EBP1 regulates organ size through cell growth and proliferation in plants

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Plant organ size shows remarkable uniformity within species indicating strong endogenous control. We have identified a plant growth regulatory gene, functionally and structurally homologous to human EBP1. Plant EBP1 levels are tightly regulated; gene expression is highest in developing organs and correlates with genes involved in ribosome biogenesis and function. EBP1 protein is stabilised by auxin. Elevating or decreasing *EBP1* levels in transgenic plants results in a dose-dependent increase or reduction in organ growth, respectively. During early stages of organ development, EBP1 promotes cell proliferation, influences cell-size threshold for division and shortens the period of meristematic activity. In postmitotic cells, it enhances cell expansion. EBP1 is required for expression of cell cycle genes; CyclinD3;1, ribonucleotide reductase 2 and the cyclin-dependent kinase B1;1. The regulation of these genes by EBP1 is dose and auxin dependent and might rely on the effect of EBP1 to reduce RBR1 protein level. We argue that EBP1 is a conserved, dose-dependent regulator of cell growth that is connected to meristematic competence and cell proliferation via regulation of RBR1 level.

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Introduction

Morphogenesis in plants is largely postembryonic, and along with organ growth is influenced by environmental factors,

such as light or nutrient availability (Ingram and Waite, 2006). Moreover, plant growth is intimately connected to the capacity of source organs to produce assimilates. This regulatory mechanism determines the yield potential for harvested organs in agricultural crops. In potato (*Solanum tuberosum*), the separation of source and sink organs illustrates the long-distance regulation of organ growth through the interplay of assimilates such as sucrose and other growth factors produced in the source and sink organs (Bologa *et al*, 2003).

The capacity for growth of plant organs is determined by zones of proliferating cells, called meristems. When cells leave the meristematic zone they begin to exit the cell cycle and undergo differentiation that is accompanied by increases in cell size. Cell expansion is facilitated by the loosening of crosslinks between cell wall polymers accompanied by water uptake to vacuoles, and frequently by endoreduplication of DNA (Sugimoto-Shirasu and Roberts, 2003). Therefore, the timing of the transition between proliferative growth and cell expansion/differentiation, largely determines the final cell number and so the size potential of the organ. Differences in organ size between closely related species, such as rapeseed and *Arabidopsis* or differential responses to environmental conditions tend to reflect cell number rather than cell size variation (Beemster *et al*, 2003).

The control of cell size is best understood in yeast where the attainment of a cell size threshold triggers the initiation of cell division. This coordination is thought to be regulated through the translational machinery which, in turn, is determined by the nutritional state (Jorgensen and Tyers, 2004). In plants, remarkably uniform cell sizes in both meristematic regions and young developing organs also indicate the existence of an intricate regulation of cell growth and cell division. However, their coordination is poorly understood (Beemster *et al*, 2003). A mechanism for such coordination could occur via the TCP transcription factors that coregulate the expression of genes coding for the translational apparatus and cell cycle genes (Ingram and Waite, 2006).

In multicellular organisms, it is debated whether the factors determining growth impose their influence on organs as a whole or whether they regulate growth and proliferation at the cellular level (Tsukaya and Beemster, 2006). Moreover, cells in multicellular organisms do not proliferate logarithmically. Their growth and proliferation requires coordination with other parts of the organism mediated by growth and mitogenic factors that may impinge on growth regulation (ribosome biogenesis) and cell cycle control (DNA replication) separately (Conlon and Raff, 1999).

The nucleolus is the main site of ribosome biosynthesis. In humans, EBP1, the ErbB-3 epidermal growth factor receptor binding protein has been shown to be a nucleolar dsRNA binding protein; forming part of the ribonucleoprotein complexes via association with different rRNA species (Squatrito *et al*, 2004). EBP1 was also shown to associate with mature ribosomes and to block the stress-induced phosphorylation of

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the eukaryotic initiation factor 2 alpha (eIF2 α) and thus presumably sustain protein translation (Squattrito *et al*, 2006). Furthermore, EBP1 is a nuclear cell survival factor that together with the protein kinases, Akt and PKC inhibit apoptosis (Ahn *et al*, 2006). Surprisingly, ectopic expression of EBP1 decreased proliferation rate and induced cellular differentiation in cultured human breast carcinoma cell lines (Lessor *et al*, 2000). As the effect on proliferation was linked to its nucleolar localisation in human fibroblasts, it was suggested that EBP1 may represent a new link between ribosome biosynthesis and cell proliferation (Squattrito *et al*, 2004).

Studying tuber organogenesis in potato upon sucrose induction, we have identified *StEBP1*, the potato homologue of the human *EBP1* gene. We show, via modulating the level of EBP1 in potato and *Arabidopsis*, that this gene regulates plant organ growth, effects the expression of different cell cycle genes and influences RBR1 protein level. Furthermore, we demonstrate that auxin regulates the protein stability of EBP1 through which it may influence plant growth.

Results

Induction of gene expression during potato tuber initiation

Potato tubers are formed at the termini of stolons as a result of internal and external cues such as light period and assimilate supply. We have used an *in vitro* system to study the molecular mechanisms that control tuber development and growth (Bachem *et al*, 1996). In the course of this work, a transcript-derived fragment was identified (TDF1044) that showed a transient elevation in abundance during early stages of tuberisation (Supplementary Figure 1A). The expression pattern of the gene represented by TDF1044 was confirmed by microarray data (Supplementary Figure 1B; Kloosterman *et al*, 2005).

Northern analysis was carried out on tissues of different developmental stages using the TDF1044 as a probe (Supplementary Figure 1C). Its expression is correlated with growth and cell division activities of a range of organs. This ubiquitous expression profile suggests that this gene is functionally not limited to tuber development, but correlates with actively growing tissues.

TDF1044 is homologous to the human EBP1, a conserved cell proliferation and cell growth-related protein

In order to identify the full-length potato mRNA corresponding to TDF1044, a cDNA library derived from swelling stolon tips was screened (Taylor *et al*, 1992). The isolated 1.5 kb full-length cDNA codes for a 43 kDa protein and shows sequence similarity (69%) to the human EBP1 (Yoo *et al*, 2000). The gene was named *StEBP1* accordingly. Two major groups of ESTs were identified in the current potato TIGR-EST database; one is showing 100% homology (TC126314), whereas the other (TC128561) shares 87% similarity with *StEBP1*. It is not clear whether the second group of ESTs are derived from an allele within the tetraploid potato or from another gene family member. The *StEBP1* protein shows high similarity (89%) to the *Arabidopsis* G2p protein encoded by a single copy gene at locus At3g51800 (unigene10184), that we name

Figure 1 *StEBP1* inhibits colony formation and represses E2F-mediated transcription in MCF7 human cell line. Colony formation was inhibited when cells were transfected with varying amounts (A) of the full-length *StEBP1* and (B) the truncated Δ -*StEBP1* plasmids and compared to the control CMV-10 construct. (C) Expression of *StEBP1* or the Δ -*StEBP1* repressed the activity of the E2F1 promoter-luciferase reporter when compared to the CMV-10 control. The data are shown as a ratio between the firefly and the control cotransfected *Renilla* luciferase activities; relative luciferase unit (RLU). Error bars represent standard deviations. (D) *StEBP1* protein expression in transfected human cells was detected with the FLAG-M2 monoclonal antibody. Molecular mass (50 kDa) is indicated.

AtEBP1. The structurally and functionally conserved amino-acid sequences are shown in Supplementary Figure 2.

The functional conservation of *StEBP1* was tested by studying the effect of its overexpression on colony formation (Figure 1A and B) and on E2F-dependent gene expression (Figure 1C) in the MCF-7 human breast cancer cell line. Cells were transfected with increasing concentrations (0.25, 0.5 and 1 µg) of plasmid coding for the full-length protein (Figure 1A) or a truncated version of the protein starting from the second in-frame ATG (Figure 1B). As a control, 1 µg of the vector p3xFLAG-CMV10 was used. The full-length *StEBP1* significantly ($P < 0.05$) reduced colony formation in a dose-dependent manner when compared to the vector alone (Figure 1A). The colony inhibition with *StEBP1* is similar to that found with the human EBP1 (Lessor *et al*, 2000). Interestingly, transfection with the truncated *StEBP1* resulted in a more pronounced inhibition of colony formation (Figure 1B).

To test whether *StEBP1* can repress the expression of E2F-dependent genes, human cells were cotransfected with the *StEBP1* constructs together with an E2F1-luciferase reporter (Cress and Nevins, 1996). The truncated version of *StEBP1* was significantly more potent in suppressing E2F1 promoter activity than the full-length version (Figure 1C). The presence of the *StEBP1* proteins was verified using Western blot analysis (Figure 1D). From these results, we conclude that *StEBP1* is conserved both structurally and functionally with the human EBP1 protein.

EBP1 regulates plant growth in a dose-dependent manner

To elucidate the function of the *EBP1* gene, its expression was altered in potato and *Arabidopsis* plants. From around 100

antisense *Stebp1(as)* potato lines, 11 showed growth retardation. Three independent lines, chosen for further characterisation, were smaller in their final height, as well as the tuber yield was lower than in the nontransformed control (Supplementary Figure 3A, B).

Of 51 regenerated RNA interference lines, *Stebp1(RNAi)*, 11 showed a similar but more pronounced phenotype than the antisense lines. Plants such as the *Stebp1-12(RNAi)* (Figure 2A and E) were severely dwarfed and showed hardly any growth on soil. Lines, such as *Stebp1-13*, *-14* and *-67(RNAi)* had a medium phenotype and were stunted and grew slower during the entire course of their development, when compared to control plants (Figure 2A and E). Other lines, such as *Stebp1-65(RNAi)* did reach a similar height to the wild type (Figure 2E).

Quantitative real-time PCR (qRT-PCR) was carried out to determine whether these alterations to the phenotype were directly correlated to the reduction of the corresponding mRNA levels. As shown in Figure 2C and Supplementary Figure 3, the level of the *StEBP1* mRNA was 1.5–2-fold less in the *Stebp1(as)* lines, whereas it decreased between 8 and 12-fold compared to the endogenous wild-type level in the different *Stebp1(RNAi)* lines. Comparison between the *Stebp1(as)* and *Stebp1(RNAi)* lines should, however, be drawn independently as the plants were grown at different times and environmental conditions.

Leaf size and morphology were altered both in the *Stebp1(as)* and the *Stebp1(RNAi)* lines. Wild-type potato has a compound leaf structure (Figure 2F and G). Leaves of the *Stebp1(RNAi)* lines have a reduced number of leaflet pairs, more comparable to younger leaves in the control plants. The total surface area of the leaves was reduced compared to the wild type, although the individual leaflets from the top nodes were somewhat larger (Figure 2F and G and Figure 4A). The wild-type leaf lamella is smooth, whereas in contrast, the morphology of the individual leaflet in the silenced lines was convex with edges curling downwards, giving a folded, compact structure (Figure 2G, i-13). At later stages of development, the tops of the plants were foreshortened and deformed resulting in zigzag internodal stem growth with unopened curled leaves turning towards the stem (Figure 2F, i-13). The size of individual tuber and tuber yield per plant were also reduced and tuber morphology was abnormal (Figure 2H and I).

Plants with elevated expression of *StEBP1* showed normal development, but reached a greater final height compared to the control (Figure 2B and E). Overexpressed *StEBP1* protein in the different *Stebp1(oe)* lines was detected on Western blots via the myc-epitope (Figure 2D). The increase in *StEBP1* transcript level is shown in Supplementary Figure 3D.

EBP1 was also silenced and overexpressed in *Arabidopsis*. Eighteen out of 80 kanamycin-resistant T1 *Atebp1(RNAi)* lines displayed distorted growth, 10 of these did not reach maturity and only three produced homozygous T1 seeds. In a similar way, we also obtained three homozygous *Atebp1(oe)* lines. As shown in Figure 3A, the reduction of endogenous *ATEBP1* mRNA resulted in smaller plants, whereas the increase in *EBP1* led to enlarged plant size compared to the wild-type Columbia control. Images of three parallel plants from each transgenic line and measurements of the canopy area convincingly show the size differences (Supplementary Figure 4). At the seedling stage, a delay in leaf initiation and

distorted leaf shape were characteristic of the silenced lines (Figure 3B). Using qRT-PCR, we found a four-fold reduction of *AtEBP1* level in a representative *Atebp1(RNAi)* line (Figure 3C) whereas the *Atebp1(oe)* lines contained significantly higher levels of both *EBP1* mRNA and protein than the control (Figure 3D and E, respectively). These results are in good agreement with the data obtained for the potato *Stebp1* transgenic lines.

***StEBP1* regulates both cell number and cell size in developing leaves**

Leaves positioned along the potato stem represent consecutive developmental stages, and therefore provide a suitable experimental system to study the developmental regulation of organ growth. The size of the leaflets in the wild-type plant gradually increases from the sixth to the 12th node. In the *Stebp1(RNAi)* lines, leaflets at the sixth node were slightly larger than the equivalent control. At subsequent developmental stages, leaf growth ceased between leaf nodes 8 and 12 in lines *Stebp1-13(RNAi)* and *Stebp1-67(RNAi)*, whereas leaves in *Stebp1-65(RNAi)* continued to grow, although their size stayed behind the equivalent wild type (Figure 4A).

To determine the basis of the observed organ size differences, cell size was measured in the leaves of these lines (Figure 4, Supplementary Table 2). The cell size increased gradually between sixth and 12th leaves in the wild type. In the sixth nodal leaf of the *RNAi* lines, the cell size was ~30% larger. In 8–12th node leaves, the size of the pavement cells remained smaller in the *Stebp1-13(RNAi)* and *Stebp1-67(RNAi)* lines compared to the wild type, but was similar in line *Stebp1-65(RNAi)*, correlating with the degree of silencing (Figure 2C). In summary, in young developing leaves, cell size becomes larger in the *Stebp1* silenced lines, whereas at later stages, during expansion growth, the cell size falls behind the wild type. Using statistical analysis, the differences in cell size were significant here and in subsequent data sets. Numerical presentation of the data is summarised in Supplementary Table 2.

In order to better understand the relationship between leaf and cell size, an index for the total cell number in the investigated leaflet was calculated by dividing the leaflet surface area with the average cell size. We were aware of the fact that the size of cells in different areas of the leaves is variable, but we standardised our sampling procedure as much as possible. The total cell number per leaflet gradually increased with the developmental stages in the wild type. The comparison of several data sets revealed that the total cell number per leaflet generally reaches a plateau of around 10^6 cells per leaflet. We thus assume that after this number, no more cell division occurs. Leaf growth then continues through cell expansion (Figure 4A and B). In contrast, in *Stebp1-13(RNAi)* and *Stebp1-67(RNAi)* lines there was no increase in the total cell number between sixth and 12th leaves, indicating that leaf growth in these lines is attributed to cell expansion across all the developmental stages. In line *Stebp1-65(RNAi)*, the total cell number initially increased from leaves sixth to eighth, after which it levelled off.

Interestingly, when *Stebp1-13(RNAi)* was grown under greenhouse conditions, these plants showed similar stability in the total cell number, but an abnormal cell expansion led to a larger leaf size (Supplementary Figure 5). The phenomenon of counteracting the block in cell proliferation by cell

expansion is known as compensation (Horiguchi *et al*, 2006). Our experiments show that compensation can be environmentally dependent.

During differentiation, pavement cells gain a more complex shape, becoming longitudinally expanded featuring a lobed structure. The shape complexity was quantified by

calculating the 'shape factor' ($4\pi \text{ area/perimeter}^2$), which is defined as 1 for a perfect circle and decreases for more complex shapes. During development, wild-type cells adopt more complex shapes as they differentiate (Figure 4A and B). Pavement cells in the *Stebp1(RNAi)* lines were arrested in cell division and although they expanded, the complexity of their shape did not increase (Figure 4C and D, sixth and 12th leaves, respectively).

We analysed leaves of *Stebp1(oe)* lines in order to learn whether StEBP1 is sufficient to drive cell and organ growth. These lines had a larger leaflet surface area when compared to the corresponding leaflets in the controls (Figure 4B). Surprisingly, the cell size of the overexpression lines in the

youngest sixth leaf was around half of the size of the wild-type cells whereas at later stages cell sizes surpassed the wild type, in parallel with the accelerated organ growth. The total cell number per leaflet reached a higher level at an earlier stage than in the wild type, indicating that the switch between meristematic and expansion growth is shifted to an earlier developmental stage (Figure 4B). Overexpression of the *StEBP1* also brought forward differentiation (Figure 4B and D). In summary, elevated StEBP1 level leads to an increase in the number of cells at early stages of leaf development, ceasing later on in development when further organ growth occurs via a boost in cell expansion and differentiation.

Figure 3 Altered expression level of *EBP1* results in changes in growth habit in *Arabidopsis* transgenic lines. (A) Silencing of the *AtEBP1* causes growth retardation in *Atebp1(RNAi)*, referred as Ati-1, whereas overexpression of the *StEBP1* in *Atebp12(oe)* line, labelled as AtOE12 leads to larger plants compared to the control wild-type (wt) under greenhouse conditions. (B) Silencing of *AtEBP1* delays leaf initiation and alters leaf morphology, as shown by representatives of *Atebp1-1(RNAi)*, and *Atebp1-5(RNAi)* lines compared to the wild type photographed 10 days after germination. (C) Silencing of the *AtEBP1* expression is shown for the representative line *Atebp1-1(RNAi)* and (D) the elevated expression of *StEBP1* is shown in the overexpression lines (AtOE-12, AtOE-19, AtOE-43). RNA for qRT-PCR was isolated from the second to fourth leaves of the same set of plants shown in (A). The expression level of *EBP1* is standardised to the level of the *Arabidopsis actin2* gene. The relative expression is given as a ratio compared to the endogenous *EBP1* transcript, set to unit 1 in the control. (E) The presence of *EBP1* in *Atebp1(oe)* lines (AtOE-12, AtOE-19, AtOE-43) was confirmed with Western blot analysis using myc antibody. Molecular mass (55 kDa) is indicated. Error bars represent standard deviations. Asterisk indicates a cross-reacting protein band with the myc antibody. As a loading control, amido-black staining of the corresponding membrane is shown.

Figure 2 Alteration in expression of the *StEBP1* effects growth in *S. tuberosum* transgenic lines. (A) Silencing of the *StEBP1* inhibited growth in height of the *Stebp1(RNAi)* lines; i-12, i-13, i-14, i-67 whereas (B) overexpression of *StEBP1* in *Stebp1(oe)* lines, OE-3, OE-5, OE-16 led to increased height compared to the control wild-type (wt) under greenhouse conditions. (C) Silencing of *StEBP1* mRNA level in the antisense *Stebp1* lines (as-23, as-78, as-81) and *Stebp1(RNAi)* lines (i-12, i-13, i-14, i-65, i-67) was determined by qRT-PCR. The level of expression is shown as a ratio compared to the wild type given the value of 1. (D) The elevated level of *StEBP1* was detected using the myc-antibody in protein extracts of the overexpression lines (OE-3, OE-5 and OE-16). Molecular mass (55 kDa) is indicated. (E) Potato plants with strong (i-12), intermediate (i-13) and a weak phenotype (i-65) of the *Stebp(RNAi)* lines are compared to the wt and to the *Stebp1(oe)* line (OE-5) grown in the climate chamber. (F) The morphology of the apical area at different developmental stages and (G) the leaf morphology of the *Stebp1-13(RNAi)* is compared to the control (wt). (H) Average weight of tubers per plant in the *Stebp(RNAi)* lines. (I) Tuber yield for four representative plants of wt and i-13 are shown. Error bars represent standard deviations. Bars on F and I = 10 cm.

Figure 4 Leaf size, cell size, total cell number per leaflet (cell number index) and cell shape factor in the *Stebp1* transgenic lines. (A) *Stebp1(RNAi)* lines (i-13, i-65, i-67) and (B) *Stebp1(oe)* lines (OE-3 and OE-5) compared to the wild type (wt). Columns represent values for each successive leaflet from the 6th, 8th, 10th and 12th nodes and coloured in shades of grey. The error bars refer to standard deviations of the values except in the cell number index, where the standard error was calculated. (C) Representative images from the adaxial epidermal cell layer of the sixth leaflet and (D) of the 12th leaflet from the *Stebp1-13(RNAi)*, wild type and *Stebp1-5 (oe)* are shown for illustration. Bar = 100 μM.

EBP1 regulates RBR1 protein level and the expression of cell cycle regulators in an auxin-dependent manner

To understand how cell division is arrested during leaf development when the *StEBP1* mRNA level is reduced, the expression of critical cell cycle regulators of the G1 to S and G2 to M transitions were followed. QRT-PCR was carried out using primers for the potato homologues of the *Arabidopsis* *CYCD3;1* (Dewitte *et al*, 2003), *RNR2* (ribonucleotide reductase) (Chaboute *et al*, 2000), *CDKB1;1* (Boudolf *et al*, 2004) genes. Two independent lines, *Stebp1-13* and *-67(RNAi)* and a control plant were chosen to detect the expression in the apex (pool of the meristem, the first and second nodal leaves) and in leaves from the sixth and tenth nodes. The expression of different genes was quantified as the difference in the cycle numbers (Δ CT) in the qRT-PCR

experiments between the gene of interest and the constitutive *Ubiquitin* gene (Figure 5A). In the apex of young, developing plants, no significant difference was detected in the expression level of the *CDKB1;1* and *RNR2* genes, whereas the level of *CYCD3;1* mRNA was somewhat lower in the *Stebp1(RNAi)* plants compared to the control (Figure 5A). However, in young developing leaves, the abundance of all the three cell cycle regulators was reduced compared to the control (Figure 5A). Although the expression of these cell cycle genes naturally diminishes as leaves develop, their relative levels in the *Stebp1(RNAi)* lines remained below the control even in the fully developed leaves (Figure 5A). Thus, the effect of *StEBP1* gene silencing reduces the expression of cell cycle regulators in a developmentally dependent manner.

Figure 5 Altered level of StEBP1 affects the expression of cell-cycle-related genes. **(A)** The expression levels of *CYCD3*, *CDKB1;1* and *RNR2* mRNAs were determined by qRT-PCR in the apex (meristem, first and second leaves sampled together) and in the sixth and 10th leaves taken from the *Stebp1-13(RNAi)*, *Stebp1-67(RNAi)* and the wild-type control. The expression levels are given as a difference in cycle numbers during qRT-PCR of the genes of interest and *Ubiquitin* (Δ CT). **(B)** The expression levels of these cell-cycle-related transcripts in the *Stebp1-5(oe)* line and the wild type were similarly determined as in (A) in the meristem sampled together with the first leaf primordium (M+1), and in the second, third, fifth and ninth leaves. Regulation of the promoters of **(C)** *CYCD3;1*, **(D)** *CDKB1;1*, **(E, F)** *RNR2* by altered EBP1 expression in transfected *Arabidopsis* cells cultured either in the presence of auxin (+) or in its absence (-). StEBP1 stands for the transfected StEBP1 expression construct under the control of CaMV promoter and iAtEBP1 labels the silencing construct below the graphs. Promoter activities were determined through their fusion construct to the GUS reporter. The inset in **(F)** illustrates the increasing amount of c-myc-tagged StEBP1 protein as a result of the elevation in concentration of the transfected plasmid, detected by the c-myc antibody. Molecular mass of the protein is 55 kDa. The error bars represent standard deviations.

The expression of these cell cycle regulators showed the opposite tendency in the *Stebp1-5(oe)* line (Figure 5B). To detect subtle changes during early development, we examined expression in the meristem sampled together with the first leaf and in the leaves from nodes second, third, fifth and ninth. The *CYCD3;1* expression in the meristem increased approximately 60%, compared to the wild type, whereas at later stages it followed the same level as the control. The expression level of the *CDKB1;1* increased 2–3 times during early leaf development whereas in leaf 9 it was equal to the corresponding wild-type level. An elevated expression of the *RNR2* was present in all leaves of the *Stebp1-5(oe)* line that were analysed.

To follow the transcriptional control of the genes *CYCD3;1*, *CDKB1;1* and *RNR2*, their promoter regions were cloned and fused to a GUS-GFP reporter. The activity of these promoters was analysed by measuring β -glucuronidase (GUS) activity after transfection into *Arabidopsis* protoplasts derived from a suspension culture. As the growth hormone, auxin positively regulates the cell cycle, these experiments were carried out in either the absence or the presence of auxin. When no auxin was added, the promoters had no, or a very low residual activity. With the addition of the hormone, the activity rose to different levels depending on the promoter used (Figure 5C–E). These results show that exogenously applied auxin stimulates the expression of these cell cycle regulated promoters in cultured cells.

To investigate the effect of elevated *StEBP1* expression a construct of *StEBP1* under the control of 35S CaMV promoter was cotransfected with plasmids carrying *CYCD3;1*, *CDKB1;1*

and *RNR2* promoters fused to the *GUS* reporter gene (Figure 5C–F). In all cases, in the presence of auxin and an excess of StEBP1, a further increase of GUS activity was found when compared to applying auxin alone. In contrast, *StEBP1* overexpression did not alter the promoter activities when cells were cultured in the absence of auxin (Figure 5C–F).

Subsequently, the requirement of AtEBP1 in the auxin-dependent activation of cell cycle promoters was tested. For this, the expression of the *AtEBP1* was silenced using the *AtEBP1(RNAi)* construct. We found that silencing of *AtEBP1* completely abolished the activation of *RNR2* promoter by auxin (Figure 5E), indicating that AtEBP1 is required for the auxin-dependent promoter activity. To analyse the dose dependency, quantity of StEBP1 was varied by transfecting increasing amounts (5, 10 and 15 μ g) of *StEBP1* plasmid with the *RNR2*-promoter construct into cells cultured with auxin (Figure 5F). This transfection series resulted in a corresponding accumulation of StEBP1 protein (Figure 5F, inset) and a proportional activation of the promoter activity (Figure 5F).

As auxin was required for EBP1 to regulate the expression of cell cycle promoters, we examined its effect on EBP1 protein level. We found that EBP1 protein accumulated in the presence of auxin, whereas it was hardly detectable in cells cultured for 2 days in its absence, despite of being expressed under the control of the constitutive 35S CaMV promoter (Figure 6A). To study post-translational regulation, cells were cultured for a shorter 16 h period either in the presence or absence of auxin, and subsequently the *de novo* synthesis of proteins was blocked using cycloheximide (CHX). At this time point (T0), the difference in EBP1 levels

Figure 6 EBP1 protein level is regulated by auxin (2,4-D) through its stability and affects the quantity of RBR1 protein. (A) *Arabidopsis* cells transfected with the Myc-StEBP1 construct and cultured for 2 days in the presence (+) or absence (-) of 2,4-D. (B) *Arabidopsis* cells transfected with the Myc-StEBP1 construct and cultured for 16 h in the presence or absence of 2,4-D after which CHX was added (T0) and sampled for the indicated times (CHX + 2,4-D, or CHX - 2,4-D, respectively). StEBP1 was detected on Western blots in (A, B) with the c-myc antibody. Molecular mass (55 kDa) is indicated. (C) Transfection of *Arabidopsis* cells with 0, 5 and 10 µg Myc-StEBP1 plasmid (-, +, ++), together with 5 µg RBR1 construct (+). StEBP1 was detected by the c-myc antibody and RBR1 with the RBR1-specific antibody. (D) Silencing of AtEBP1 in the *Atebp1* (RNAi) lines (Ati-1, Ati-5, and Ati-39) effects the endogenous levels of RBR1 and CDKB1;1. The RBR1 is detected using RBR1-antibody (molecular mass ~125 kDa) and CDKB1;1 by the CDKB1;1-antibody (molecular mass ~35 kDa). Loading control is as in Figure 3.

in the presence or absence of auxin was smaller than after 48 h (Figure 6A and B). In cells cultured with auxin, the EBP1 level remained unchanged for the course of the CHX experiment, whereas without auxin EBP1 decreased with an estimated half-life of 1 h (Figure 6B). In summary, EBP1 protein level alters in accordance with presence of the growth promoting hormone, auxin.

The promoters of the cell cycle genes examined are thought to be regulated by the E2F transcription factors and repressed by the RBR1 pathway (Chaboute *et al*, 2000; Boudolf *et al*, 2004). Therefore, we tested RBR1 protein level in *Arabidopsis* suspension cells and transgenic plants with altered EBP1 level (Figure 6C and D, respectively). We found that the overexpression of EBP1 in *Arabidopsis* cells dose-dependently reduced the endogenous RBR1 protein (Figure 6C). In contrast, the *Atebp1*(RNAi) plants had a consistently elevated RBR1 protein accumulation that was paralleled with the reduction of the CDKB1;1 (Figure 6D). The negative regulation of RBR1 level by EBP1 could provide the mechanism where modulated EBP1 level influences cell cycle promoters, and thus cell proliferation in leaves.

Discussion

EBP1 is a conserved nucleolar protein

In human cells, the EBP1 protein was shown to be part of ribonucleoprotein complexes binding to rRNA precursors and small nucleolar RNA species in the nucleoli, and to mature rRNAs of ribosomes. HsEBP1 was suggested to regulate the production and assembly of translational machinery and to regulate translation in response to cellular stresses (Squatrino *et al*, 2004, 2006). The plant EBP1 protein shows a remarkably high structural conservation to the human counterpart and contains all the essential sequences shown to be required

for the nuclear and nucleolar transport and retention, and for efficient binding to rRNA species in humans. Proteomics of the *Arabidopsis* nucleolus also identified AtEBP1 as a nucleolar protein (Pendle *et al*, 2005). We have screened for StEBP1 interacting proteins using a stolon-derived yeast two-hybrid cDNA library and found numerous putative interactors that relate to ribosome biogenesis (Horváth, unpublished results). These proteins include components of the 60S ribosomal subunit, such as the proteins L6, L7 and the elongation factor EF1A. Putative homologues of the StEBP1 interacting partners were also identified as part of the HsEBP1 complex in human cells (Squatrino *et al*, 2004). The nucleolar localisation and the interaction with ribosome biogenesis factors suggest a conserved function for EBP1 in regulation of protein synthesis and cell growth.

Expression of EBP1 correlates with active growth and protein biosynthesis

We identified *Stebp1* as an early tuberisation-induced transcript. However, its expression is not restricted to the developing tuber, but is present in all actively growing organs. Analysis of publicly available microarray data for expression of the *Arabidopsis* homologue, *AtEBP1*, also demonstrates a convincing connection with cell growth and cell division. *AtEBP1* expression is rapidly induced by sucrose and correlates with the proliferation of cell cultures (Zimmermann *et al*, 2004; Menges *et al*, 2005). Furthermore, we have found that genes coregulated with *AtEBP1* code for proteins implicated in protein synthesis, such as nucleolin, fibrillarin, ribosomal proteins and translational initiation factors (Supplementary Table 1), suggesting that EBP1 is part of a regulatory network previously described as the ribosomal biogenesis regulon in yeast (Jorgensen and Tyers, 2004).

EBP1 is a dose-dependent regulator for organ size

To learn more about EBP1 function, we modulated its expression in potato and *Arabidopsis*. In both systems, we found that growth was stunted in parallel with a reduction in *EBP1* transcript, affecting organs including roots, leaves and tubers in the case of potato. In contrast, plants with elevated levels of EBP1 were generally normal, but had increased growth habit and leaf size.

The organ size is the result of the constituent cell numbers and sizes (Ingram and Waites, 2006). The timing of the transition from proliferative growth to differentiation largely determines the final cell numbers and thus the organ size (Beemster *et al*, 2006). This transition occurs in potato leaves at nodal positions 8–10, a region beyond which cell number ceases to increase further. In lines with strong silencing of *Stebp1* expression, cell number is already slightly decreased at the sixth node, and most importantly, stagnates at this reduced level in leaves at 8–12th nodes. Thus, StEBP1 appears to regulate the developmental end point of cell proliferation. *CycD3;1* is a known cell cycle regulator that inhibits the exit from proliferation and the entry into differentiation (Dewitte *et al*, 2003). *CycD3;1* in complex with CDKA, phosphorylates RBR1 and releases the repression of genes required for cell proliferation. RBR1 was shown to regulate cell production through stem cell function in *Arabidopsis* root meristem (Wildwater *et al*, 2005). Our findings that a normal StEBP1 level is required for sustained *CycD3;1* expression in the apical meristem and that EBP1 dose dependently down regulates the abundance of RBR1 level provides a mechanism how EBP1 may regulate cell proliferation and organ growth.

StEBP1 overexpression does not influence the final cell number, but brings forward the developmental stage at which the final cell number is reached. In the overexpression lines of *Argos* (Hu *et al*, 2003) and *Aintegumenta* (Mizukami and Fischer, 2000), the increase in cell number and organ size is proportional, the period of cell proliferation and cell growth is remaining to be linked and extended. In the *CycD3;1* overexpression line, the meristematic activity is also prolonged leading to a greatly increased cell number but cell proliferation becomes dissociated from cell growth resulting in smaller cells and stunted plants. In all these cases, however, the cell number exceeds the control limit, which contrasts with our results on *EBP1* overexpression, suggesting that EBP1 controls organ size by a different regulatory pathway from *Argos*, *Aintegumenta* and *CycD3;1*. *CycD2;1* overexpression accelerates plant growth and development through simultaneous stimulation of cell growth, cell proliferation and development and thus has similarities to EBP1 (Cockcroft *et al*, 2000). Cyclin D in *Drosophila* is known to be a growth driver (Datar *et al*, 2000). The similarity between *CycD2* and EBP1 further extends to their expression, where both rapidly respond to growth signals, such as sucrose.

StEBP1 limits cell size in proliferating cells

The second cellular parameter that influences organ size is cell size. This is uniquely regulated in meristems during cell division and in postmitotic differentiating cells via organ-specific cell enlargement. Reduced levels of *StEBP1* leads to increased cell size in the *RNAi* lines, whereas overexpression of *StEBP1* results in reduced cell size in developing young leaves. Two alternative explanations can be given for the effect on cell size increase in the *RNAi* lines. Either the

cells stop dividing at an earlier stage and then expand for a longer period or cells divide at a larger cell size threshold. We favour the later explanation as in the *EBP1*(*oe*) lines in young leaves cell sizes are reduced. Furthermore, altered EBP1 levels oppositely affects cell sizes in young and old leaves, indicating two distinct mechanisms, one possibly acting on cell division, the other on cell expansion.

The homeostasis of cell size in proliferating cells is a dynamic balance between cell growth and division. This process is best understood in budding yeast, where, in order to pass Start in the G1 phase, several requirements must be fulfilled such as growth to a critical cell size, nutrient sufficiency and attainment of a critical translational rate. There is a powerful nutrient repression of Start, by which the critical cell size threshold can be reset at each cell division depending on the nutrient availability (Jorgensen and Tyers, 2004). In plants, however, EBP1 stimulates rather than represses cell division allowing cells to divide at a lower cell size. Recently, it was found that genes coding for translational components and cell cycle genes are coregulated through the TCP transcription factors, suggesting a positive connection between cell growth and cell division (Li *et al*, 2005). Cell division is spatially restricted to meristems and temporally controlled by developmental and environmental cues. An example of this is the sprouting of lateral shoots from buds, a process that is under developmental and environmental control, regulated by auxin and dependent on the simultaneous upregulation of protein translation and cell cycle genes by the TCP transcription factors (Tatematsu *et al*, 2005).

EBP1 is required for the full activation of cell cycle genes

The connection between protein translation and cell proliferation is further substantiated by our results, showing that EBP1 is required for the auxin-dependent activation of genes involved in three separate phases of the cell cycle, the G1 phase-specific *CycD3;1*, the S-phase-specific *RNR2* and the mitosis-specific *CDKB1;1*. *StEBP1* overexpression stimulated these promoter activities dependent on the presence of auxin, whereas reduced levels of *StEBP1* abolished their activation. The promoters of these genes contain E2F-binding elements (Chaboute *et al*, 2000; Boudolf *et al*, 2004). We have found that elevated EBP1 levels dose-dependently downregulated the endogenous RBR1 protein that could lead to the release of the E2F-dependent transcription of these cell cycle genes and thus, to increased proliferation and reduced cell size.

Auxin is a plant hormone that plays a pivotal role in plant growth and development. Previously, we have found that auxin regulates cell proliferation by influencing the stability of the E2FB transcription factor (Magyar *et al*, 2005). Here we show that auxin also has an effect on StEBP1 stability, its half-life was drastically reduced when cells were cultured without auxin. Thus, auxin may promote plant growth by simultaneously influencing the stability of both translational regulators such as EBP1 and cell cycle regulators such as E2FB.

It is also possible that EBP1 coregulates protein synthesis and cell cycle through the RB-E2F pathway. Cavanaugh *et al* (1995) have shown that differentiation of a human cell line is accomplished by the accumulation of the RB protein in the nucleolus, correlating with changes in the ribosomal RNA synthesis. RB associates directly with the transcription factor upstream binding protein, the key regulator of rRNA synthesis and with TFIIB, involved in transcription driven by

RNA polymerase III. It was shown that HsEBP1 through its C-terminal end binds RB protein, and contains a nuclear localisation signal in the same region (Xia *et al*, 2001). Although all these motives are conserved in the plant EBP1, we were unable to detect *in vivo* interaction with RBR1 in plant cells in pull-down experiments (Magyar, unpublished result). Consequently, the question whether EBP1 regulates proliferation and cell-growth-related gene expression by direct binding to RBR1 remains open.

EBP1 positively regulates expansion growth of leaf cells

In potato, we found that leaves from the 10th to –12th nodes continue to grow by cell expansion. This process is compromised in *SteBP1(RNAi)* plants leading to decreased cell size, whereas overexpression of *EBP1* drives further cell expansion, giving rise to larger cells with proportionally larger leaves. Thus, elevated levels of EBP1 conversely regulates cell size during meristematic and expansion growth, decreasing the size at which cells divide whereas positively influencing cell expansion. Similarly, in *Drosophila* the cellular response to CycD-Cdk4-driven growth varied according to cell type. In undifferentiated proliferating wing imaginal cells, CycD-Cdk4 caused accelerated cell division whereas in differentiating postmitotic eyes and salivary gland cells, CycD-Cdk4 caused cell enlargement (Datar *et al*, 2000).

During cell expansion, pavement cells on the adaxial leaf surface gain a gradually more complex lobed structure (Fu *et al*, 2005). In the *SteBP1(RNAi)* lines, cells remain ovate whereas elevated StEBP1 levels resulted in more complex cell shape, indicating that in parallel to cell expansion, cell shape is also effected by EBP1 levels. Enhanced growth through EBP1 initially promotes cell proliferation but simultaneously advances cell differentiation, thus the total cell number within organs is unchanged.

Despite its obvious importance, only a handful of genes that have an impact on plant growth or fruit size are known. Thus, the understanding of basic growth-control mechanisms, the identification and characterisation of genes controlling plant body size, development, growth kinetics and growth habits is an ongoing practical need. Discovering the mechanisms how EBP1 regulates organ growth and plant cell size, advances our knowledge in this field.

Materials and methods

Expression analysis in plant tissues

RNA isolation from axillary buds grown *in vitro* and potato plant tissues for Northern analysis was carried out as described previously (Bachem *et al*, 1996). Primers with two selective nucleotides *TaqI* (TC) and *AseI* (GC) were used for cDNA-AFLP (Bachem *et al*, 1996). During qRT-PCR in potato, *Ubiquitin*, and in *Arabidopsis*, *Actin2* transcript was used for normalisation. Potato primers to cell-cycle-related sequences were designed based on the sequence of *A. thaliana*. The list of the *Arabidopsis* genes, their potato homologues and all primer pairs are listed in the Supplementary data. For qRT-PCR, total RNA was isolated from 2–2.5-month-old potato plants, from leaves described in the Results and from *Arabidopsis* transgenic lines, from the pool of the 2nd–4th nodal leaves using the RNA-Easy Plant Mini kit from both plant systems (Qiagen, Hilde, Germany). QRT-PCR was carried out as described by Kloosterman *et al* (2005). The reactions were repeated in triplicate at least twice with independent cDNAs. Relative quantification of the target RNA expression level and standard deviation was performed using the comparative Ct method according to the User Bulletin #2 (ABI PRISM 7700 Sequence Detection System, December 1997, Applied Biosystems).

Methods used in human cell culture

The full-length and the truncated version of *StEBP1* were cloned into the mammalian expression vector (p3xFLAG-CMV-10, Sigma) under the control of the CMV promoter. The colony forming assay with human breast carcinoma cell line MCF-7 (Lessor *et al* 2000) and E2F1 reporter assays were carried out according to Zhang *et al* (2005). All transfection experiments were carried out in triplicate wells and repeated three times.

Vectors, amplified fragments and cloning

Cloning of the tobacco *RNR2* promoter was carried out as described by Chaboute *et al* (2000). To clone the *CYCD3;1* promoter genomic DNA of *A. thaliana* var. Columbia was amplified. StEBP1 cDNA was used to clone the antisense—and in a tail-head/tail orientation the silencing constructs. In the overexpression construct, the coding region was translationally fused at its N-terminus to the 2xmyc sequence coming from the pBS SK-2xmyc vector (Magyar *et al*, 2005). To clone *AtEBP1* for silencing, the sequence related to the clone NM180348 was amplified. The StEBP1(as) was cloned into pBI121vector. Otherwise, pENTR™/D-TOPO® Cloning Kit (Invitrogen) was used and fragments were recombined into the GATEWAY binary-vector system (Karimi *et al*, 2002).

Generation and analysis of transgenic plants

Transformation of the potato (*var. Karnico*) and *Arabidopsis* Col-0 was carried out using *A. tumefaciens* cocultivation. In total, around 150 transgenic lines carrying the silencing constructs and 50 overexpression lines were analysed in consecutive years in the greenhouse in 3–5 repeats. Growth in height was followed on plants with a single stem in 2-week intervals. Lines with altered phenotype were re-examined under highly controlled conditions (160 µmol/m²/s light intensity, 16 h light/8 h dark photoperiod, 18°C/16°C light/dark). In *Arabidopsis* several independent, homozygous T1 transgenic lines were used in further studies.

Transient expression in Arabidopsis protoplast and GUS assays

Protoplasts were prepared as described by Magyar *et al* (2005). Around 10⁶ protoplasts were transformed with plasmid DNA (5 µg) and incubated either in the presence (1 µM) or absence of auxin for 16–24 h unless otherwise indicated. To determine the GUS enzyme activity, the β-glucuronidase (GUS) Reporter Gene Activity Detection Kit (Marker Gene Technologies, Inc., The University of Oregon, Oregon, USA) was used. All experiments were carried out in triplicate and performed independently at least twice.

Epidermal peels and cell measurement

The youngest, not yet fully developed compound leaf (sixth node leaf); young, fully developed leaves (eighth and 10th leaf) and an older, fully developed leaf (12th leaf) were collected from plants grown under controlled conditions. Cell measurements were taken from the adaxial epidermal cell layer of the first opposing leaflet pair of the compound leaf. Similar region of the lamina was analysed for every measurement. Tissues for cell size measurements were prepared according to Taylor *et al* (2003) and were observed under a DMLB microscope (Leica, Wetzlar, Germany) fitted with differential interference contrast optics. Cells were photographed from at least five different positions of the blade section and on average 100 cells were analysed. Cell outlines were traced and parameters such as cell area, perimeter and shape factor were calculated with the public domain image analysis program ImageJ (version 1.33; <http://rsb.info.nih.gov/ij/>). This program was also used to measure the total canopy of *Arabidopsis* transgenics. For statistical analysis to compare mean cell size, the REML procedure in Genstat was used, giving an overall Wald test for any mean being different. Pair-wise comparisons were made by least significant differences based on the estimated standard errors. The average cell area and the leaf surface area were taken to calculate the cell number index.

Protein extraction and protein gel blot analysis

Immuno-blotting was performed as described previously (Magyar *et al*, 2005). The Myc-StEBP1 protein was determined by immuno-blot analysis with monoclonal c-myc antibody (9E10) purchased from Roche Diagnostics. The polyclonal antiCDKB1;1 antibody was used in 1:2000 dilution as described previously (Magyar *et al*, 2005). A cDNA fragment encoding the C-terminal 236 amino acids

of the *AtRBR1* was amplified and cloned in pET28a (Novagen). For protein production, the plasmid was transformed into *Escherichia coli* strain BL21DE3 Rosetta (Novagen) and the expressed hexahistidine-tagged RBR C-terminal polypeptide was purified under denaturing conditions on Ni²⁺-NTA beads (Qiagen). Protein was renatured by stepwise dialysis, concentrated and used to immunise hens (Agrisera, Sweden). IgY was isolated from the egg yolks with the Eggcellent kit (Pierce) and used for Western blots at a dilution of 1:7000.

Transfected protoplasts were cultured for 16 h in the absence or presence of auxin (1 µM) and afterwards treated with CHX (100 µM) to block protein synthesis. Total proteins were extracted and analysed in equal amounts (10 µg).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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