UNIVERSITY OF SZEGED
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Summary of Ph.D. Thesis

The role of plant (Arabidopsis) activator E2F transcription factors in seed development and cell cycle regulation

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INTRODUCTION

Plant growth has exceptional importance for human civilization, yet we are only starting to gain an understanding of its mechanisms. It is well known, that plant growth is restricted to meristematic regions where sufficient cells need to be continuously generated to build the developing plant body. The key factor in growth is the duration of cell proliferation and the timing of the exit from proliferation to cell expansion and differentiation. The current view is that entering or leaving the cell cycle are regulated by an evolutionarily conserved transcriptional mechanism called E2F-RB (Magyar et al., 2008). In Arabidopsis, a single RETINOBLASTOMA-RELATED protein (RBR) has been identified and that can form complexes with three E2F transcription factors (E2FA, E2FB, E2FC, Magyar et al., 2016). Plant E2Fs have been classified as transcriptional activators (E2FA and E2FB), or transcriptional repressor (E2FC), however most of these data were derived from overexpression studies (De Veylder et al., 2002; del Pozo et al., 2006; Magyar et al., 2005; Sozzani et al., 2006; Magyar et al., 2012).

Cell proliferation in plants is strongly regulated during embryo development, where oriented divisions take place during morphogenesis in a highly predictable manner. Morphogenesis is followed by maturation, but how genes involved in cell proliferation and maturation are coordinated during embryo and seed development is not exactly known.

Seed development of plants consist of two consecutive stages; first is morphogenesis, where cell division is the major event; second is maturation phase, where the embryo reaches its final size and seed storage reserves accumulate (Holdsworth et al., 2008; Lau et al., 2012; Sun et al., 2010). The final step of embryogenesis is to develop desiccation tolerance and seed dormancy (Devic and Roscoe, 2016). From an agricultural point of view, these are all very important processes that determine the quality of seeds. Key genetic factors controlling seed maturation are LEC1 TF and three related B3 domain transcription factors LEC2, FUS3 and ABI3 (Lotan et al., 1988; Stone and et al., 2001; Luerssen et al., 1988; Giraudat et al., 1992). While B3 TFs together activate the expression of seed storage reserve protein genes (Kroj et al., 2003), LEC2 TF regulates the expression of WRI1 TF, which target genes are involved in fatty acid synthesis (Baud et al., 2007).

Recently, the dimerization partner (DP), RB, E2F and the MuvB (DREAM) multiprotein complex emerged as a novel unifying regulator in the control of animal cell cycle. Changes in the composition of various animal DREAM complexes shift the balance from quiescence towards proliferation and determine the expression levels of mitotic genes

(Sadasivam and DeCaprio, 2013). Homologues of DREAM components have been identified in plants but whether they form DREAM-like complexes have not been determined yet.

It is paramount to advance plant biotechnology to further boost biomass production and crop yield. To do this effectively, it will be necessary to understand how the molecular machinery that determines yield parameters operates. We can conclude here that E2F-RBR regulation is central in coordinating different developmental phases during seed development and the RBR-E2F network is important both for the extent of seed growth and accumulation of seed storage reserves and should be considered as an important breeding target to increase crop yield.

OBJECTIVES

1. Characterize the activator E2F transcription factors (E2FA and E2FB) during seed and embryo development. How unique and overlapping are their functions? How are they expressed at mRNA and protein levels in the developing siliques and seeds? What characterizes their protein distribution in the developing embryo?

2. What regulatory role do the activator E2F transcription factors play during seed and embryo development in particular to the regulation of cell division? How do the expression of cell cycle genes change in the *e2fa-2*, *e2fb-1* single or the *e2fa-2/e2fb-1* double mutant seeds and siliques? How do embryo and embryonic organ size alter and how do cell number and cell size change in these *e2f* mutant embryos?

3. An E2F-binding sequence can be identified in the promoter region of seed maturation genes (*LEC2* and *WRI1*), which suggests direct regulatory role for E2F transcription factors in the control of these genes. We investigate whether E2F factors regulate non-cell cycle genes in the developing seed and silique, and if so, what effect does this have on the developing embryo and seed?

4. Animal E2F proteins function in multicomponent so-called DREAM protein complexes, and their primary function is transcriptional repression. We examine whether plants also contain a DREAM complex with a similar composition to animals. What proteins do plant E2Fs interact with?

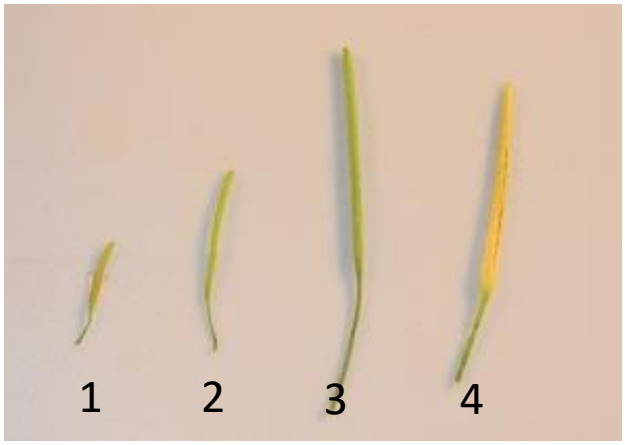
5. E2FB was also found in rapeseed (Brassica napus), one of the closest relatives of Arabidopsis. How similar is the rapeseed E2FB to that of Arabidopsis? We generated transgenic Arabidopsis lines expressing the brassica E2FB under the control of its own promoter. Does the rapeseed E2FB express similarly to the Arabidopsis relative? What proteins does rapeseed E2FB interact with?

USED METHODS

1. Plant material and silique collection

Arabidopsis, Brassica, Arabidopsis transformant and Arabidopsis mutant lines
Arabidopsis thaliana Columbia (Col-0)
Brassica napus cv. Westar
pgE2FA-vYFP (Őszi és mtsai., 2020)
pgE2FB-vYFP (Őszi és mtsai., 2020)
pgRBR-CFP (Őszi és mtsai., 2020)
pgE2FA-GFP (Magyar és mtsai., 2012)
pgE2FB-GFP (Őszi és mtsai., 2020)
pgMYB3R3-GFP (Kobayashi és mtsai., 2015)
pgMYB3R4-GFP (Kobayashi és mtsi., 2015)
e2fa-1: MPIZ-244 (Berckmans és mtsai., 2011b)
e2fa-2: GABI-348E09 (Berckmans és mtsai., 2011b)
e2fb-1: SALK_103138 (Berckmans és mtsai., 2011a,b)
e2fb-2: SALK_120959 (Horváth és mtsai., 2017)
e2fa-2/e2fb-1 (Heyman és mtsai., 2011)
Generated Arabidopsis transformant lines
pWRI1-CFP
p^{mutE2F}WRI1-CFP
pLEC2-CFP
p^{mutE2F}LEC2-CFP
pgBrE2FB-vYFP

Table 1. List of used and generated Arabidopsis and Brassica lines.



Silique stage	Days after pollination
1.	2-3
2.	4-7
3.	8-12
4.	13-17

Figure 1. Silique samples were collected with different sizes representing distinct seed developmental stages.

Representative pictures of the developing silique samples were collected with four sizes; the few mm long S1, the 0,4-0,6 cm long S2, the full size siliques with green seeds (S3) and the yellow silique containing brown dry seeds refer as S4. Timing of seed development days after pollination (DAP) are indicated on the right side in the table.

2. Generation of plasmid constructions (pWRI1-CFP; p^{mutE2F}WRI1-CFP; pLEC2-CFP; p^{mutE2F}LEC2-CFP; pgBrE2FB-vYFP)

2.1. Purification of genomic DNA using CTAB extraction method

2.2. Generation of genomic clones using Phusion DNA polymerase

2.3. Purification of PCR products with polyethylene glycol (PEG)

2.4. Cloning by Gateway^{TM} (Invitrogen) method using homologous recombination

2.5. BP cloning reaction

2.6. LR cloning reaction

2.7. Generation of site-specific mutations using the method of Quick change mutagenesis

3. Transformation and amplification of plasmid DNA in E. coli (XL-1 Blue) competent cell

4. Check of selected bacterial clones by colony PCR

5. Isolation of plasmid from bacterial cultures

6. Transformation and amplification of plasmid DNA in *Agrobacterium tumefaciens* (pSoupAgricola /GV3101) cells

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

8. Western immunoblot analysis

9. Protein interaction studies by immunoprecipitation

10. RNA extraction (CTAB-LiCl method) and quantitative RT-PCR

11. Chromatin immunoprecipitation (ChIP)

12. Mass spectrometric analysis of proteins

13. Embryo preparation, microscopic examinations

RESULTS

1, In spite of the partial requirement of these activator E2Fs to fully promote cell cycle genes, cell number in the mature embryos of the single and double E2FA and E2FB mutants was comparable with the control WT. These findings demonstrate that E2FA and E2FB are only partially required for the expression of cell cycle genes during embryonic cell division, and the reduced expression of these cell cycle genes does not manifest in reduced cell proliferation.

2, We show here that cell cycle genes still express even after the completion of the proliferation phase in the developing seeds of double *e2fa-2/e2fb-1* mutant. This shows that these two E2Fs function as repressors on cell cycle genes as seed development progresses into the maturation phase. In the *e2fa-2/e2fb-1* double mutant embryo, however, we have not seen significant increase in cell number, indicating the requirement for additional components besides E2FA and E2FB downstream of RBR, likely E2FC, to repress cell proliferation during the maturation phase of embryogenesis. E2FA and E2FB regulates S-phase genes in redundant manner, but only E2FB activates the expression of the G2-M phase specific *CDKB1;1* gene.

3, Contrary to E2FA and RBR, both the transcript and the protein of E2FB could be detected in dry seeds. Based on these findings, E2FB may have an unknown, but RBR-independent regulatory role at this stage of seed development.

4, We found that both *LEC* genes were prematurely up-regulated in the *e2fa-2/e2fb-1* double mutant. In addition, we show that the *LEC2* gene could be directly regulated by E2Fs through an E2F-binding site during the maturation phase. Additionally, *LEC2* expression was also prematurely activated in the *e2fb-1* mutant suggesting that E2FB regulates *LEC2* but earlier than E2FA. In agreement, expression of *LEC2* became de-regulated when its E2F-binding sequence in its promoter region was mutated, and showed a nearly maximum level of expression already during the morphogenic developmental phase.

5, The most important seed storage reserve proteins, 2S albumin and 12S globulin, became prematurely accumulated at the proliferating phase of seed development in the *e2fa* and *e2fb* simple and double mutants. Based on these data, we hypothesize that E2Fs in complex with RBR prevent premature accumulation of seed storage proteins.

6, The embryonic *LEC2* and *WRI1* genes were found to be expressed in the root meristem. Interestingly, the *LEC2* is differently regulated by E2Fs in the embryo and in the root; repressor in the former, and activator in the later. Based on these findings, the regulation of a specific gene by E2Fs could depend on developmental context.

7, We purified DREAM-like protein complexes from plants, consisting of components similar with animal DREAM complexes. However, contrary to animals, where exclusively repressor E2Fs form DREAM complexes, in Arabidopsis besides the repressor E2FC, the activator E2FB was also identified in complex with DREAM components. In young dividing leaf, E2FB binds to activator MYB3R4 protein, whereas in the already differentiated leaves, repressor E2FC associates with repressor MYB3R3 protein indicating functional differences between these E2F containing DREAM complexes.

8, Transgenic Arabidopsis plants were generated with the rapeseed E2FB genomic clone. Rapeseed and Arabidopsis E2FB proteins show very similar expression patterns in the root meristem. Interestingly, both plant E2FB accumulate at the highest level in post-mitotic root cells. Based on this, E2FB may also play an important role in cell elongation and differentiation. By identifying the interactors of rapeseed E2FB protein we found RBR, DPA, and DPB as well as ALY3 proteins. All these data suggest that rapeseed E2FB has very similar properties to the Arabidopsis E2FB protein.

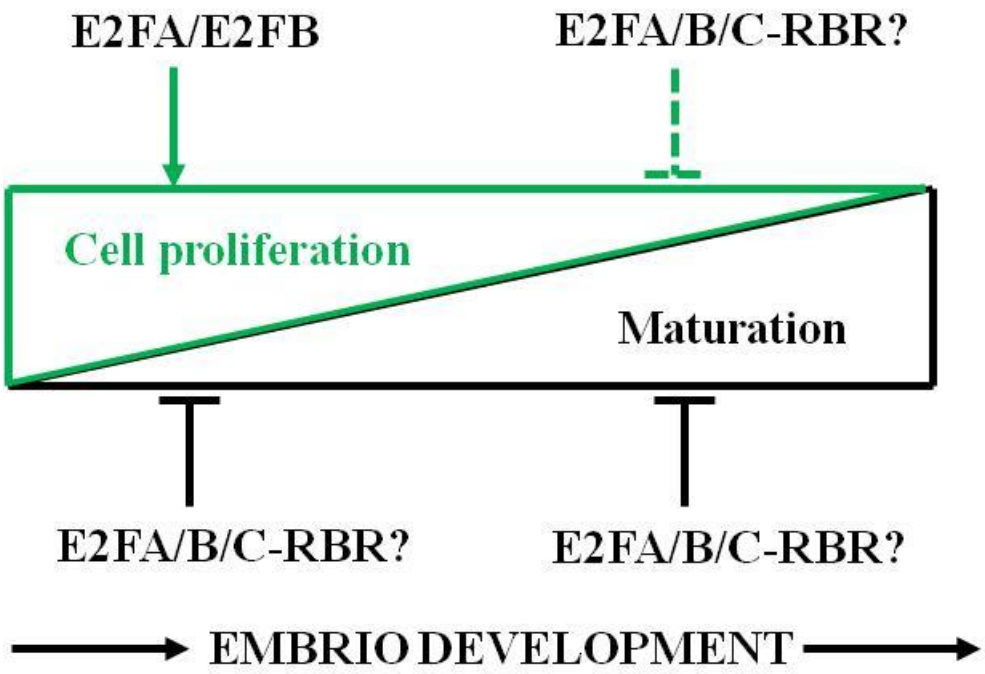


Figure 2. Model explaining the functions of activator E2Fs during seed and embryo development.

The proliferative morphogenic (green triangle) and the differentiation-related maturation (black triangle) phases are the two major and oppositely regulated phases of seed and embryo development. Activator E2Fs are required for the full activation of cell cycle genes in the morphogenic developmental phase, whereas in the subsequent maturation phase they are involved in the repression of cell proliferation, probably together with E2FC and in complex with RBR to establish quiescence. The maturation programme is inhibited in the proliferative phase by activator E2Fs through either repression of the expression of maturation genes such as *LEC2* or inhibition of the accumulation of the SSPs 2S albumin and 12S globulin. Activator E2Fs also tune the expression of maturation genes during the differentiation phase of seed development, and E2FC and RBR might also participate in this regulation.

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