organisms depend on the correct spatiotemporal coordination of cell proliferation, cell differentiation, and cell specialization (Coffman 2004; Ramirez-Parra and others 2005). In plants, most cell cycle studies have been carried out on the model plant *Arabidopsis thaliana*. At present, several plant genomes have been sequenced, and the majority of the cell cycle regulators that function in mammals and yeast have been identified, suggesting that cell division control might be conserved among the eukaryotic organisms. The retinoblastoma-E2F/DP (RB/E2F/DP) pathway is one of the major regulators that control and couple cell division with differentiation in both animals and plants (Gutierrez 2005; Korenjak and Brehm 2005; van den Heuvel and Dyson 2008; Poznic 2009; Olson and others 2010).

E2F/DP proteins are transcription factors that play a crucial role in regulating several pathways related to cell division, DNA repair, and cell differentiation (Ahlander and Bosco 2009; Berckmans and De Veylder 2009; Chen and others 2009). E2F activity is modulated by the retinoblastoma protein (RB) that interacts with E2F proteins,

promoting the formation of repressor complexes. The retinoblastoma protein mediates this repression by binding and blocking the transactivation domain of E2F factors and by recruiting chromatin-modifying repressive complexes to promoters containing E2F sites (Müller and Helin 2000; Macaluso and others 2006). This repression is relieved by phosphorylation of the RB by cyclin-dependent kinases (CDK) (Boniotti and Gutierrez 2001), modification that blocks the ability of RB to interact with E2Fs and by ubiquitin-dependent degradation of E2F repressor factors (Jurado and others 2008, 2010), allowing the function of positive E2F factors (Cooper and Shayman 2001).

E2F/DP family proteins have been identified in different plant species (Ramirez-Parra and others 1999; Sekine and others 1999; Albani and others 2000; Olson and others 2010). The *Arabidopsis thaliana* genome encodes six different E2Fs (E2FA–E2FF) and two DPs (DPA–DPB) proteins (de Jager and others. 2001; Gutierrez and others 2002; Mariconti and others 2002; Shen 2002; Vandepoele and others 2002). The *Arabidopsis* E2Fs can be divided into two groups that differ both structurally and functionally: E2FA–E2FC possess all the features of typical E2Fs, including a DNA-binding domain, a DP heterodimerization domain, a transactivation domain, and an RBR-binding region. E2FA and E2FB function as activators of E2F-responsive genes that promote cell division (De Veylder and others 2002; Rossignol and others 2002; Magyar and others 2005; Sozzani and others 2006), whereas E2FC, which has a truncated transactivation domain that interacts with RBR, is a repressor of cell proliferation that controls the balance between cell division and endoreduplication and is degraded through the ubiquitin pathway by the auxin-binding protein SKP2A (del Pozo and others 2002, 2006; Jurado and others 2008, 2010). E2FA in conjunction with DPA promotes cell proliferation, induces ectopic cell division and DNA endoreduplication, and severely affects plant development (De Veylder and others 2002). E2FB plays a role in regulating auxin-dependent cell division. In *Arabidopsis*, E2FB is one of the key cell cycle targets for auxin action to settle on entering into the proliferative program. Auxin positively regulates the accumulation and the stability of E2FB protein (Magyar and others 2005). Overexpression of *E2FB* in *Arabidopsis* plants leads to striking modification of plant development, explained in part by the promotion of cell division and reduction of the cell cycle. In addition, *E2FB*-overexpressing plants show slightly enlarged cotyledons that contain almost twice the number of epidermal cells, which are of smaller size than wild-type (wt) plants cells. These transgenic plants also show up-regulation of the E2F-responsive S-phase genes such as *RNR1* and *MCM3* (Chaboute and others 2002; Stevens and others 2002), as well as the G2/M marker genes *CYCA2;1* and *CDKB1;1*. In tobacco Bright Yellow-2 (BY-2) cells, coexpression of *E2FB*, but not *E2FA*, with DPA increases cell cycle rate and promotes cell division, even in the absence of auxin (Magyar and others 2005). Based on these data, it has been proposed that E2FB stimulates cell division by promoting the entry into G1 and G2 and shortening the transitions between G1 and S and between G2 and M phases (Magyar and others 2005).

In the present study we have analyzed the effect of ectopically expressing the *Arabidopsis E2FB* gene into tomato plants ($E2FB^{OE}$). We have found that *E2FB* expression accelerates tomato plant development. $E2FB^{OE}$ plants germinate and flower significantly earlier than wt and also generate more and bigger fruits, with the total fruit yield significantly higher than in wt plants.

Materials and Methods

Plant Material and Growth Conditions

The plants used in this study were domesticated tomato (*Solanum lycopersicum* cv. Micro-Tom). All plants were grown in a growth chamber with a 16 h light/8 h dark photoperiod at 24°C or in a greenhouse with a 16 h light/8 h dark photoperiod at 28°C. Plants were irrigated periodically with a commercial fertilizing solution.

DNA Constructions

The full-length *Arabidopsis thaliana* cDNA (1411 pb) of the *E2FB* gene (At5G22220) was amplified with specific primers At5g22220F (5′-GGAGATAGAACCATGTCTGA AGAAGTACC-3′) and At5g22220R (5′-CAAGAAAGC TGGGTCGCTACCTGTAGGTGGC-3′) and subsequently cloned into the gateway vector pDONR221 (Invitrogen). The *E2FB* cDNA was transferred by LR recombination (Invitrogen) into the gateway binary vector pGWB2 (Nakagawa and others 2007) to express it under the control of constitutive cauliflower mosaic virus (CaMV) 35S promoter. The 35S::E2FB construct was introduced into *Agrobacterium tumefaciens* (C58C1 strain) and used for transformation of tomato plants using the method described by McCormick and others (1986). To generate the tomato CYCB1-GUS line, the PCYCB1::CYCB1-GUS construct (Colón-Carmona and others 1999) was introduced in tomato plants by *Agrobacterium*-based transformation (see below).

Agrobacterium-based Tomato Transformation and Plant Regeneration

The transformation of tomato was carried out basically as described by McCormick and others (1986) with

modifications. Seeds were surface-sterilized by immersing them for 10 min in 75%/0.01% NaCl/Tween20 solution and then washing three times with sterile distilled water. After sterilization, seeds were germinated in the darkness in a tissue-culture chamber at 24 ± 2°C, on solid medium consisting of MS (Murashige and Skoog) inorganic basal salts supplemented with 2% sucrose and solidified with 0.7% bacteriological agar (Pronadisa, Spain). After 2–3 days, when the radicle emerged and curved into the medium, the containers were incubated in the same chamber with 16 h light/8 h dark photoperiod conditions. Cotyledons from in vitro-grown seedlings were used as explants. Cotyledons were excised from 8 to 10 day germinated seedlings and incubated overnight at 24 ± 2°C on 90 mm Petri dish plates containing a MS-based medium supplemented with zeatin 2 mg/l and a thin layer of BY-2 tobacco suspension (tobacco feeder layer). *Agrobacterium tumefaciens* C58C1 strain containing a gene construction was cultured overnight for transformation of these explants the next day. The explants were mechanically damaged and carefully submerged and shaken during 3 min in the *Agrobacterium tumefaciens* culture and returned to the plates with the tobacco feeder layer and then incubated at 24 ± 2°C for 2 days. After cocultivation, the explants were transferred to a Petri dish with MS-based selective regeneration medium containing 2% sucrose, Claforan® (cefotaxime) 250 mg/l, kanamycin 50 mg/l, and zeatin 2 mg/l. Every week the explants were subcultured under the same conditions, reducing zeatin concentration (1 and 0.5%, respectively). The first callus appears about 3 or 4 weeks after infection and culture on regeneration and selection medium. Subsequently, when the regenerated shoot stems elongated to 2–4 cm, individual shoots were excised from calli and transferred to the rooting medium (MS salts and vitamins with 2% sucrose, Claforan 250 mg/l, kanamycin 50 mg/l, and IAA 0.5 mg/l). The seedlings were transferred to fresh rooting medium every 15 days. Rooted seedlings were adapted to soil and transferred to a growth chamber at 24 ± 2°C with a photoperiod of 16 h of light and 8 h of darkness.

Analysis of Transgenic Plants

To identify plants that harbor the *E2FB* transgene, DNA extracted from calli-derived tomato plants (T0) was analyzed by PCR assay using the REDExtract-N-Amp™ Plant PCR Kit (Sigma). The primers used for amplification were E2FB-ATG: 5′-ATGTCTGAAGAAGTACCTCAA C-3′ and E2FB-STOP: 5′-TCAGCTACCTGTAGGTGATC TC-3′. The PCR products were resolved in agarose gel and visualized with SYBR® Safe DNA gel stain (Invitrogen).

Semiquantitative and Quantitative RT-PCR Analysis

Total RNA isolated as described above was used for semiquantitative RT-PCR using the Maxime™ RT-PCR PreMix Kit (iNtRON Biotechnology, Korea). Amplification was carried out using 200 ng of total RNA as template (primer sequences are available upon request). The amplified RT-PCR products were analyzed by agarose gel electrophoresis.

To analyze gene expression by quantitative RT-PCR in dividing tissues (shoot apical meristem and the small true leaves surrounding the meristem), total RNA was extracted from 14-day-old wt or E2FB 6.2 plants grown in MS medium. To analyze gene expression in mature tissue, total RNA was extracted from 5-week-old mature leaves of control plants (harboring the promoter of the *Arabidopsis* SKP2B fused to the GUS reporter) or E2FB^{OE} (lines 3.3 and 6.2) plants. Total RNA was extracted using the TRIzol® Plus RNA Purification Kit (Invitrogen).

Total RNA was treated with RQ1 RNase-Free DNase (Promega) to eliminate contamination by genomic DNA. RNA (2 μg) was converted to single-strand cDNA using the AMV reverse transcriptase and oligo-dT (Promega), following the manufacturer's instructions. Quantitative PCR analyses were performed in a 7300 Real-Time PCR system (Applied Biosystems) using the FastStart Universal SYBR® Green Master (ROX) (Roche) detection system. PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, plus a dissociation stage. Melting curve analysis was performed to confirm target-specific amplification. Quantification was standardized to tomato ubiquitin mRNA levels. At least two biological replicates were utilized in every experiment and all analyses were performed in triplicate. Primers used for real-time analysis are listed in Supplementary Table 1.

RNA Extraction and Microarrays

Wild-type and E2FB 6.2 plants were grown in MS medium for 14 days. Total RNA was extracted from the shoot apical meristem and from the small true leaves surrounding the meristem using the TRIzol Plus RNA Purification Kit (Invitrogen). These plants were grown in three different locations in the growth chamber, and after RNA extraction, these three different RNAs were mixed in one pool (hybridization pool). We prepared three independent biological samples (3 hybridization pools) that were used for three transcriptomic analyses. The RNA yield and quality were determined by bioanalyzer analysis. For microarray analysis, total RNAs were processed for use on TOM1 array, which was developed by the Center for Gene Expression Profiling (CGEP) of the Boyce Thompson

Institute for Plant Research (http://bti.cornell.edu/CGEP/CGEP.html). These arrays contain probe sets for approximately 12,000 expressed sequences. RNA amplification and labeling were performed basically as described in Adie and others (2007). Slides were TOM1 cDNA microarray from Cornell University (Center for Gene Expression Profiling: http://ted.bti.cornell.edu/cgi-bin/TFGD/order/home.cgi). Prehybridization was performed at 42°C for 30–45 min in 5 × SSC, 0.1% SDS, 10 mM EDTA, and 1% BSA. Slides were rinsed five times with distilled water. Hyper5- and Cy3-amplified RNA fragmented probes were mixed (200 pmol of each label) with 20 µg of PolyA (Sigma) and 2 mM EDTA in a final volume of 80 µl of hybridization buffer (50% formamide, 5 × SSC, 0.1% SDS). The probe was denatured at 95°C for 5 min and applied to the slide using a LifterSlip (Erie Scientific, USA). Slides were then incubated at 42°C for 16 h in hybridization chambers (Arrayit Corp., USA). After incubation, slides were washed twice with 0.5 × SSC and 0.2% SDS for 5 min each at 42°C, twice with 0.1 × SSC for 5 min, and finally in 0.05 × SSC for 5 min. Slides were dried by centrifugation at 563*g* for 1 min. Images from Cy3 and Hyper5 channels were equilibrated and captured with a GenePix® 4000B microarray scanner (Molecular Devices, USA) and spots were quantified using GenePix software (Molecular Devices). Background correction and normalization of expression data were performed using LIMMA (LIMMA is part of Bioconductor, an R language project). First, the data set was filtered based on the spot quality. A strategy of adaptive background correction was used that avoids exaggerated variability of log ratios for low-intensity spots. For local background correction, the "normexp" method in LIMMA to adjust the local median background was used. The resulting log ratios were print-tip loess normalized for each array. To have similar distribution across arrays and to achieve consistency among arrays, log-ratio values were scaled using as scale estimator, the median-absolute value. Linear model methods were used for determining differentially expressed genes. Each probe was tested for changes in expression over replicates by using an empirical Bayes moderated *t*-statistic. To control the false discovery rate, *P* values were corrected by using the method of Benjamini and Hochberg (1995). The expected false discovery rate was controlled to be less than 5 or 10% where specified.

Morphological Measures

For the germination assays, 30 seeds of each transgenic line ($E2FB^{OE}$ 6.2 and $E2FB^{OE}$ 3.3) and wt, which were collected at the same time and under the same growth conditions, were placed in sterile plastic containers (10 seeds per container) in an MS medium (Duchefa Biochemic) supplemented with 2% sucrose and solidified with 0.7% bacteriological agar (Pronadisa) at 24–26°C and with a photoperiod of 16 h light/8 h dark. The germination velocity was analyzed at different days, considering that a seed germinated when the root protruded through the seed coat, and the results were expressed as percentage of the seeds germinated. The time required for each transgenic and wt lines to reach the complete expansion of the cotyledons (just before the emergence of the true leaves) was also analyzed. These observations were realized at 5, 7, and 11 days after germination and the results were represented as the percentage of seedlings with fully open cotyledons at different days.

The aerial part (hypocotyl length and cotyledons area) and root length of 18-day-old seedlings growing in MS medium, just before they were transplant to soil, were measured.

Floral Buds and Open Flowers Quantification

The numbers of floral buds and open flowers were quantified in plants that had been growing 18 days in MS medium and 24 or 30 days in soil. Results are expressed as the percentage of plants with floral buds and open flowers with respect to the total number of plants in the experiment. A minimum of 15 plants for wt plants and 30 for the transgenic lines were analyzed.

Ploidy Analysis

The samples were prepared from the first two leaves of internode 4 of 3-month-old plants, with the main nerve eliminated, and processed according to the protocol of Galbraith and others (1983). The extracted nuclei were filtered with 60 and 40 µm filters and then stained with 50 µg/ml propidium iodide (Sigma) for 45 min. Ploidy measurements were performed as described by del Pozo and others (2006). The histograms were generated in the flow cytometer FACSCalibur (Bioscience).

Leaf Cell Dimensions

To determine the leaf cell dimensions, fragments in the same position in the leaf of internode 10 were sectioned. These leaf sections were dehydrated in a successive series of ethanol (10, 30, 50, 70, and 90%), being incubated for 15 min in each one. For further use, the leaf sections were rehydrated by being incubated for 15 min in 70, 50, 30, and 10% ethanol, and finally mounted on slides in a solution containing sodium chlorhydrate-glycerol-water (8:1:1). Epidermal cells were analyzed by Nomarsky microscopy and several pictures were taken with a digital camera (Leica DFC300FX). Cell areas were calculated using

IMAGE J software (http://rsb.info.nih.gov/ij). Data correspond to the main value of at least 200 cell areas from 20 different sections. Data values were statistically analyzed using the Student *t* function.

Quantification of Fruit Productivity

The productivity of tomatoes per plant was calculated by measuring all tomato fruits harvested from 15 individual plants at different times. Results are expressed as the median of tomatoes collected per plant for each line. The sizes of the tomatoes (length and diameter) were measured individually with a vernier caliper. In addition, all fruits were individually weighed on an analytical balance just after collection to calculate their fresh weight. Data are expressed as the percentage of tomatoes of each line distributed throughout different weight ranges (0–2, 2–4, and >4 g).

E2F Site Identification

To identify E2F sites, the genomic sequence of each gene, containing 4 kbp upstream and 4 kbp downstream from the ATG, was retrieved from the Sol Genomic Database (http://solgenomics.net/tools/blast/index.pl). We searched for E2F sites in these genomic sequences (3 possible variants of the consensus in sense or antisense orientation: TTNSSCGSS, TNTSSCGSS, or NTTSSCGSS, where S stands for C or G and N stands for any nucleotide), using the serial cloner program by designing six restriction sites that contain these motifs. The number and position from the ATG of these E2F sites are given in Supplementary Table 1.

Results

Generation of E2FB-expressing Tomato Plants

Coordination between cell proliferation and differentiation is crucial for almost all aspects of plant growth and development, including fruit formation and size. We wanted to analyze whether manipulation of cell division might have positive effects on agronomic traits. In this work we decided to ectopically express *Arabidopsis E2FB* rather than *E2FA* because it was reported that overexpression of *E2FA* led to small, dwarf, and unhappy plants, whereas *E2FB* accelerated cell division with few undesirable phenotypic consequences, except for root growth (Sozzani and others 2006). When we started this work, we could not identify a clear *E2FB* homology in the tomato genome. For that reason, and to avoid co-suppression effects, we decided to express the *Arabidopsis E2FB* cDNA under the control of a constitutive promoter. The tomato genome sequence has recently been released. We have searched for E2F-like proteins and identified five different ones (Supplementary Fig. 1a). These tomato E2F-like proteins contain the conserved DNA-binding domain, the marked box, the leucine zipper, and the putative RBR binding site (Fig. 1a). Based on the predicted amino acid sequences (http://solgenomics.net/tools/blast/index.pl) of these E2F-like proteins and the *Arabidopsis* E2Fs proteins, we have generated a phylogenetic tree (Fig. 1b), in which we can infer the E2FA or E2FC tomato orthologies, but not that for E2FB.

To assess the effect of an increased level of *E2FB* in tomato, we generated transgenic tomato plants that ectopically express *Arabidopsis E2FB* cDNA by *Agrobacterium*-based transformation ($E2FB^{OE}$). We regenerated five independent transgenic tomato lines that were tested for insertion of the transgene by PCR. We analyzed the *E2FB* expression levels in these lines by quantitative RT-PCR (Fig. 2a). A homozygous T2 generation was established by selection in kanamycin-containing medium. The expression levels in this T2 generation were analyzed by semi-quantitative RT-PCR and found to be similar to that in the previous generation (data not shown), suggesting that the expression of the transgene is stable. Finally, we chose two independent *E2FB*-expressing transgenic lines that showed a high or medium expression level, E2FB 3.3 and E2FB 6.2, respectively, for further phenotypic analyses.

Ectopic Expression of *E2FB* Alters Cell Division

Because E2Fs are transcription factors that regulate cell cycle gene expression (de Jager and others 2001; Gutierrez and others 2002; Shen 2002; Vandepoele and others 2002; Sozzani and others 2006), we decided to study the effect of expressing *E2FB* on gene expression using microarray analyses (see below). However, because the majority of the cell cycle genes were not present in the Micro-Tom1 array, we conducted quantitative RT-PCR of some well-known cell cycle-regulated genes that contain E2F sites in their promoters (Supplementary Fig. 1b), such as *LePCNA*, *LeRNR1*, *LeCYCB1*;*4*, *LeKNOLLE*, and *LeHISTONE H4*. We analyzed their expression using RNA extracted from proliferating tissue (shoot meristem and small true leaves surrounding the meristem) of 14 day-old seedlings or from mature leaves of 5-week-old plants. These cell cycle genes were significantly repressed in the mature leaves and to a lesser extent in dividing areas of *E2FB*-expressing plants compared to wt (Fig. 2b, c). To analyze this effect in vivo, we generated a tomato line that expresses the CYCB1-GUS reporter under the control of the CYCB1;1 promoter (Colón-Carmona and others 1999). As in *Arabidopsis*, only the CYCB1-GUS protein is stable and therefore detectable

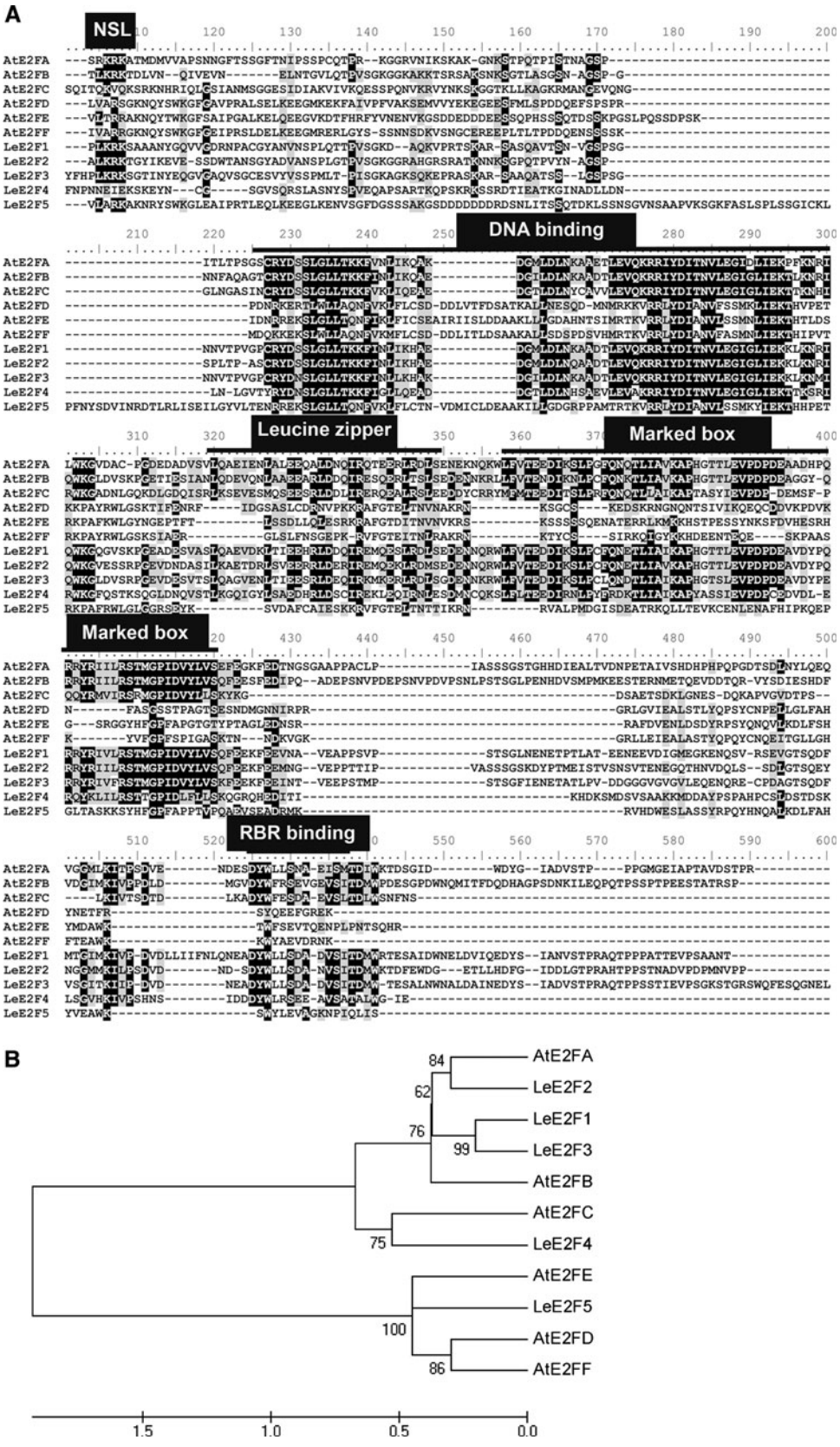

Fig. 1 E2F-like proteins in tomato. **a** Alignment of the six *Arabidopsis* and five tomato amino acid sequences of E2F proteins and identification of conserved functional domains. Identical residues are in *white letters* on a *black background* and amino acid similarities are shown with a *gray background*. The highly homologous DNA-binding domain and the conserved regions (marked box, leucine zipper, and RBR binding pocket), which are found only in the AtE2Fa, AtE2Fb, and AtE2Fc proteins, are indicated. The putative NLS regions are also indicated. **b** Phylogenetic tree showing the relationship between tomato and *Arabidopsis* E2F proteins. Multiple alignments were used to construct a rooted neighbor-joining tree, based on sequence similarity, using the MEGA5 program

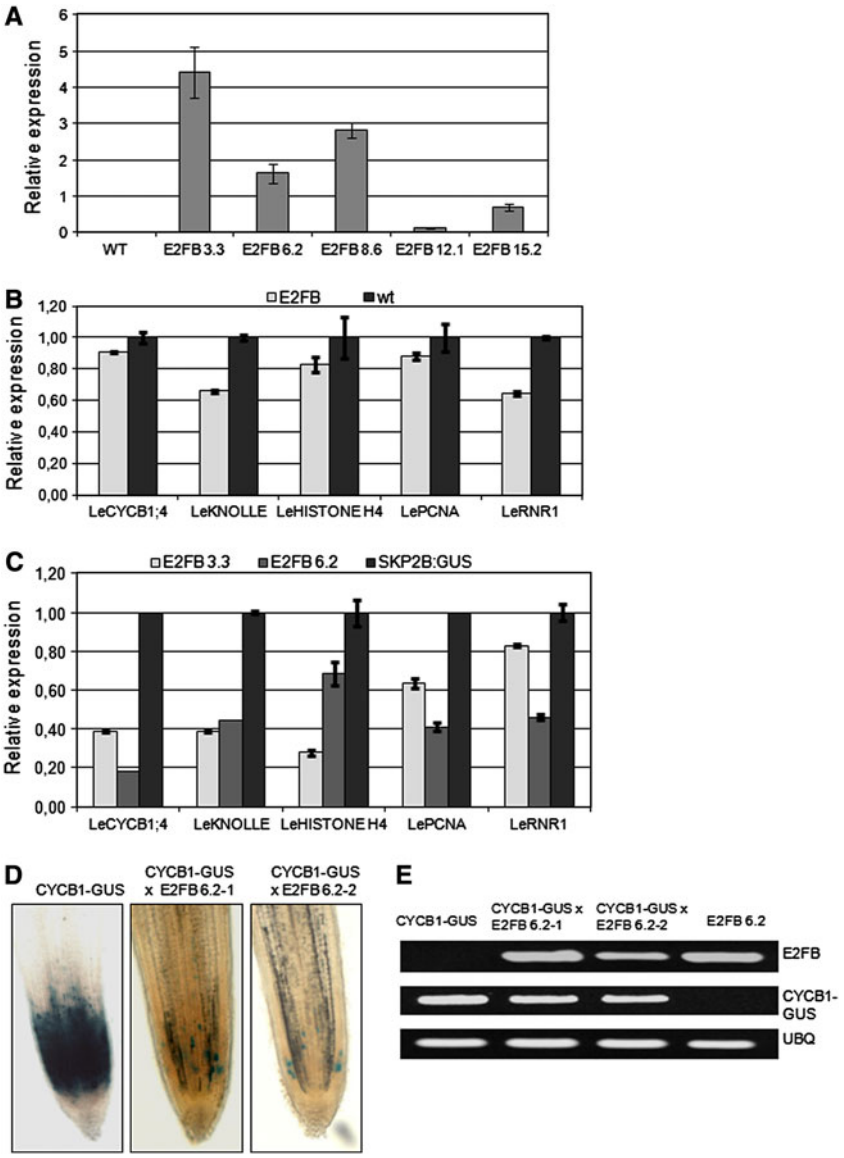

Fig. 2 Ectopic expression of *E2FB* reduces the expression of cell cycle genes. **a** The expression of *Arabidopsis E2FB* was analyzed by qRT-PCR in five independent transgenic plants. The expression was normalized to the ubiquitin gene expression and shown as arbitrary units. **b** Total RNA was isolated from the shoot meristem and small true leaves surrounding the meristem from control wild-type or *E2FB*-expressing transgenic plants ($E2FB^{OE}$ 6.2). These are the same samples that we used for the microarray experiment. The *Y* axis represents the relative mRNA expression value of different cell cycle genes that contain E2F sites in their promoters normalized against the *UBIQUITIN* (*UBQ*) gene in each case. The expression of the cell cycle genes in the $E2FB^{OE}$ plants was relative to the expression in the control (value 1). Values are the average of at least three different measurements performed on two independent cDNAs. **c** Total RNA was isolated from 5-week-old mature leaves from control plants, which harbor the *Arabidopsis* SKP2B promoter fused to the GUS reporter gene (*P2B*:*GUS*) or $E2FB^{OE}$ plants. The *Y* axis represents the relative mRNA expression value of different cell cycle genes normalized against the *UBIQUITIN* (*UBQ*) gene in each case. The expression of the cell cycle genes in the $E2FB^{OE}$ plants was relative to the expression in the control (value 1). Values are the average of at least three different measurements performed on two independent cDNAs. **d** *E2FB* controls G2/M transition in tomato. Representative pictures of 10-day-old CYCB1-GUS and CYCB1-GUS/$E2FB^{OE}$ root meristems stained for GUS activity. **e** Expression level of *E2FB* and *CYCB1-GUS* in CYCB1-GUS, CYCB1-GUS/$E2FB^{OE}$, and $E2FB^{OE}$ plants analyzed by semiquantitative RT-PCR. As a loading control, the expression of the *UBIQUITIN* (*UBQ*) gene was analyzed

by GUS staining during the G2/M transition (Fig. 2d). We crossed this marker line with the E2FB 6.2 plant (CYCB1-GUS/E2FB). We analyzed the F1 seedlings of these crosses by GUS staining and we found that expression of *E2FB* significantly reduced the number of stained G2/M cells in the root meristem in 10 of 12 different analyzed crosses (Fig. 2d). This might be due to a reduction in the expression of CYCB1 or to a faster degradation of the CYCB1 protein. To discern between these two possibilities, we analyzed by semiquantitative RT-PCR the expression level

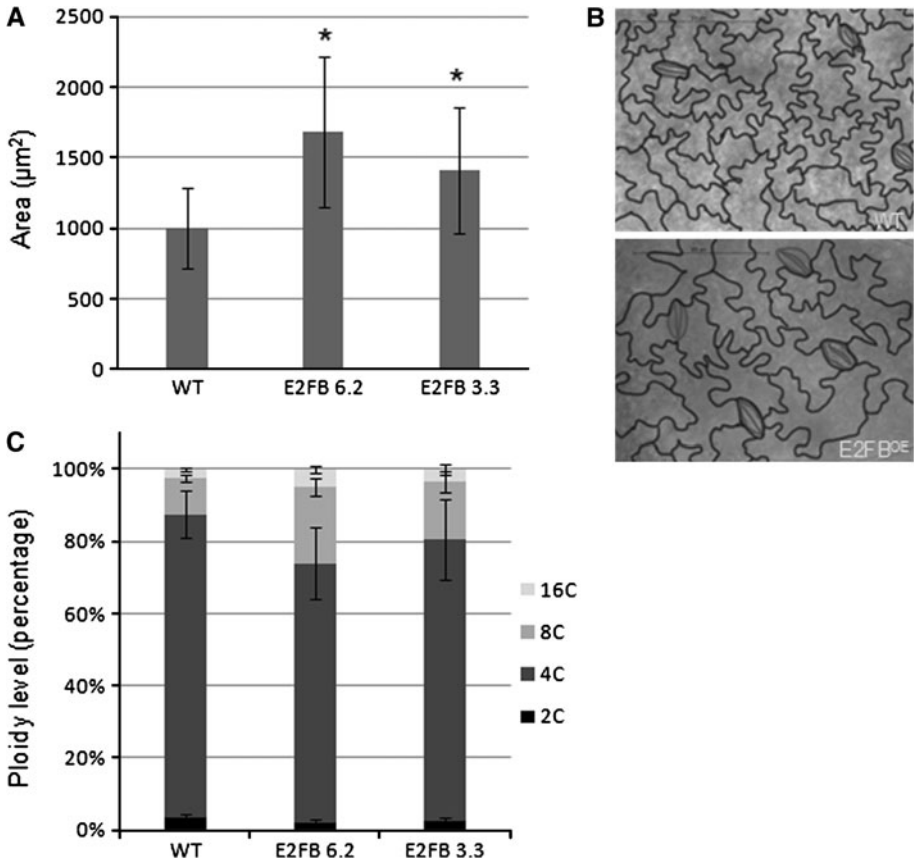

Fig. 3 Ectopic expression of *E2FB* increases cell size. **a** Area sizes of adaxial epidermal cells of the first leaf of internode 10 of wild-type (wt) and E2FB^{OE} plants. Error bars represent SD; $n \geq 200$. *Statistically significant difference for two E2FB^{OE} lines compared with wt values as determined by Student's *t*-test ($P < 0.001$). **b** Representative picture of adaxial epidermis of wt and E2FB^{OE} leaves. The cell margins and stomata were highlighted for better observation. **c** Percentage distribution of ploidy level from leaves of 3-month-old wt and two E2FB^{OE} line plants

of the *CYCB1-GUS* transcript in these seedlings. Both *E2FB* and *CYCB1-GUS* transcripts are expressed to similar levels as in the parental lines (Fig. 2e), suggesting that expression of *E2FB* might affect the degradation time of CYCB1. In other words, if the G2/M transition is shortened, the window of time in which GUS staining can be detected is also reduced.

In *Arabidopsis*, overexpression of *E2FB* led to more but smaller cells in the cotyledons, which were slightly larger than the cotyledons of controls, suggesting a positive role of *E2FB* in cell division (Sozzani and others 2006). *Arabidopsis E2FB*-overexpressing plants also showed a modest increase of DNA content compared to control plants (Sozzani and others 2006). However, expression of *Arabidopsis E2FB* in tomato led to dissimilar phenotypes. Thus, expression of *E2FB* in tomato increased the cell size of mature leaves (Fig. 3a, b). To analyze whether ectopic expression of *E2FB* in tomato plants amends the DNA content, we quantified ploidy levels in mature leaves by flow cytometry analysis. Most of the nuclei in mature leaves of wt plants had a ploidy level of 4C. However, *E2FB*-expressing lines exhibited a modest, but significant, increase of nuclei, with a ploidy level of 8C and 16C (Fig. 3c). However, because *E2FB*-expressing plants have an accelerated development (see below), these phenotypes, that is, bigger cell size and modest increase in ploidy level, might be the result of wt and *E2FB*-expressing plants being in a different developmental stage rather than a direct effect of E2FB.

Phenotype of *E2FB*-expressing Tomato Plants

To evaluate the effect of expressing *E2FB* on cell cycle and development in tomato plants, we analyzed the phenotype of *E2FB*-expressing transgenic lines. First, we examined the effect of *E2FB* expression on germination velocity. About 30 seeds of wild-type Micro-Tom (wt) and 30 seeds of two independent E2FB^{OE} lines, which were collected at the same time and under the same conditions in the greenhouse, were sowed in containers containing MS plus 2% sucrose and grown in a chamber for 3, 7, and 10 days. We found that the percentage of germinated seeds was higher in the E2FB^{OE} lines than in wt (Fig. 4a). It is remarkable that at 7 and 10 days after planting (DAP), 100% of the seeds of the two transgenic E2FB^{OE} lines were germinated, whereas only 50% of the wt seeds germinated, suggesting that expression of *E2FB* increases the germination efficiency in our conditions. In addition, we quantified the number of seedlings that were already germinated which showed fully expanded cotyledons at different times (5, 7, and 11 DAP). We found that the cotyledons of E2FB^{OE} grew faster than those of wt plants (Fig. 4b). Eleven DAP all-transgenic seedlings showed totally

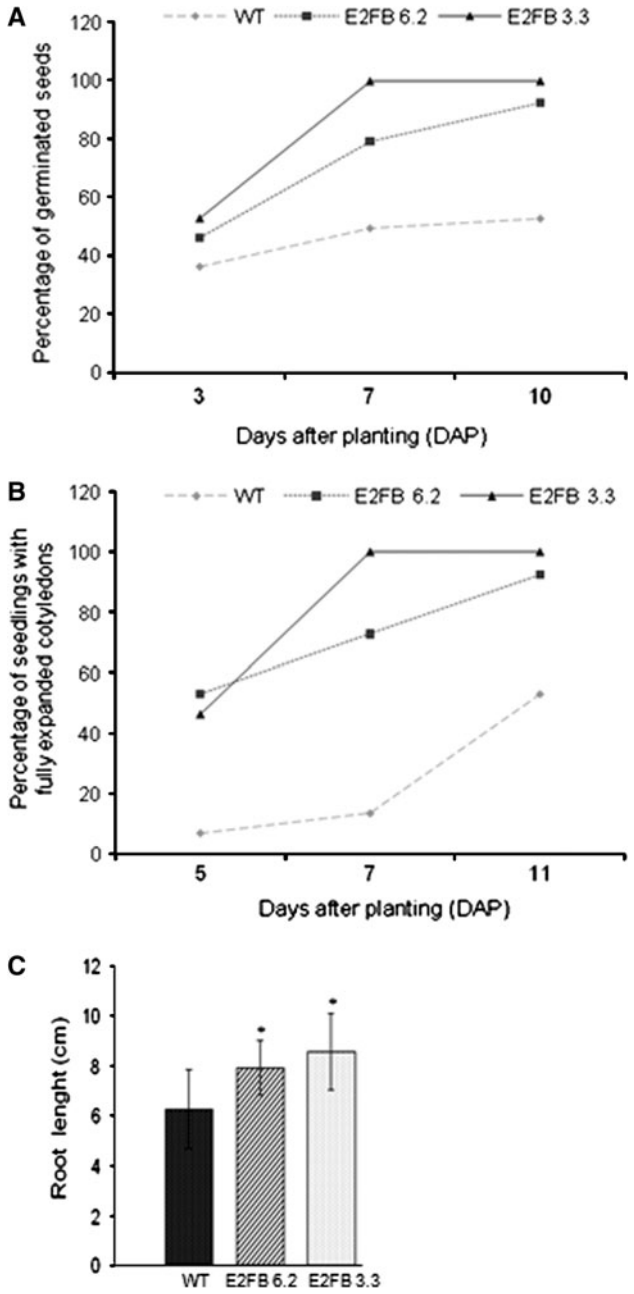

Fig. 4 Ectopic expression of *E2FB* improves seed germination and regulates root growth. **a** Percentage of germinated seeds at different times. Wild-type Micro-Tom (wt) and two transgenic lines that expressed E2FB protein (E2FB^{OE} 3.3 and E2FB^{OE} 6.2) were analyzed. The analyses were carried out at 3, 7, and 10 days after planting (DAP); $n = 30$. **b** Percentage of wt and E2FB^{OE} seedlings with fully expanded cotyledons. The observations were realized at different times after germination. **c** Root length of wt and two independent E2FB^{OE} lines grown in MS medium for 18 DAP. *Error bars* represent SD; $n \geq 15$; cm, centimeters

expanded cotyledons, whereas only 52% of the germinated wt seedlings showed the same stage of cotyledon development, suggesting that ectopic expression of E2FB accelerates tomato plant growth and development.

Before transplanting to soil (18-day-old seedlings grown in MS medium in a chamber), the aerial parts (quantified as hypocotyl length and cotyledon area) of wt and E2FB^{OE} plants were similar in size (data not shown), but the roots of E2FB^{OE} were longer than those of wt (Fig. 4c). When we observed the development of these plants in the greenhouse, we found that E2FB^{OE} plants in general grew faster than wt (Fig. 5a), although at the end of their life cycle, both genotypes reached similar sizes and stages of development.

To analyze whether these plants also have affected an important agronomic trait, such as the flowering time, wt and E2FB^{OE} plants were grown in the greenhouse for 24 or 30 extra days after transplanting (DAT) from the in vitro containers. Then, we counted the number of plants that showed at least one floral bud or one open flower. We found that the percentage of plants with floral buds or open flowers at 24 days was significantly higher in the E2FB^{OE} plants than in the wt plants (Fig. 5a, b). Later, at 30 DAT, all E2FB^{OE} and wt plants showed at least one floral bulb, but E2FB^{OE} plants developed a higher number of open flowers than wt plants (Fig. 5a, b). Taken together, our data indicate that *E2FB* ectopic expression accelerates plant growth and the emergence of inflorescences and flowers.

E2FB-expressing Plants Increase Fruit Yield

To analyze the effect of expressing *E2FB* on tomato fruit production, we harvested all fruits from each plant at 3, 4, and 5 months. As shown in Fig. 6a, the number of fruits per plant was significantly higher in *E2FB*-expressing plants than in wt. At 3 months, E2FB^{OE} plants produced almost 30 times more fruit than wt plants. Later, at 5 months, significant but reduced differences in the number of fruits collected were still found; the average number of fruits per E2FB^{OE} plant was 16, whereas the average per wt plant was 10 (Fig. 6a). When total productivity was analyzed, we found that E2FB^{OE} yielded about 1.6 times more fruit than wt plants (Fig. 6b). In addition, the size and weight of all harvested fruits were individually analyzed. E2FB^{OE} fruits were significantly bigger, in both length and diameter, than wt fruits (Fig. 7a). We also weighed each individual tomato harvested. Because standard deviations of the samples were high, we decided to group the values in weight ranges: 0–2, 2–4, and >4 g. In the 0–2 g range, wt had the highest percentage of tomatoes (45% of total), whereas E2FB^{OE} fruits represented only 30% (Fig. 7b). In the 2–4 g range, the distribution of percentage was reversed. In this range, the average number of E2FB^{OE} tomatoes (60%) was slightly higher than that of wt fruits (55%). It is remarkable that we did not find any wt fruit that weighed more than 4 g, but about 10% of the fruits harvested from E2FB^{OE} plants weighed more than 4 g. Taken

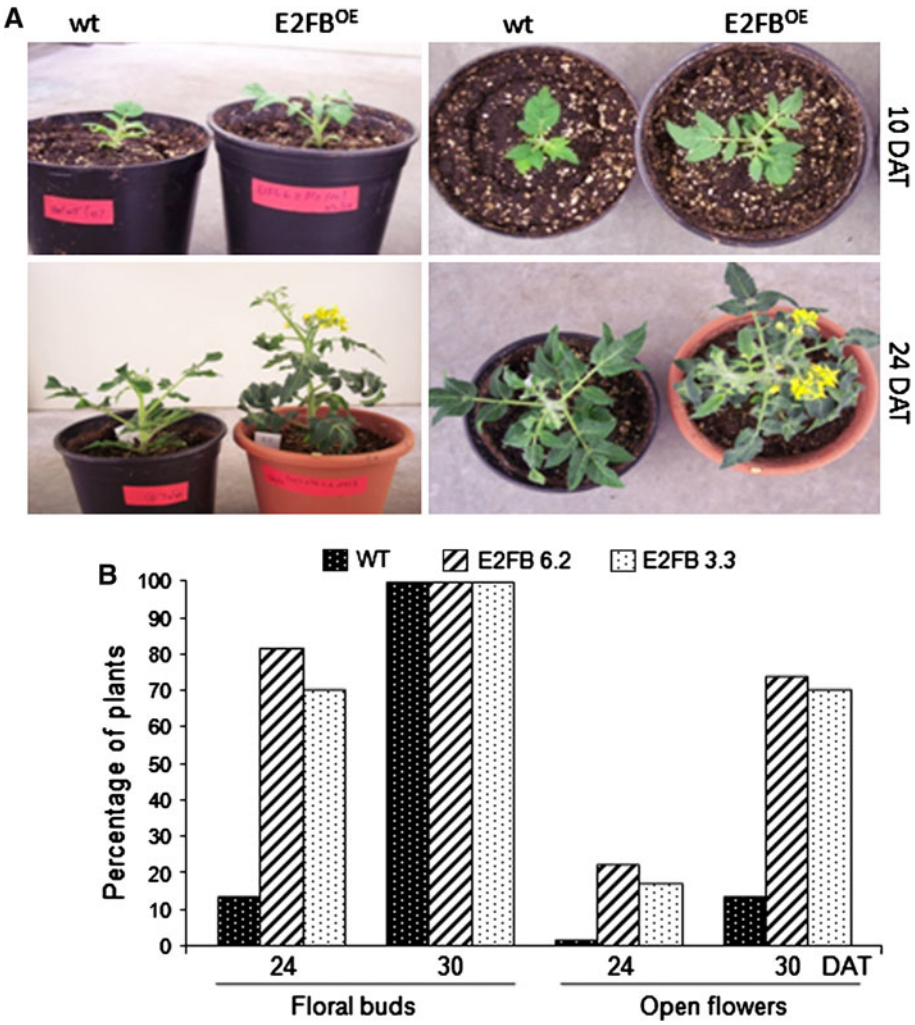

Fig. 5 Ectopic expression of *E2FB* accelerates plant development. **a** Representative photograph of wt and E2FB^{OE} plants grown in soil at 10 and 24 DAT (days after transplanting). **b** Percentage of plants that showed at least one floral bud and open flower at 24 or 30 DAT

together, we can conclude that ectopic expression of *E2FB* leads to higher fruit yield, producing bigger and heavier fruits than wt plants.

E2FB Expression Modifies Gene Expression

E2FB is a cell cycle transcription factor that regulates cell cycle and development genes (Magyar and others 2005; Sozzani and others 2006). To better understand the role of *E2FB* when ectopically expressed in tomato plants, we carried out a transcriptomic analysis using the Micro-Tom1 arrays. At a stringency level of $P < 0.005$, we found that ectopic expression of *E2FB* led to slight changes in gene transcription (Supplementary Table 1) compared to the changes that overexpression of *E2FA* induced in *Arabidopsis* (de Jager and others 2009). This is likely because the Micro-Tom1 array does not cover all the tomato genes and, in addition, the majority of the cell cycle genes are not represented in this array. Nevertheless, we found several genes that were upregulated or downregulated by expressing *E2FB*. These differentially expressed genes belong to diverse functional categories, with ethylene response and defense genes highly represented among the upregulated ones. According to the digital expression data (http://ted.bti.cornell.edu/cgi-bin/TFGD/array_data/home.cgi), a large number of the induced genes in the E2FB^{OE} plants were not expressed in wild-type young seedlings, but they were expressed in further developmental stages such as flower or fruit development (Supplementary Table 1). The induction of MAD-box transcription factors that have been related to flower and fruit development rather than seedling growth (Supplementary Table 1) is especially interesting. To determine whether these up- or downregulated genes might be direct targets of E2F factors, we searched for E2F binding sites in their promoter regions (Supplementary Table 1). We found that over 50% of the upregulated genes contain E2F sites. This percentage is higher than expected because in *Arabidopsis* the number of promoters (considering 3000 bp upstream from the ATG) that contain at least one E2F site is about 20%. However, we found only that 16% of the downregulated genes have at least one E2F site in their promoter regions.

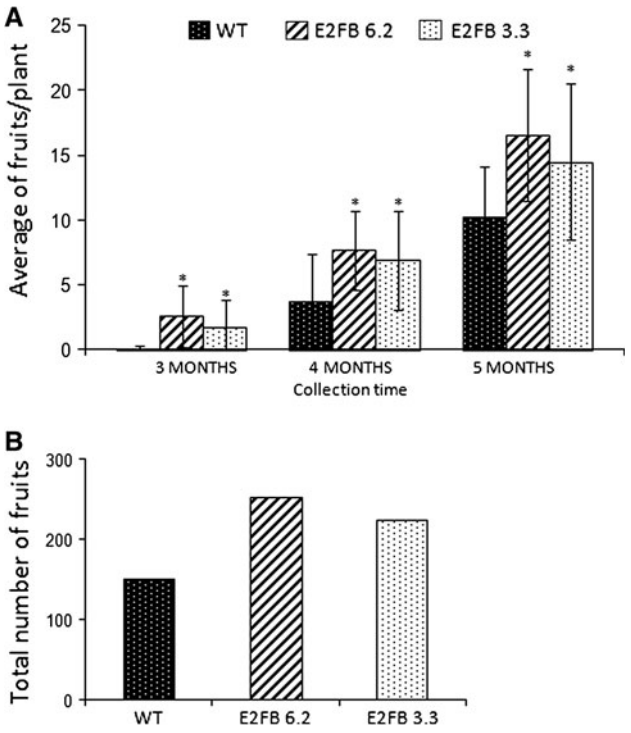

Fig. 6 Ectopic expression of *E2FB* increases the number of fruits. **a** Average of fruits per tomato plant. *Error bars* represent SD; $n = 15$. *Statistically significant difference for E2FB^{OE} compared with wt values as determined by Student's *t*-test ($P < 0.01$). **b** Total number of fruits collected from 15 plants of each genotype after 5 months of growing in the greenhouse

Discussion

In this study we have analyzed the effect of the ectopic expression of the *Arabidopsis* cell cycle transcription factor *E2FB* in tomato plants. Our data show that an increased level of *E2FB* accelerates plant growth and development, although both *E2FB*-expressing plants and wild-type plants reached a similar size at the end of their life cycle. Nevertheless, it is remarkable that ectopic expression of *E2FB* improved economically important traits such as flowering time and fruit yield. E2FB^{OE} plants develop inflorescences about a month earlier than wt plants. This clearly contributes to a faster formation and collection of fruits. Expression of *E2FB* also leads to a significant increase in fruit yield, an aspect that is important for crop improvement.

The E2F family of transcription factors regulates cell division and cell differentiation by modulating, positively or negatively, the expression of different genes (Stevens and La Thangue 2003; Vlieghe and others 2003). In the model plant *Arabidopsis thaliana*, plants that overexpress *E2FB* develop shorter roots than wt plants (Sozzani and others 2006). However, when *E2FB* is expressed in tomato plants, we have found that roots are longer than in wt. It was proposed that in *Arabidopsis* accumulation of *AtE2FB* antagonizes cell elongation and could delay root cell differentiation. In this work we showed that ectopic expression of *E2FB* generates longer roots than wt tomato plants.

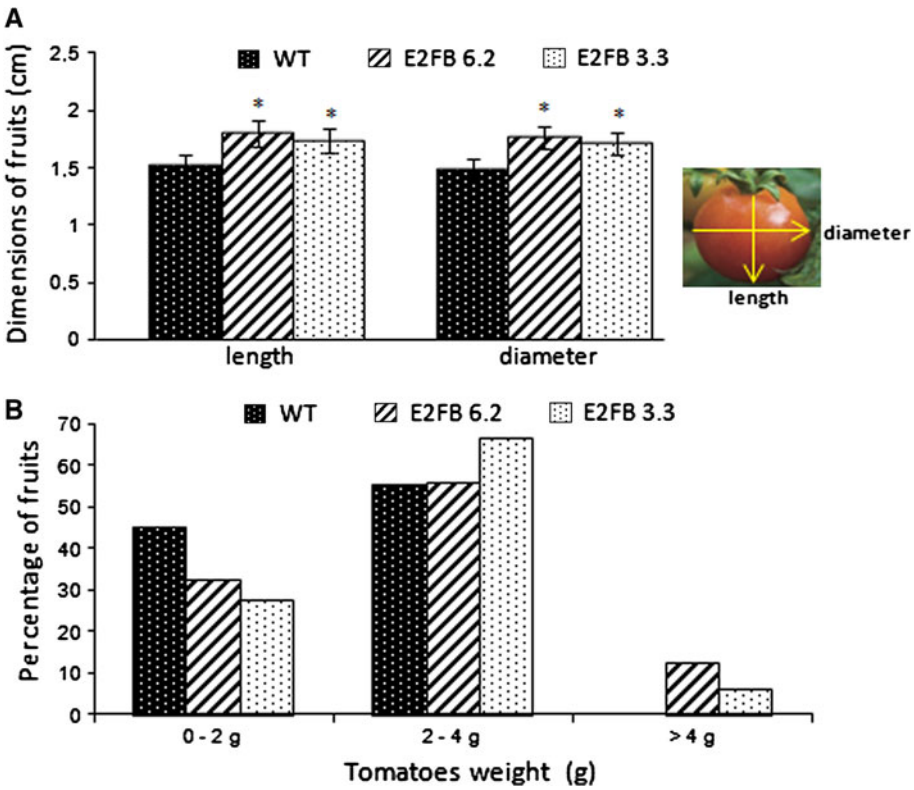

Fig. 7 Ectopic expression of *E2FB* increases fruit yield. **a** Length and diameter of all tomatoes harvested from plants grown in the greenhouse for 5 months were measured with a vernier caliper. *Error bars* represent SD; $n = 224$ for wt; $n = 360$ for E2FB6.2; $n = 356$ for E2FB3.3. *Statistically significant difference for E2FB^{OE} compared with wt values as determined by Student's *t*-test ($P < 0.001$). **b** Percentage of all harvested fruits ($n = 224$ for wt, $n = 360$ for E2FB6.2, $n = 356$ for E2FB3.3) distributed by their weight in three different ranges (0–2, 2–4, or >4 g). All tomatoes were immediately weighed after harvesting

These differences in root growth might be attributed to the fact that different genes are regulated in the two species. Indeed, we found that ectopic expression of *E2FB* reduces the level of cell cycle genes, opposite to the effect found in *E2FB*-overexpressing *Arabidopsis* plants (Sozzani and others 2006). We have shown that ectopic expression of *E2FB* reduces the CYCB1;1 protein level, a marker of the G2/M transition, likely due to a shortened G2/M phase. This reduction of the cell cycle might cause these cells to differentiate faster, leading to faster development of $E2FB^{OE}$ than wt plants.

Using microarray analysis, we found that ectopic expression of *E2FB* leads to slight changes in gene transcription, probably because the majority of the cell cycle genes are not represented in the array and because of the number of genes covered. We found that ectopic expression of *E2FB* leads to a reduction of cell cycle gene expression. It has been proposed that cell cycle genes such as *PCNA* are E2F-dependently repressed in differentiated mature cells (Egelkrout and others 2001). Thus, it is possible that the ectopic expression of *Arabidopsis E2FB* in tomato plants interferes with tomato E2F genes or interacts differently with endogenous tomato RBR, favoring cell cycle gene repression rather than activation. On the other hand, expression of *E2FB* seems to promote the expression of a large number of genes involved in defense and flowering and fruit formation. At this moment, we do not know whether the expression of these kinds of genes is a consequence of the faster development promoted by expressing *E2FB* or is a direct regulation of E2F factors. It is remarkable that more than 50% of the upregulated genes in $E2FB^{OE}$ plants contain an E2F site in their promoters, suggesting that they can be direct E2F target genes. The fact that many upregulated genes belong to different functional categories outside of the cell cycle genes is not surprising. It has been shown that in mammals and in *Arabidopsis*, the expression of genes involved in cell proliferation (DNA replication, mitosis, apoptosis), but also genes related to cell differentiation and development, is modified in response to altered E2F levels (Ishida and others 2001; Vlieghe and others 2003; Ramirez-Parra and others 2004). Unexpectedly, overexpression of E2FA/DPA increases the expression of, in addition to cell cycle genes, a set of genes related nitrate assimilation, likely due to a nitrate drain in the plants as a consequence of increased nucleotide biosynthesis (Vlieghe and others 2003). Transcriptomic and physiological analyses have shown that overexpression of E2FC increased the expression of genes involved in C compounds and carbohydrate metabolism and light signaling, suggesting a role for E2FC in other developmental processes in addition to cell division (del Pozo and others 2002; de Jager and others 2009). These data indicate that E2F factors also regulate, in addition to cell cycle genes, genes involved in cell differentiation.

DNA endoreduplication is a widespread process in eukaryotes. In plants, endoreduplication often occurs in cell types that undergo specialized differentiation, and it has also been directly correlated with cell size (Sugimoto-Shirasu and Roberts 2003). In fact, some crops, including coffee, potato, and banana that have higher yields than their native relatives have higher content in DNA per nuclei, which usually correlates to their increased cell sizes. Overexpression of *E2F* transcription factors in *Arabidopsis* differently alters the ploidy level. Although overexpression of *AtE2Fa-DPa* or *AtE2Fe/DEL1* substantially changed the ploidy levels (De Veylder and others 2002; Vlieghe and others 2005), overexpression of *E2FB* did not induce a relevant modification in the ploidy level in *Arabidopsis* plants (Sozzani and others 2006). In tomato, we have found that ectopic expression of *E2FB* leads to a modest increase in the ploidy level. It is well known that ploidy level increased with maturation of the leaves (Gutierrez and others 2002; Zoschke and others 2007). Because $E2FB^{OE}$ tomato plants reach maturation earlier than wild type, it is possible that the leaves analyzed by flow cytometry had slightly higher levels of ploidy because they were in a more developmentally mature stage than the wt leaves and contained higher levels of DNA.

Although cell division is an essential process for plant growth and development, very little is known about the control and function of cell cycle regulators in tomato. In the last few years, the importance of cell division with respect to fruit size has been shown. WEE1 is a cell cycle kinase that negatively regulated by phosphorylation the activity of CDC2. Plants that express WEE1 in antisense orientation produced smaller tomatoes than wild type (Gonzalez and others 2007). These authors proposed that this phenotype is due to smaller cell size rather than cell division potential. Another example is found in plants that overexpress IMA, a Mini Zinc Finger protein that negatively regulates cell division (Sicard and others 2008). Tomato plants that overexpress IMA showed a severe reduction in plant size and smaller flowers and fruits than wild type. The fw2.2 QTL was one of the few QTLs known to control fruit size in tomato (Frary and others 2000; Nesbitt and Tanksley 2001). The *fw2.2* locus was cloned and found to encode a plant-specific protein that regulates cell division in the fruit (Frary and others 2000; Nesbitt and Tanksley 2001; Cong and Tanksley 2006). It has been demonstrated that FW2.2 physically interacts with LeCKIIβ1, which shows a strong homology to the regulatory subunit of the cell cycle protein CKII kinase (Cong and Tanksley 2006).

We presented data that show that ectopic expression of a positive regulator of cell division, *E2FB*, in tomato plants accelerates plant growth and development. $E2FB^{OE}$ plants form flowers significantly earlier than wt plants and

produce more and bigger fruits, increasing the fruit yield almost twofold. Our data also support the idea that regulation of cell division is important to control plant development and fruit production in agronomic crops and, therefore, cell cycle genes are good candidates to manipulate to improve crop productivity.

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