Summary of Ph.D. Thesis

The functional characterization of the *Arabidopsis* E2FB transcription factor

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PUBLICATIONS

Full papers related to the Thesis:

1. <u>**Őszi E**</u>, Papdi C, Mohammed B, Petkó-Szandtner A, Leviczky T, Molnár E, Galvan-Ampudia C, Khan S, Juez EL, Horvath B, Bögre L, Magyar Z (2020) E2FB Interacts with RETINOBLASTOMA RELATED and Regulates Cell Proliferation during Leaf Development. Plant Physiology 182(1):518-53

   Q1 IF: 6,420

2. Leviczky T, Molnár E, Papdi C, <u>**Őszi E**</u>, Horváth GV, Vizler C, Nagy V, Pauk J, Bögre L, Magyar Z (2019) E2FA and E2FB transcription factors coordinate cell proliferation with seed maturation. Development. 26;146(22)

   Q1 IF: 5,611

3. <u>**Őszi E**</u> (2016) Study of the Retinoblastoma (RB) and E2FB controlled developmental processes in transgenic *Arabidopsis* plants. Original title: A növényi Retinoblasztoma (RB) és az E2FB által szabályozott fejlődési folyamatok tanulmányozása transzgenikus *Arabidopsis* növényekben. PEME XIII. PhD. - Konferencia ISBN: 978-615-5709-00-5, 81-86

Full paper, not involved in the Thesis:

1. Natan E, Endoh T, Haim-Vilmovsky L, Flock T, Chalancon G, Hopper JTS, Kintses B, Horvath P, Daruka L, Fekete G, Pál C, Papp B, <u>**Őszi E**</u>, Magyar Z, Marsh JA, Elcock AH, Babu MM, Robinson CV, Sugimoto N, Teichmann SA (2018) Cotranslational protein assembly imposes evolutionary constraints on homomeric proteins. Nature structural and molecular biology 25(3):279-288

   Q1 IF: 12,109

INTRODUCTION

Plant biological and agricultural research is facing serious challenges nowadays. Global warming leads to unfavourable change of climate, making agricultural production more difficult, while human population is steadily increasing, and the area of arable land cannot be extended further. Therefore, solutions to maximize crop yield are needed, for which the understanding of molecular processes regulating plant growth and development is crucial. Plant development and growth is regulated mainly on the level of cell division and cell expansion. These activities are determined by a developmental genetic program, but are strongly influenced by environmental changes as well. In the developing and growing plants, the spatial and temporal regulation of cell divisions is under the control of complex regulatory pathways, but the exact mechanisms are not known yet. Plant hormones play pivotal roles in the regulation of growth and development. Auxin is particularly important by regulating the activity of cell proliferation in a concentration dependent manner. Entering and exiting the cell cycle is regulated by an evolutionary conserved transcriptional mechanism, called E2F-RB regulation, named after its participating components. The *Retinoblastoma* (*RB*) was the first tumour suppressor cloned from mammalian cells, while the first adenovirus E2 factor (E2F) has been identified based on its ability to form complex with RB. In spite of the obvious differences between plant and animal lifestyle and development, this regulatory mechanism is surprisingly well conserved in plants too (Figure 1). In model plant *Arabidopsis*, there is a single RETINOBLASTOMA RELATED protein (RBR), which regulates the activities of three E2F transcription factors. These E2F transcription factors are classified as activators (E2FA, E2FB) or repressor (E2FC) and they are the primary effectors of RBR (Figure 1). According to the canonical model, the activator E2FB stimulates the expression of genes required for cell cycle entry, and RBR inhibits E2FB by forming complex with E2FB. Emerging new data indicate that the activator E2Fs could function as repressors in complex with RBR, suggesting that the E2F-RBR regulation is much more complex than originally thought. Apart from their redundant functions, E2Fs have individual roles, and they can have tissue- and organ-specific functions as well. A deeper understanding of the function of E2F transcriptional factors brings us closer to the ability of modifying regulatory mechanisms behind plant growth and development, and thereby offering new opportunities for breeding programmes of major crops in the near future.
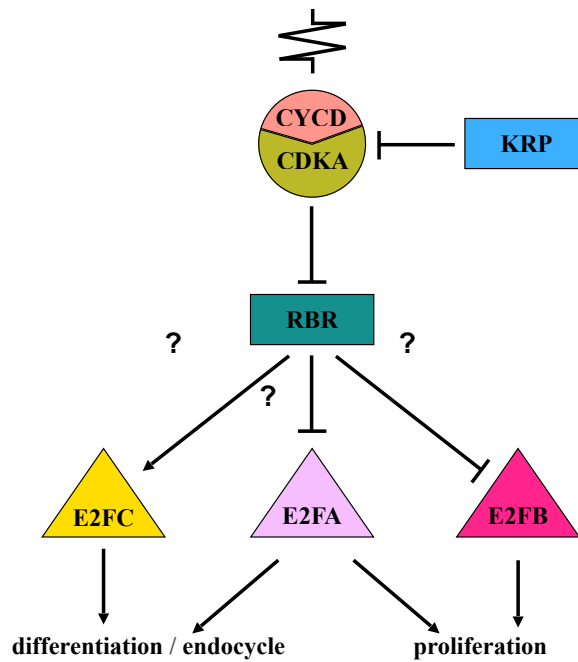
Figure 1. The *Arabidopsis* E2F-RBR transcriptional regulatory model
Under growth-stimulating conditions (in nutrient-rich environment or in the presence of growth-promoting plant hormones like auxin and kinetin) RBR kinase (CYCD-CDKA) is activated and cells enter into proliferation, whereas under growth-inhibitory conditions (nutrient- or light-limited conditions, or in the presence of inhibitory hormones such as abscisic acid (ABA)), RBR kinase remains inactive due to CDK inhibitors (KIP-Related Proteins, KRPs). The activated CYCD-CDK kinase phosphorylates RBR, and this form is unable to bind and inhibit activator E2F transcription factors (E2FA and E2FB). *Arabidopsis* has a single RB-related protein (RBR), forming complexes with all the three E2F transcription factors, but the developmental and cell cycle regulatory function of these E2F-RBR complexes is not fully known (indicated by question marks).

OBJECTIVES

1)   To determine the role of E2FB in division and differentiation, we used the first leaf pair of *Arabidopsis* plants as a model organ, where development is thoroughly characterized and easy to monitor. We were looking for answers to the following questions:

- What is the regulatory role of E2FB in entering or exiting cell division during leaf development?
- How do the quantitative characteristics of leaf epidermal cells change in a developing leaf if the amount and/or activity of E2FB is increased or decreased?
- RBR and CYCD3;1 are the primary regulators of E2FB and also potential E2F target genes. Does E2FB directly regulate these genes through their E2F binding sites, and if it does, what is its regulatory role?
- During leaf development, what characterises the formation of the E2FB-RBR protein complex, and how does this complex change when the amount of E2FB protein is increased?
- How does RBR phosphorylation change during leaf development?
- What is the effect on leaf development of an E2FB mutant, that has lost its abilities either to activate target genes or bind to RBR, but retains its DNA-binding activity?

2)   We attempted to inhibit RBR function specifically in the E2FB expression domains by using an artificial microRNA targeted RBR under the control of E2FB promoter (pE2FB::amiRBR), in order to release E2FB from RBR repression. We identified a transgenic line with macroscopic changes related to RBR silencing mutants, and with novel phenotypes as well. The molecular mechanisms behind these phenotypes have been extensively studied.

3)   In the developing leaf, transcription factor E2FB was detected not only in the nucleus, but surprisingly in close association with the plasma membrane as well. What could be the role of E2FB in the plasma membrane? Does the E2FB have membrane-specific interacting partners? What are these? Could E2FB affect the function of these membrane components?

USED METHODS

1. Production of plasmid constructs and transgenic plants:

    - Different E2F translational constructs labeled with fluorescent tags: pgE2FB-GFP, pgE2FA-GFP, pgE2FB-3xvYFP, pgE2FA-3xvYFP

    - Overexpressor E2FB and DPA constructs coexpressing in transgenic lines: p35S:HA-E2FB-DPA^{OE}

    - E2FB and E2FA deletion mutant constructs: HA-E2FB$^{\Delta RBR}$/DPA, GFP-E2FA$^{\Delta RBR2}$, GFP-E2FB$^{\Delta RBR2}$, E2FB$^{\Delta MB}$ (Fig. 2)

    - E2F specific T-DNA insertion mutant lines: *e2fb-1* [SALK_103138], *e2fb-2* [SALK_120959], *e2fa-1* [MPIZ-244], *e2fa-2* [GABI-348E09], *e2fab* double, complemented *e2fb-2*[pgE2FB- GFP] (Fig. 2)
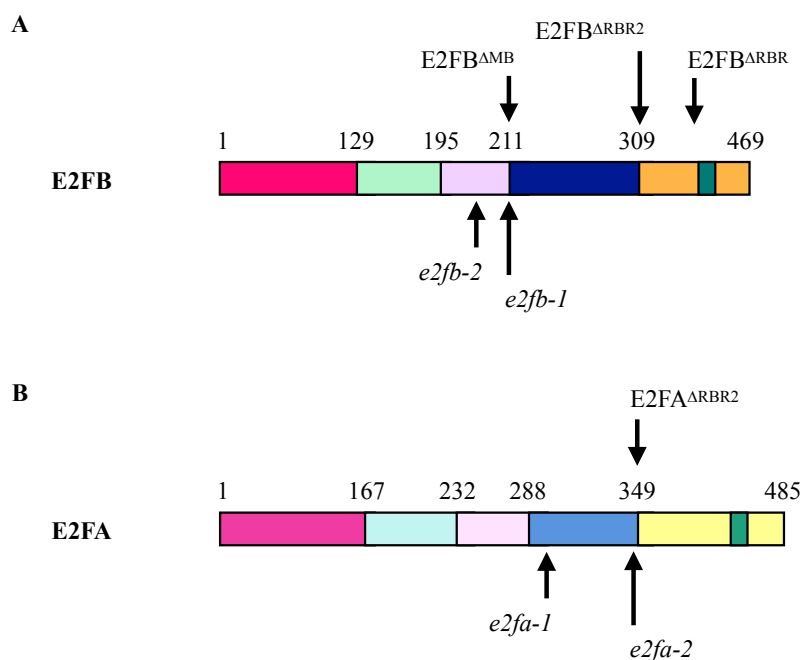
Figure 2. E2FB and E2FA mutants used in this work
A) E2FB and **B)** E2FA deletion constructs and the T-DNA insertion mutants with their corresponding domain organizations. Different colours indicate different domains. Cyclamen = N-terminal region, mint green = DNA binding domain, pink = dimerization domain, blue = marked box, yellow = transactivation domain, greenish-blue = RBR binding domain. In the T-DNA insertion *e2fa* and *e2fb* mutant lines we used arrows to indicate the insertion site (shown on the lower side), whereas in the case of deletion constructs (denoted by Δ), arrows indicate the beginning of deletions (shown on the upper side).

    - Constructs expressing an artificial microRNA targeting RBR, under the control of specific E2F promoters, aiming to inhibit RBR in the E2F expressing cells and tissues: pE2FC::amiRBR and pE2FB::amiRBR

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- Constructs expressing PIN3 in fusion with a fluorescent tag either under the control of its own promoter (pgPIN3-GFP), or the strong viral promoter 35S::PIN3-RFP.

2. Transformation of *Arabidopsis thaliana* plants with floral dip method

3. Transient transformation of *Nicotiana benthamiana* leaves with agroinfiltration method

4. Microscopic imaging:

- Agarose imprints of leaf epidermis observed under DIC light microscope
- Lugol staining of root cap columella cell layers
- EdU (5-Etinil-2-DeoxiUridin) staining of root meristem, visualized under confocal laser microscope
- Imaging of the leaf epidermis in transgenic lines expressing E2Fs in fusion with different fluorescent tags, by using confocal laser microscopy

5. Kinematic analysis of the leaf: measurement of cell size, determining circularity index, total cell number in leaf epidermis, stomatal index, stoma number/leaf

6. Flow cytometry studies

7. Immunoblot analysis

8. Protein interaction studies by immunoprecipitation

9. *In vitro* histone H1 kinase activity assay

10. Membrane preparation with differential centrifugation

11. Chromatin immunoprecipitation (ChIP)

12. Quantitative reverse transcription PCR (RT-qPCR)

13. Thermal asymmetric interlaced PCR (TAIL-PCR)

14. Root gravitropic response test

15. Application of single-cell auxin transport system for Agroinfiltrated *Nicotiana benthamiana* leaves and transgenic *Arabidopsis* plants

NEW SCIENTIFIC RESULTS

During my PhD work I have systematically analysed the function of activator E2FB in various transgenic lines including different T-DNA insertion *e2fb* mutants, dominant negative overexpression lines and in Arabidopsis plants, where E2FB protein could be monitored during organ development in its own expression domain. We have followed gene expressions, purified E2FB-containing protein complexes, identified interaction partners and analysed mutant phenotypes as the consequence of modified E2FB activities. We have focused on the function of E2FB in the developing leaf as it is a great model system for organ development. Based on these studies, we could draw the following conclusions:

1)   We confirmed that E2FB is closely regulated by RBR. Increased E2FB level, either by expression driven its by own promoter or ectopically together with DPA, further elevates the amount of E2FB-RBR repressor complex, leading to reduced cell number and smaller leaf.

2)   We found that elevated and ectopic overexpression of E2FB leads to increased RBR level. This autoregulatory loop enforces the repression, which ensures that cell proliferation is kept under control and thus that increased E2FB level does not lead to tumorous growth. RBR repression of cell proliferation through inhibiting E2FB is suppressed by RBR phosphorylation, and E2FB positively regulates the regulatory cyclin subunit (CYCD3;1) of the RBR-kinase (CDKA;1) as well (Fig. 3 A).

3)   By analysing the effect of ectopic co-expression of an E2FB dominant negative mutant (E2FB$^{\Delta RBR}$) with DPA, we observed overproliferation of small cells, likely belonging to the stomata lineage in the leaf epidermis. Accordingly, E2FB could function as co-repressor in complex with RBR in cell type specific manner in the developing leaf (Fig. 3 B).

4)   The protein levels of both E2FB and RBR decline as leaf development proceeds. During this transition phase from cell proliferation to differentiation, the E2FB-RBR complex is important for exiting cell proliferation and to establish quiescence. When E2FB escapes from RBR repression after the transition phase, differentiated cells reenter cell division, which is the case when E2FB level is elevated with expression driven by its own promoter. Accordingly, E2FB could function either as activator or repressor in leaf cells depending on the developmental stage (Fig. 3A).

5)   Reduced expression of cell cycle genes in E2FB mutants was not manifested at their corresponding protein levels indicating the implication of compensatory mechanism. We found negative correlation between the protein levels of E2FA and E2FB, and they have different impact on the level and activity of RBR protein. In *e2fb* mutant, E2FA protein did not bind to E2FB targets, and could not associate with its specific interactors. Our data indicate that they might compensate each other, but not through direct substitution.
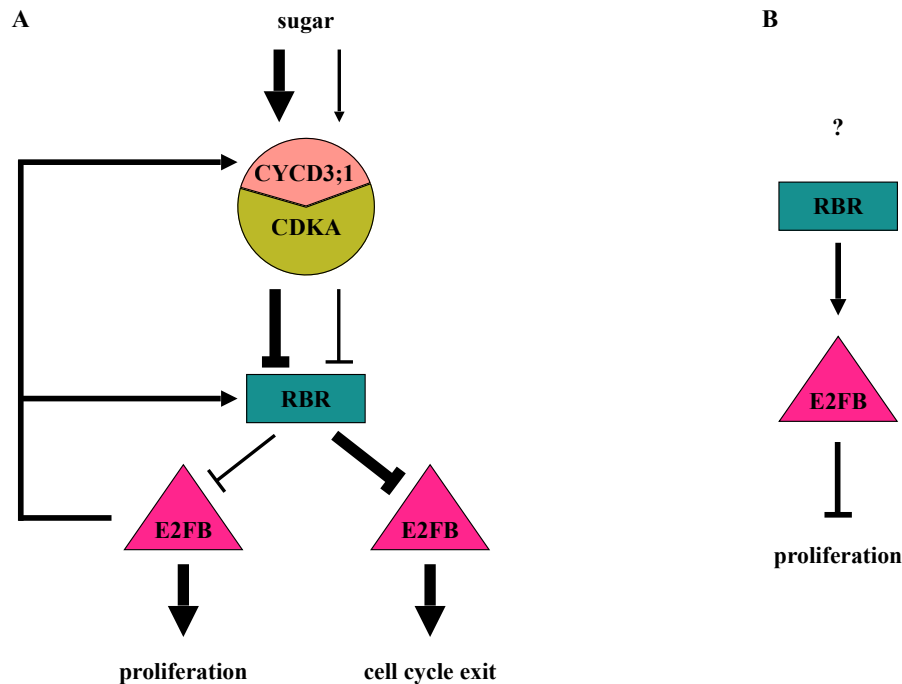
Figure 3. Model explaining the functions of E2FB during leaf development
E2FB has three different activities, and each is dominant at different leaf developmental stages (A) or in different cell types (B). **A)** Activator E2FB is in its RBR-free form, characteristic of young leaves consisting of mostly proliferating cells. The young meristematic leaf is a nutrient-rich sink tissue, where E2FB is released from the repression of RBR by the CYCD3;1-regulated RBR kinase in a sucrose (Suc)-dependent manner. E2FB controls the activity of RBR by using CYCD3;1 activity to regulate RBR transcriptional and protein level, as well as phosphorylation status. In leaf cells where the growth-promoting signal is weakened, the protein levels of both E2FB and RBR decrease, and RBR becomes more active (less phosphorylated) to bind and inhibit E2FB. This repression is important to establish quiescence in leaf cells committed to differentiation. **B)** In developing leaves, E2FB also forms a repressor complex with RBR in meristemoid leaf cells to corepress their divisions. How this repression is regulated by upstream signal(s) is hitherto unknown.

6)   E2FB protein was detected predominantly in the nucleus of nearly all leaf cells, regardless whether they were proliferating or differentiating. Interestingly, we observed the association of E2FB protein with the plasma membrane as well. We discovered that E2FB forms complex with membrane proteins, including auxin efflux PIN3 and PIN7. Our data indicate that E2FB could influence the activity of PIN3, and therefore has an impact on the transport of auxin, and could regulate the cellular concentration of this plant growth hormone (Fig. 4).
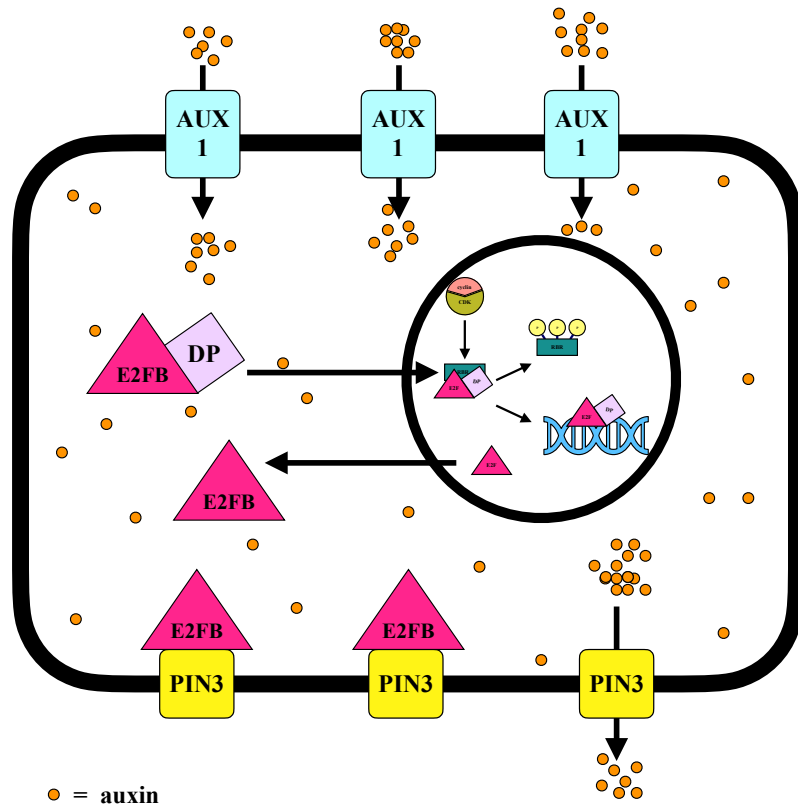
Figure 4. A hypothetical model describing the function of E2FB in the regulation of auxin transport
Auxin activates cell division, presumably through activation of RBR kinases (e.g. CYCD3;1-CDKA;1). Inactivation of RBR by phosphorylation resulted in RBR-free E2FB, which activates cell cycle genes. The model assumes that CDK also inactivates the DP partner after the completion of S-phase. The monomeric DP-free form of E2FB might be excluded from the nucleus and binds to the PIN3 auxin efflux carrier proteins located in the plasma membrane. By making complex with PIN3, the auxin outflow is inhibited, and consequently the intracellular auxin level increased. Elevated auxin level may stimulate the synthesis of DP proteins resulting in the formation of E2FB-DP heterodimer. E2FB-DP is targeted into the nucleus, decreasing the level of E2FB at the plasma membrane. E2FB stimulates the expression of cell cycle genes, including its own upstream negative regulator RBR, which tightly controls its activity.

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