Final Report of the OTKA research grant K-105816

Project title: *Transforming Arabidopsis research into targeted rapeseed breeding for improved growth and yield.*

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Introduction

The E2F-Rb pathway is remarkably conserved in plants regulating the balance between cell proliferation and differentiation. Keeping this balance is central to plant growth and development. Our previous results revealed that E2FB, the main candidate for transcriptionally activating cell cycle genes in *Arabidopsis* is a key dose dependent regulator of cell production in meristems. Thereby E2FB can boost biomass, while in seeds it regulates development through other target genes, including WRINKLED1 (WRI1) and LEAFY COTYLEDON2 (LEC2). Thus E2FB can be a promising breeding target for crop improvement. Here we further study the regulatory role of E2FB during plant development in the model plant *Arabidopsis*, and in its closest relative in the rapeseed, which is an important crop plant. We have decided to repeat the same experiments in rapeseed we have done in Arabidopsis aiming to improve growth and oil content. Accordingly, we modulate the levels of E2FB, WRI1 and LEC2 in rapeseed by introducing extra genomic copies identified from the rapeseed. In addition, we have further studied the function of E2FB in *Arabidopsis*; we show that the activator E2FB could function as transcriptional repressor in cell type specific manner regulating the division of meristemoid cells. Recently it was demonstrated in animals, that E2F and Rb proteins function in multiprotein complexes called DREAM to regulate cell cycle and development. By using mass spectrometry we identified different DREAM-like complexes in Arabidopsis for the first time and suggested that they have either repressor or activator functions (Kobayashi et al., 2015).

Results

1. E2FB regulates cell proliferation and differentiation. Gene regulatory E2FB function in growth.

Previously we have shown that E2FB functions as transcriptional activator on cell cycle genes (manuscript in preparation). Plants lacking E2FB in *e2fb* T-DNA insertion mutants show reduced expression of cell cycle genes confirming the transcriptional activator function of E2FB. However these mutant plants develop fairly normal indicating that E2FB function is not essential for cell cycle progression. In addition, the double homozygous loss of function *e2fa/e2fb* (*e2fa-2/e2fb-1*) mutant that lack both activator E2Fs with transactivation function are still viable and develops into fertile plants. These results are in complete agreement with current findings in the animal cell cycle fields where the function of activator E2F transcription factors was demonstrated not to be necessary for normal cell proliferation. Interestingly however, another *e2fa* mutant allele (*e2fa-1*) where the T-DNA is inserted further upstream into the so called MARKED box region could not produce double homozygous plants when combined with the *e2fb* loss of mutants (our unpublished data). This demonstrated a potential novel role for the MARKED box region in the function of plant E2Fs. Animal studies confirmed that the MARKED box domains of both E2F and DP are important to provide contact with the Rb protein and our results show that this interaction domain to form protein complexes is important for E2FA and E2FB functions. Thus contrary to the expectation the transactivator function of the so called activator E2Fs in *Arabidopsis* is not essential for the control of cell cycle but their complexes with RBR play crucial regulatory role in plant development.

By expressing an E2FB mutant (p35S::HA-E2FB$^{\Delta RBR}$/DPA) unable to transactivate and making complex with RBR we discovered that E2FB can also form repressor complex with RBR but cell type specific manner controlling the division of small meristemoid-like cells in the leaf. In contrast to meristemoid like cells, pavement cells found to be enlarged in comparison to the wild type control leaf indicating that they prematurely stop dividing in the mutant leaf. On the basis of our data we suggest that E2FB has different roles in these leaf epidermal cells; it operates as an activator in the pavement cells, and functions as a co-repressor in complex with RBR in meristemoid leaf cells belong to the stomata lineage.

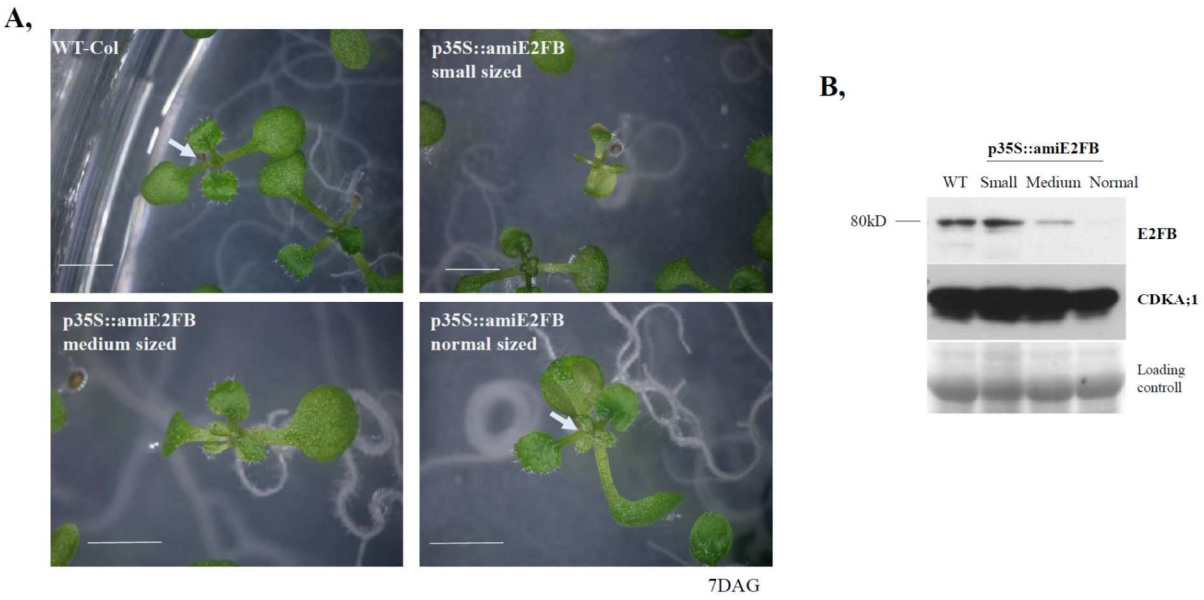


Figure 1. **Reducing E2FB level by using microRNA based method targeting a sequence in the 3' non-translated region of E2FB change growth rate of transgenic *Arabidopsis* plants.** The artificial microRNA was produced by the strong viral promoter p35S (p35S:amiE2FB). (A) Transgenic lines expressing the amiE2FB show different growth-related phenotype. (B) Total protein was isolated from pulled seedlings of a particular phenotype and the E2FB protein level was determined in western-blot by using E2FB-specific antibodies. Anti-CDKA;1 antibody against the PSTAIRE region was used as control. The abundant Rubisco small subunit was stained with Ponceau-S on the membrane and showed as equal loading. Arrows show the newly form leaves in WT-Col, and in normal sized transgenic seedling.

Previously we produced *Arabidopsis* lines with extra copy of E2FB gene. Importantly, the lines with modest elevation of E2FB compared to endogenous level showed a clear growth advantage to wild type, while lines with high E2FB levels were growth retarded (manuscript in preparation). In contrast, the complete lack of E2FB in the *e2fb* mutant lines did not show dramatic changes in growth. Than we asked whether decreasing E2FB level has any effect on growth. For this reason transgenic *Arabidopsis* plants were generated where we introduced an artificial microRNA under the control of the strong viral promoter 35S targeting the 3' prime region in E2FB. We have identified a single T-DNA insertion line (p35S:amiE2FB) where we could detect growth-related phenotypes ranging from very small retarded to normal sized wild type looking plants (Figure 1). Interestingly, the growth rate of normal sized transgenic plants was increased as the newly formed leaf developed faster and make flower earlier in time in comparison to the similar aged wild type control (Figure 1A). Similar change in growth rate was observed in transgenic *Arabidopsis* plants overexpressing CyclinD2 (CYCD2), a negative

regulator of RBR (Cockcroft at al., 2000). Surprisingly, these phenotypes were found inversely correlated with E2FB protein level (Figure 1B). By crossing an E2FB variant within the amiE2FB line insensitive to the artificial microRNA we confirmed that the reduced E2FB level resulted in the growth related phenotypes. These data confirms that E2FB is a growth regulator controlling both organ size and growth rate. In addition, we suggest that there is different readings of the E2FB expression levels in the developing plants. The very low E2FB level in the amiE2FB line resulted in similar effect on growth than the zero E2FB level in the *e2fb* mutants. In contrast, only a slight change either up or down in E2FB protein level caused dramatic changes in growth. We think that E2FB is present in different complexes with transcriptional activator and repressor functions, and might the balance between these complexes regulates plant growth.

We have also recognized that RBR protein accumulates according to the level of E2FB, and we also have seen that RBR follows E2FB in its complex to regulate its activity. That indicates that E2FB directly stimulates RBR. We confirmed that RBR expression was elevated or decreased according to the level of E2FB. In addition, the phosphorylation level of RBR was also changed according to the level of E2FB expression. Previously we have seen that RBR is phosphorylated on conserved site by CYCD3;1-dependent CDKA;1 kinase, which is the canonical CDK in Arabidopsis. CYCD3;1 expression was also elevated or repressed in ectopic E2FB or e2fb mutant lines. These data indicates that E2FB regulates the expression of its up-stream regulators, RBR and CYCD3;1, and thereby controls its own activity. To determine the genes bound by E2FB on the genomic scale, we performed ChIP followed by deep sequencing (ChIP-seq). For this purpose we have used a transgenic line where we introduced the E2FB-GFP under the control of its native promoter in the *e2fb-2* mutant lacking any E2FB which could bind to target DNA sequences. Previously we have seen the enrichment of E2FB-GFP containing complexes indicating that E2FB-GFP is functional. Parallel we used E2FA or E2FC as well as RBR genomic lines in fusion with GFP. Immunoprecipitation was carried out by GFP-magnetic beads. DNA libraries for deep sequencing were generated from the immunoprecipitated DNA fraction (ChIP DNA) and input DNA fraction, and analysed by Illumina Genome Analyzer II (data not shown). This analysis identified a number of genes that were significantly enriched in ChIP DNA compared with input DNA fraction (data not shown). Currently we are analysing these results but we could confirm that both RBR and CYCD were among the identified genes including WRI1 and LEC2 (see below).

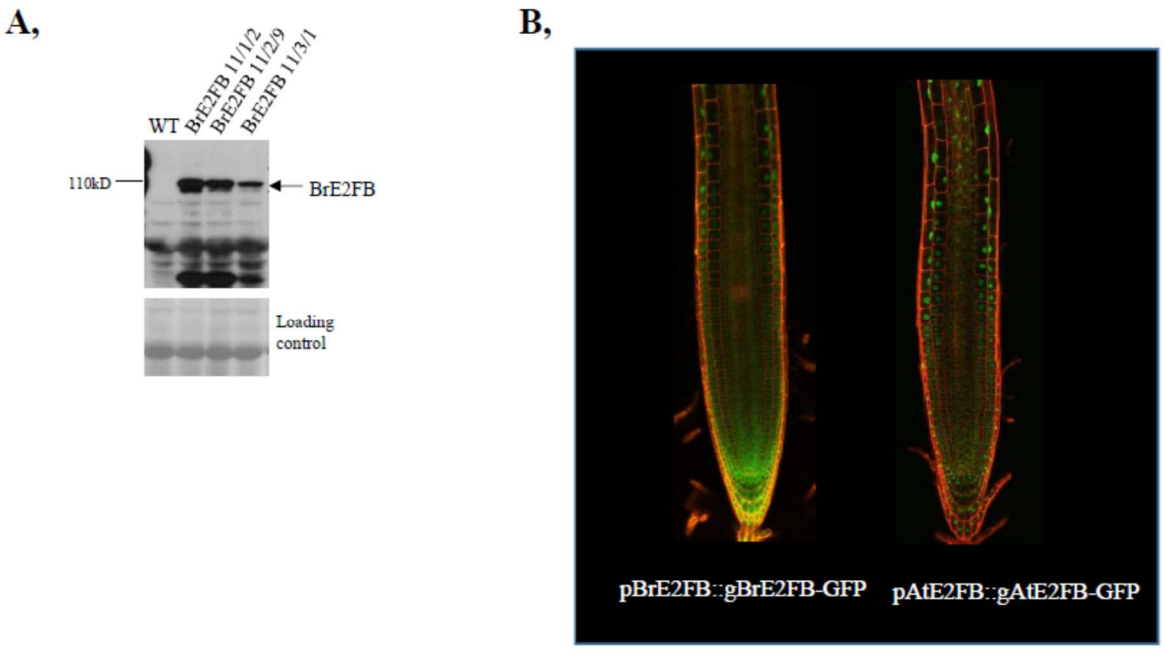
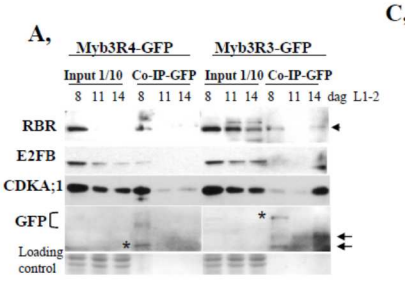


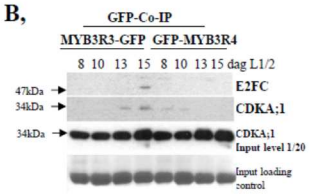
Figure 2. *Brassica* E2FB expressing under the control of its own promoter shows similar expression pattern in the *Arabidopsis* root meristem with the endogenous E2FB. (A) Total proteins from transgenic Arabidopsis plants expressing the Brassica E2FB fused with GFP under its native promoter were immunoblotted by using anti-GFP antibody. Ponceau-stained proteins were used to show equal loading. (B) Expression of BrE2FB and AtE2FB in transgenic Arabidopsis roots was analysed in confocal laser microscopy. Roots were stained with propidium iodide.

We have identified and cloned the genomic copy of rapeseed E2FB under the control of its own regulatory sequence and fused with vYFP at the C-terminus. As a first step the genomic *Brassica* E2FB clone was transformed into *Arabidopsis*. We have identified independent transgenic lines expressing the *Brassica* E2FB at various levels (Fig2A). Comparison of the expression pattern of the *Brassica* E2FB in *Arabidopsis* with the *Arabidopsis* E2FB shows considerable similarity in the root meristem (Fig 2B). Both E2F transcription factors were localized into the nuclei of the root cells, and they accumulated at higher level in post-mitotic and quiescent cells indicating that E2FB might function in these cells as cell cycle repressor. In agreement, ectopic expression of the activator E2FB-DPA heterodimer resulted in shorter root in the presence of exogenous kinetin indicating that kinetin stimulates differentiation through making repressor from the activator E2FB (manuscript in preparation).

2. Identification of proteins present in E2F-RBR complexes.

We found that the *Arabidopsis* E2Fs are making complexes with RBR and could also associate with MYB3R transcription factors (plant homologs of Drosophila dMyb or mammalian B-MYB transcription factors) in *Arabidopsis* leaves and young seedlings (Fig 3. - Kobayashi et al., 2015a; 2015b). In animals, it was shown that E2F-Rb function together with Myb transcription factors in evolutionary conserved multi-protein complexes called DREAM or LINC in human (DP, RB-like E2F, and MuvB – or LIN complex), and dREAM or MMB in *Drosophila* (RBF, E2F2 and Myb or MyB-MuvB), which represses most cell-cycle genes when cells exit cell cycle and enter quiescence. In Drosophila the complex consists of nine different proteins: dMyb, Mip130/LIN9, Mip120/LIN54, Mip40/LIN37, p55Caf1/RbAp48, E2F2, DP, Retinoblastma (Rb)-related protein (RBF1-2) and LIN52. Human cells also have conserved protein complexes, in which RBBP4, LIN9, LIN37, LIN52 and LIN54 form a stable core complex called the MuvB core. In Arabidopsis, the orthologs of these components are present except Mip40/LIN37 and LIN52, but there are five MYB3R homologs (MYB3R1-5). By using mass spectrometry based proteomics analyses of purified RBR and E2F complexes from Arabidopsis seedlings and leaves expressing GFP-tagged MYB3R3 (repressor) or MYB3R4 (activator), RBR and E2FB we have identified different DREAM-related complexes (summarized in Table 1). Our recent mass spectrometry analyses further confirmed the presence of DREAM complexes in *Arabidopsis* but also add newly identified components to this list (Table 1 – Horvath et al., 2017). The plant DREAM-like complexes differ from those in animals in that plants may have two distinct complexes containing different pairs of Myb and E2F family proteins.





C,

Identified DREAM component	*Peptide counts of label free pull downs of*				
	E2FA	E2FB	E2FC	DPA	DPB
RBR	54	59	54	21	30
E2FB	--	47	--	30	15
E2FC	--	--	81	10	4
E2FA	36	--	--	10	4
DPA	9	10	6	41	--
DPB	27	29	26	--	68
ALY3	--	5	9	6	8
MSI1	--	4	8	4	5
TCX5	--	4	5	4	--
ALY2	--	2	--	--	--
TCX6	--	1	--	--	--
MYB3R1	--	1	--	--	--

D,

Identified DREAM component	*Peptide counts of label free pull downs of*	
	BrE2FB	AtE2FB
RBR	33	59
E2FB	37	47
DPA	10	10
DPB	21	29
ALY3	3	5
MSI1	--	4
TCX5	--	4
ALY2	--	2
TCX6	--	1
MYB3R1	--	1

Figure 3. MYB3R3 and MYB3R4 both interact with RBR1 and differently associate with E2F isoforms.
A, MYB3R3-GFP and GFP-MYB3R4 both interact with RBR1 and CDKA;1, but with a different E2F isoform in Arabidopsis leaves. IP was performed with anti-GFP antibodies from protein extracts prepared from first leaf pairs of MYB3R3-GFP or GFP-MYB3R4 transgenic plants at indicated days after germination (DAG). In these transgenic plants, expression of GFP fusion proteins was driven by the corresponding native promoters. Co-IP of RBR1 and E2FB was examined by Western (specific to CDKA;1) antibodies were used. As input, 1/10 of IP was loaded. Coomassie staining of the same membrane was used as a loading control. (B) MYB3R3-GFP interacts with E2FC, but GFP-MYB3R4 does not. IP was performed with anti-GFP antibodies from protein extracts prepared from first leaf pairs of MYB3R3-GFP or GFP-MYB3R4 transgenic plants at indicated days after germination (DAG). Co-IP of E2FC and CDKA;1 was examined by Western blot analyses using anti-E2FC and anti-PSTAIRE antibodies, respectively. As input, 1/16 of IP was loaded. Coomassie staining of the same membrane was used as a loading control. C-D Tables (C) DREAM-complex interaction partners of E2FA, E2FB, E2FC, and DPA, DPB, RBR and (D) BrE2FB. Immunoprurified samples were analysed by LC-MS/MS (see details in Kobayashi et al., 2015). Numbers indicate the identified unique peptides for the respective proteins. None of these proteins were identified when the GFP-expressing control plants were analysed.

One represents putative activator complex containing E2FB associated with RBR, DPA or DPB, and the LIN9 orthologs ALY2 or ALY3 (always early), the LIN54 ortholog TCX5 or TCX6, and MYB3R1. In addition we have also seen association between E2FB and the activator MYB3R4 in young proliferating leaves and in young seedlings maintained in nutrient rich condition stimulating proliferations (Kobayashi et al., 2015). Accordingly, two activator transcription factors, the MYB3R4 and the E2FB are in common complex with RBR during the transition from G2 to M phase deep inside the cell cycle,. This is against the textbook picture as we have demonstrated that RBR could make complex with activator E2Fs inside actively

dividing cells although they are enriched in RBR-kinases. The repressor E2FC was found to associate with all these components identified with E2FB, but instead of making complex with the activator MYB3Rs it was found to associate with the repressor MYB3R3 (Kobayashi et al., 2015). Interestingly, E2FA-GFP interacts with RBR, and DP proteins but did not pull down any members of the DREAM-like complexes (like ALY2/3 or TCX, MSI1) further supporting that E2FA has different functions than E2FB. Indeed, recently we reported that E2FA and not E2FB regulates cell death in complex with RBR (Horvath et al., 2017 accepted for publication in EMBO).

We also used the rapeseed E2FB tagged with GFP expresses in *Arabidopsis* to purify its interactive partners. As the Table 2 shows the rapeseed E2FB found in similar complexes like the *Arabidopsis* E2FB including the RBR, DP proteins and ALY3 as a specific member of the DREAM complex. On the basis of this study we think that rapeseed E2FB functions in a similar way as the *Arabidopsis* one.

3. How E2FB regulates seed development and oil reserves.

The E2FB expression is peculiar during seed development (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). As an activator E2F it was expected to be high in the proliferating phase in the developing seeds however E2FB expression reaches the maximum values during the maturation phase. That indicates that E2FB might regulate the expression of non-cell cycle genes too. Our primary candidate was the WRINKLED1 (WRI1) gene since it shows overlapping expression with E2FB, and contains a putative E2F-binding site in its regulatory sequence. WRI1 belongs to the plant specific AP2 transcription family. WRI1 function was primarily coupled to seed development as in wri1 mutant the seed storage oil is reduced by 80% to the WT level. It was established that WRI1 is responsible for the activation of genes involved in carbon metabolism. WRI1 expression was suggested to be regulated by LEAFY COTYLEDON2 (LEC2), which also contains E2F consensus element in its promoter region. To follow the expression of WRI1, LEC2 we collected siliques at four different developmental stages representing seeds from early dividing (silique 1 and 2) to late maturing non-dividing stages (silique 3-4 - Figure 5A). We also monitor the expression of CDKB1;1, a G2-M phase specific cell cycle regulatory gene. As it was expected CDKB1;1 expresses at the highest level in young siliques (silique 1-2), and declined afterwards in post-mitotic maturing seeds (Figure 5B). In contrast, WRI1 and LEC2 genes were found to be the most active in the third silique representing the developmental stage when the seed storage is

synthesized and accumulated in the seeds (Figure 5C and D). We also monitored the expression of these genes in different transgenic *Arabidopsis* lines lacking the single, double E2F mutants and in ectopic E2FB expressing lines (Figure 5). The CDKB1;1 is one of the known target for E2FB. Accordingly, the expression of CDKB1;1 was reduced in two *e2fb* T-DNA insertion mutant siliques and up-regulated in ectopic E2FB expressing lines (the strong 72, and the weak 61 line expressing E2FB-GFP under its native promoter), although there were some differences (Figure 5B). These data further supports that E2FB functions as an activator on cell cycle genes in the developing seeds. In the case of WRI1, we observed a premature elevation in its expression at the silique 2 stage specifically in the *e2fb-1* line. Interestingly, WRI1 did not show

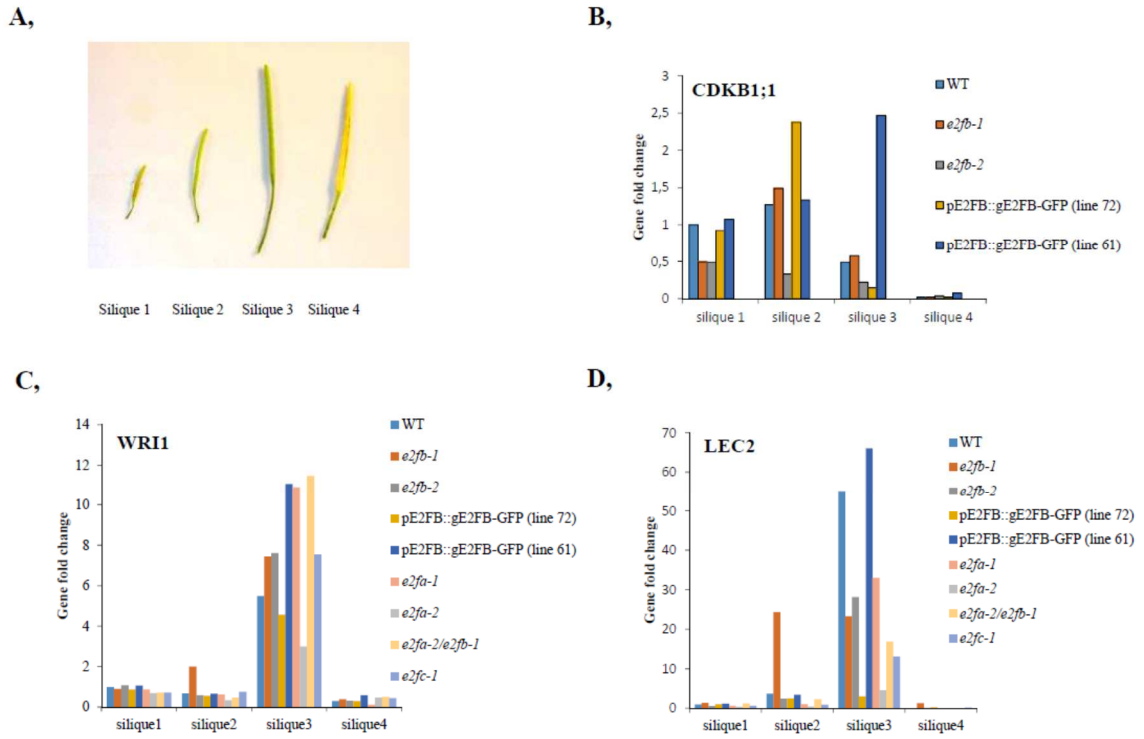


Figure 4. E2FB differently regulates the expression of the cell cycle gene CDKB1;1, and the seed maturation phase-dependent WRI1 and LEC2 genes during seed development. (A) Q-RT-PCR was carried out in siliques representing different developmental stages of seeds; (B) the expression of CDKB1;1 is the highest in the early seed developmental stages of the control wild type (WT - silique 1-2) representing the proliferative developmental stage in seed development and declined afterwards, while WRI1 (C) and LEC2 (D) expresses at the highest level in the third silique stage corresponding to the post-mitotic maturation seed developmental stage when the seed storage reserves are synthesized. Expressions of these genes were tested in siliques collected from different transgenic plants as indicated (*e2fb-1* and *e2fb-2* are two T-DNA insertion lines for E2FB; pE2FB:gE2FB-GFP line 72 and 61 representing two transgenic lines expressing high and low level of E2FB-GFP under the control of its own promoter, respectively; *e2fa-1* and *e2fa-2* are two T-DNA insertion lines for E2FA gene; *e2fa-2/e2fb-1* is the double homozygous line missing both E2FA and E2FB; *e2fc-1* is a T-DNA insertion line for E2FC).

this premature up-regulation in the single *e2fb-2* and double *e2fa-2/e2fb-1* mutants. The *e2fb-1* mutant is supposed to be a loss of function mutant retaining some of the E2F functions including the dimerization ability and the DNA-binding capability, while *e2fb-2* is more like a null mutant. Accordingly, we hypothesized that *e2fb-1* produces a truncated E2FB protein, which could not transactivate and form complex with RBR. Therefore E2FB functions as a repressor of WRI1, and controls its temporal expression at specific seed developmental phase. We also looked WRI1 expression in other *e2f* mutant lines. In two *e2fa* mutants we have seen opposite effects; it was up in *e2fa-1*, and down in the *e2fa-2* at silique 3 stage (Figure 5C).

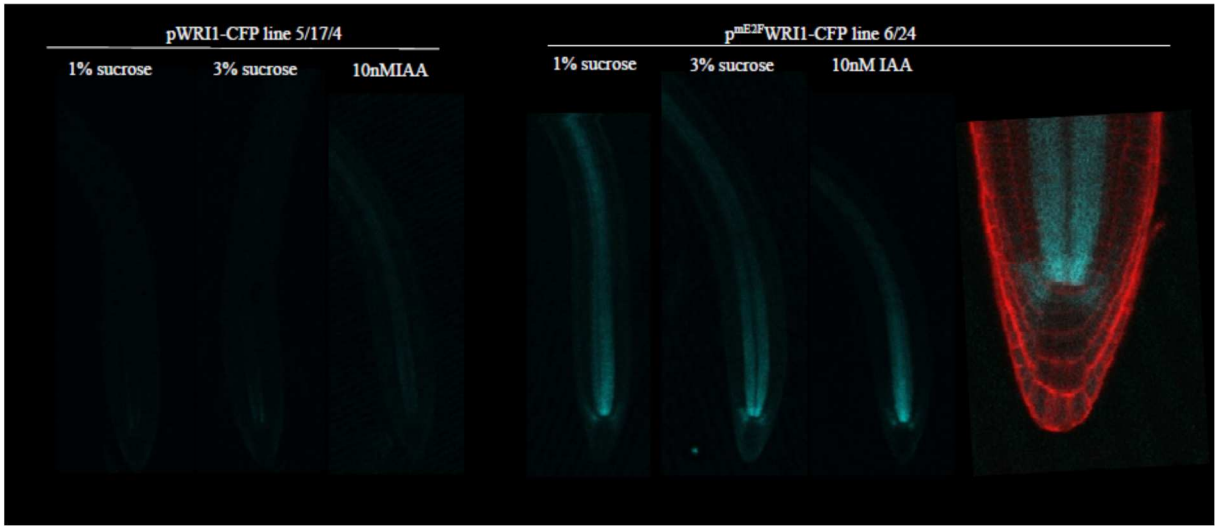


Figure 5. E2F can function as repressor on WRI1 gene. We have generated transgenic *Arabidopsis* plants expressing CFP either under the control of the wild type (pWRI1-CFP – on the left side as indicated) or the E2F-binding site mutant WRI1 promoter (p^{mE2F}WRI1-CFP – on the right side) and the CFP signal of the root tip was detected in confocal laser microscopy. Transgenic Arabidopsis seedlings were grown on vertical plates supplemented with 1% or 3% or 10nM of indole acetic acid (IAA).

These *e2fa* mutants are rather loss of function than null mutants (Horvath et al., 2017 accepted manuscript for publication in the EMBO J). We recently shown that *e2fa-2* produces a truncated protein, which could make complex with RBR through its conserved MARKED-box domain, while *e2fa-1* lost this feature (Molnár E, data not shown). Interestingly, the WRI1 expression in the *e2fa-2/e2fb-1* double mutant line was twice as high as in the control wild type in maturing silique (stage 3). These data indicates complex interconnections between individual E2Fs on the regulation of WRI1. The expression of LEC2 was also prematurely elevated in the *e2fb-1* mutant while it was repressed at various levels in the other *e2f* mutants in proliferating silique (stage 2 - Figure 5D). The strongest repression was seen in *e2fa-2* at that developmental phase, and interestingly, LEC2 expression was rather repressed in the double *e2fa-2/e2fb-1* line,

indicating that might E2FA level or activity is increased in the *e2fb-1* causing the up-regulation in LEC2. LEC2 expression peaks in silique 3 in the WT-Col0, and it was the most significantly down-regulated in the strong E2FB-GFP expressing 72 line and in *e2fa-2* mutant (Figure 5D). We know that increasing the level of a particular E2F could decrease the expression of the other E2Fs (Magyar et al., 2012, and unpublished data). These data shows that both WRI1 and LEC2 expression is regulated by E2Fs.

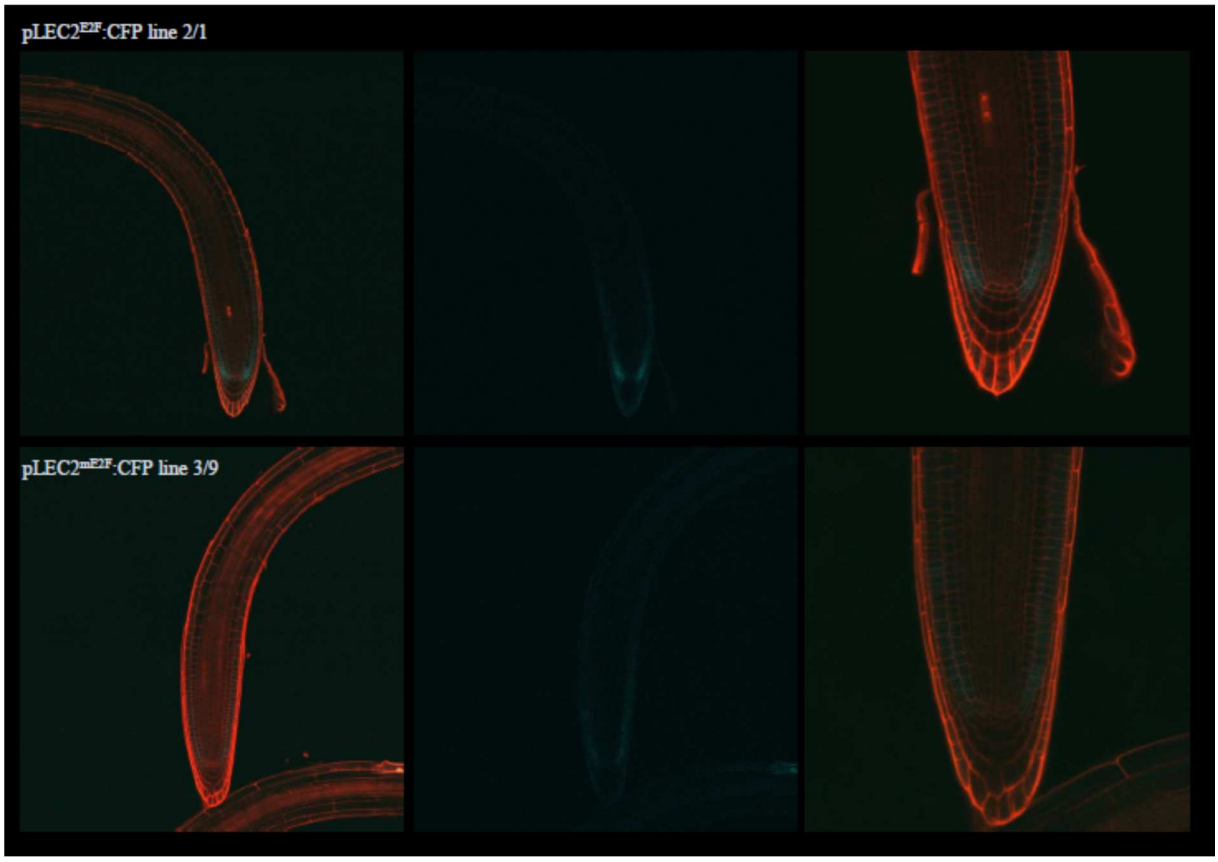


Figure 6. E2F is able to regulate the expression of LEC2 in the root meristem. Reporter CFP under the control of wild type (upper images) or E2F-binding site mutant (bottom images) LEC2 promoter was pictured under confocal laser microscopy. Roots were stained with propidium iodide.

Next we produced site directed mutations in the consensus E2F-binding sites of WRI1 and LEC2 promoters. Constructs either with the non-mutant or the mutant promoter have been made driving the cyan fluorescence protein (CFP) as reporter. Transgenic *Arabidopsis* plants have been generated and we have identified lines expressing CFP signal for each promoters by searching up to minimum 20 independent transgenic lines. Interestingly, WRI1 and LEC2 show specific expression pattern in the root meristem (Figure 6). The non-mutant pWRI1-CFP shows signal in the vasculature of the proximal root meristem, which was the strongest close to the

quiescent centre (QC). In the E2F site mutant p^{mE2F}-CFP line the signal was still the most characteristic in the vasculature but it was significantly enhanced in comparison to the non-mutant promoter and the distal root meristem was also positive (Figure 6). The CFP signal was detectible in every stem cells, but was not present in the QC cells (CFP in propidium iodide stained root tip Figure 6). Previously WRI1 expression was reported to be up-regulated by sucrose, therefore we have looked the signal in the roots grown in the presence of 3% sucrose. We have seen a slight increase in the CFP signal, in the non-mutant promoter, but there was no effect on the mutant promoter (Fig 6). Since WRI1 expression was concentrated around the stem cells niche of the root meristem we also analysed the effect of exogenous auxin on the CFP signal as auxin plays essential role in the maintenance of root meristem. Therefore we grow seedlings in the presence of auxin (10nM IAA). The non-mutant promoter showed a broader and stronger expression in the vasculature than the non-treated control. In contrast, the mutant WRI1 promoter was insensitive to auxin at this concentration.

LEC2 expression was concentrated only to few epidermal and cortex cells close to the QC as the signal faded away in distant root meristem while the E2F-site mutant promoter was much less active than the native regulator indicating that E2F activates the expression of LEC2 in the root meristem (Fig. 6). On the basis of these data, E2Fs differently regulate the expression of WRI1 and LEC2 in the root meristem; they work as repressor on WRI1, while activator on LEC2. That further supports that plant E2Fs could play either activator or repressor function depending on tissue and developmental stage. We also suggest that these plant specific transcription factors have regulatory functions not only during seed development but in the root meristem too.

To further investigate the developmental role of WRI1 and LEC2 we have generated transgenic *Arabidopsis* plants by using constructs expressing the WRI1 or LEC2 proteins fused with GFP at their C-terminal end under their native or the E2F-site mutant promoter. We have also generated transgenic Arabidopsis plants where we introduced the WRI-GFP into the previously characterized wri1-1 mutant. Neither WRI1-GFP nor LEC2-GFP give visible signal in the transgenic roots, and we also could not see these proteins on western blot (data not shown). On the transcript level however we could identified transgenic lines with different expression levels (Fig7 and data not shown). We suggested that WRI1 and LEC2 proteins are very unstable, and we tested whether inhibiting the 26S proteasome by using the chemical MG132 inhibitor could improve their stabilities. In both cases transgenic seedlings were incubated in liquid medium supplemented with MG132 for few hours, and then we looked their roots under the confocal

laser microscopy. LEC2-GFP was still not detectable but WRI1-GFP signal was observed in the root meristem in the nuclei of the ground tissue cells (data not shown, and Fig8). That indicates that WRI1 proteins move from the inner vascular root cells to the outer layers as it was reported in the case of other transcription factors like SHORT ROOT. By using an anti-GFP column we could immunoprecipitate WRI1-GFP proteins (data not shown), and the interacting partners of WRI1-GFP was analysed by using mass spectrometry (in progress).

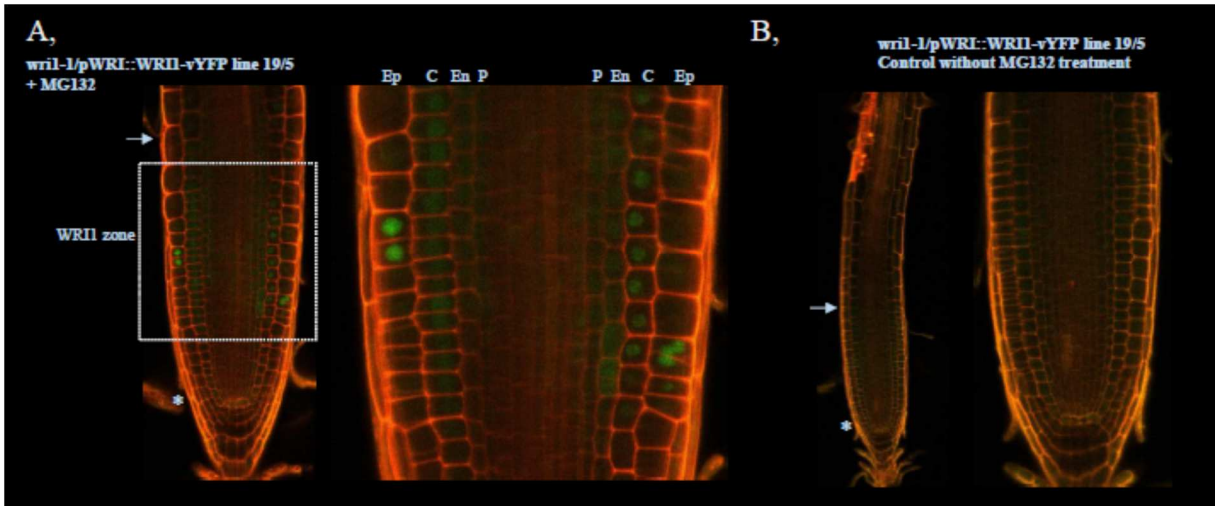


Figure 7. WRI1 proteins move radially from the inner root tissue to the ground tissue cells. Transgenic roots expressing WRI-GFP in the wri1-1 mutant were treated with MG132, the 26S proteosome inhibitor for 4 hours at 10µM concentration before the confocal microscopy images were taken (A, - left side). Untreated transgenic roots were also visualized (B,- right side). The marked areas with white boxes in the root pictures were magnified on their right side. Epidermis (Ep), Cortex (C) Endodermis (En) and Pericycle (P) cells are indicated on the top of the image.

The *wri1-1* mutant has pleiotropic phenotypes including poor germination rate, short hypocotyl, failed seedling establishment on sucrose-free medium and small, wrinkled seeds as they have very low oil content (reduced to 20%). Expression of WRI1-GFP in the mutant wri1-1 restore these phenotypes to normal, and interestingly, we got the same results when WRI1 was driven by the E2F-site mutant promoter. These data shows that WRI1-GFP is functional. Since we have identified transgenic lines expressing WRI1 at various levels first we looked whether increasing WRI1 could improve oil content both in wild type and in wri1-1 mutant background. Fatty acid composition and triacyl glycerol (TAG) level was analysed and measured by extracting transgenic seed contents and analysed further in gas chromatography (Fig 9). Interestingly, those lines where we have seen the highest WRI1 expression did not produce

more TAG but actually less than the control wild type. In contrast, we could see more TAG in seeds where the level of WRI1 was just moderately increased and in the mutant wri1-1 background (). Interestingly, elevating the level of WRI1 in the wild type did not resulted in higher TAG content. However, TAG level was increased in the WT background when WRI1 expression was under the control of its E2F-site mutant promoter. These data indicates that increasing WRI1 expression does not automatically resulted in improved oil content. We also looked whether *e2f* mutant lines have modified oil content. *E2fb-1* mutant has significantly higher TAG level than the WT control, where we have seen premature WRI1 expression during seed development (Fig. 4C). That indicates that changing the time window in WRI1 expression is probably the key for increasing oil content in the seeds through WRI1.

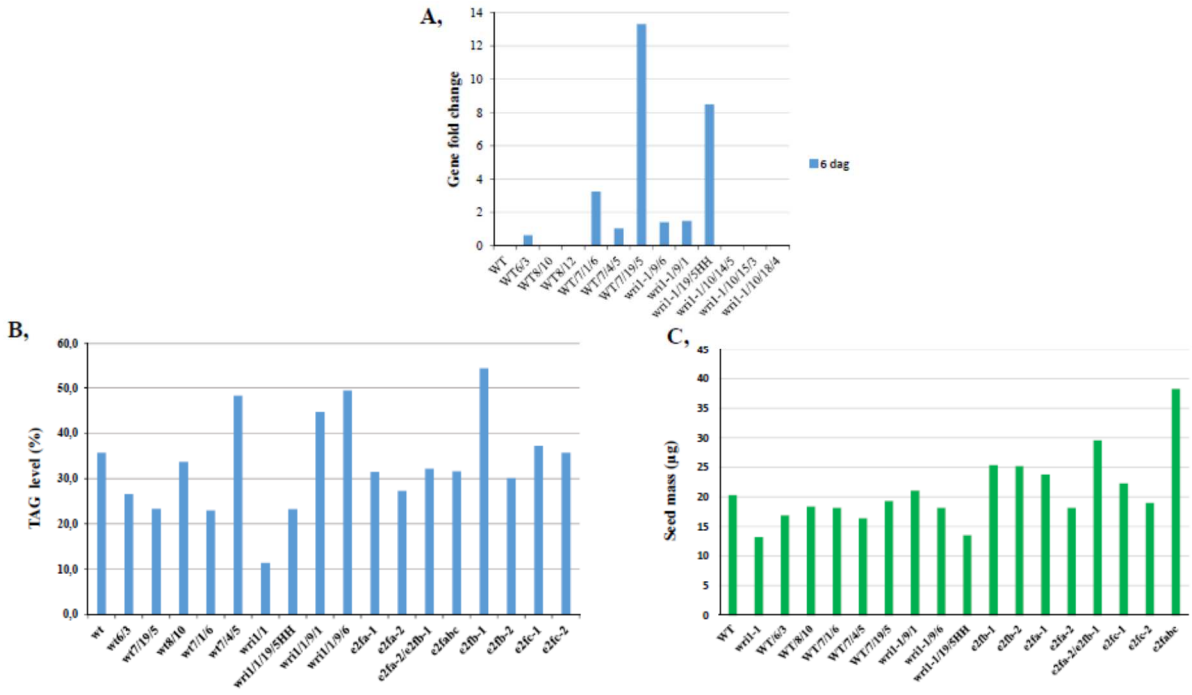


Figure 8. Modifying WRI1 expression could change TAG levels. (A) The expression of WRI-GFP (pWRI1:gWRI-vYFP) was followed by Q-RT-PCR using specific primers for the GFP-tag. Transgenic lines were generated both in the wild type Col-0 (WT) and in the *wri1-1* mutant. (B) Triacyl-glycerol (TAG) content of WT and mutant seeds including the *wri1-1* were determined by gas chromatography, and (C) seed mass of the same transgenic lines was also determined (homozygous WRI1-GFP expressing lines under the control of its own promoter either in the WT-Col; wt6/3; wt8/10 or in the wri1-1 mutant; wri1/19/5HH, wri1/1/9/1, wri1/1/9/6 or under the control of its E2F-binding site mutant promoter in WT-Col0 (wt7/19/5, wt/7/1/6, wt7/4/5). Seeds from the e2fa (e2fa-1, e2fa-2) or the e2fb (e2fb-1, e2fb-2) or the e2fc (e2fc1 and e2fc-2) single T-DNA insertion lines or the e2fa-2/e2fb-1 double, and the e2fa-2/e2fb-1/e2fc-1 triple homozygous mutant lines were also analysed in these studies.

Seed mass of the transgenic WRI1-GFP lines was not significantly improved or rather decreased but surprisingly some single *e2f* mutant seeds were heavier than the wild type seeds, and seed mass was further improved when the two activator E2Fs (e2fa-2/e2fb-1) or the three E2Fs (e2fa-2/e2fb-1/e2fc-1) were all missing (Figure 8C). However, the yield of triple *e2fabc* mutant plants was much less than the wild type indicating compensatory effect. Although the promoter mutant WRI-GFP lines could complement *wri1-1* mutant very early during seedling development, these transgenic plants showed phenotypes later during their vegetative growth as they produce smaller roots, and smaller leaves and growth retardation was more pronounced in the strong expressing lines (data not shown and Figure 9). These results show that WRI1 has function not only during seed but also in post-embryonic development. In agreement, those WRI1-expressing transgenic lines, which produce more TAG in the seeds than the WT also grow longer roots with larger meristem (Fig. 9) and develop bigger cotyledons and leaves too (data not shown).

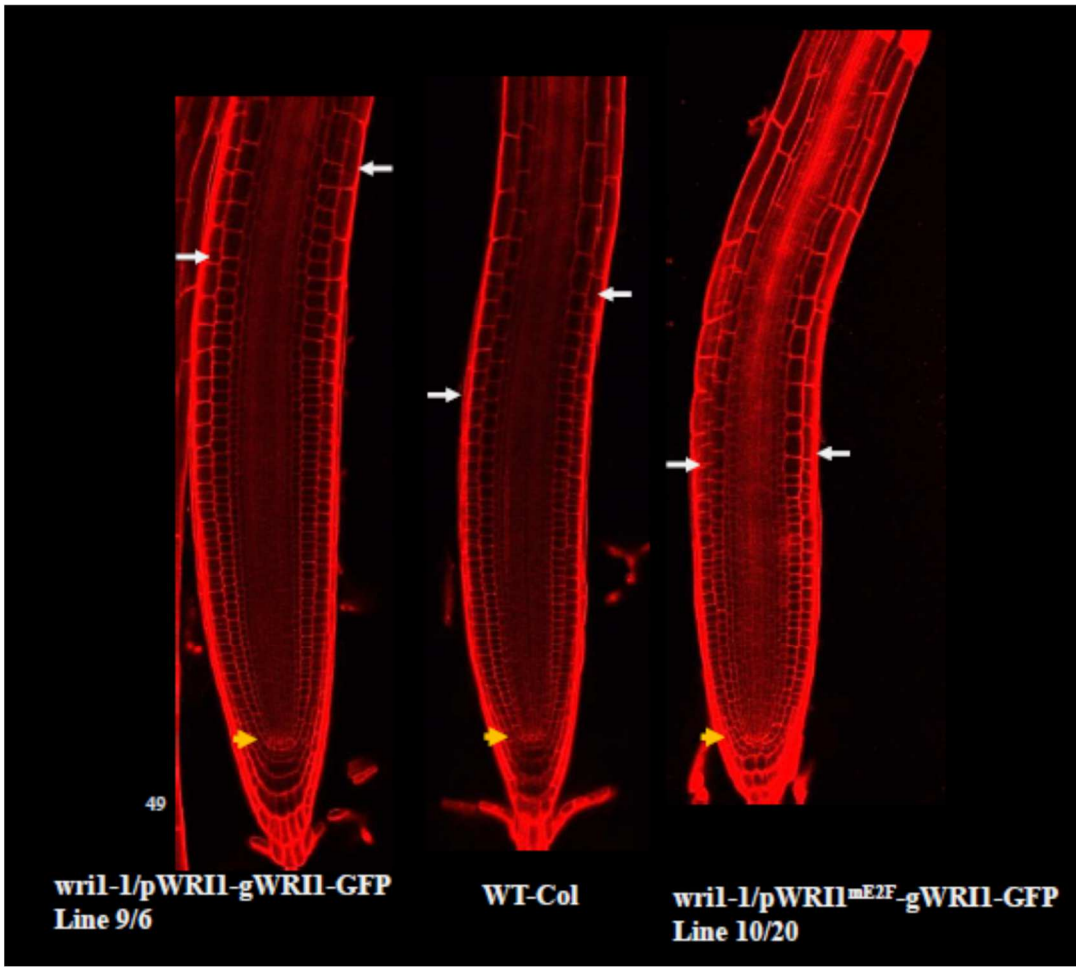


Figure 9. Increasing WRI1 level could enlarge root meristem but it depends on intact E2F site in its promoter. The roots of transgenic lines expressing WRI1-GFP under the control of its native (left side) or its E2F-site mutant (right side) promoters in the *wri1-1* mutant background were studied under confocal laser microscopy and compared to the WT control (middle root). Propidium iodide stained roots (red colour) were used for the microscopy.

Mutation of the E2F site in the LEC2 promoter resulted in opposite effect than the WRI1 as increasing the level of LEC2 in its own expression domain shortened the size of root meristem, while the mutation of its E2F-site was less effective and the root meristem was more like than the WT control (Fig. 10). These data further supports that E2F plays positive role in the expression of LEC2 gene.

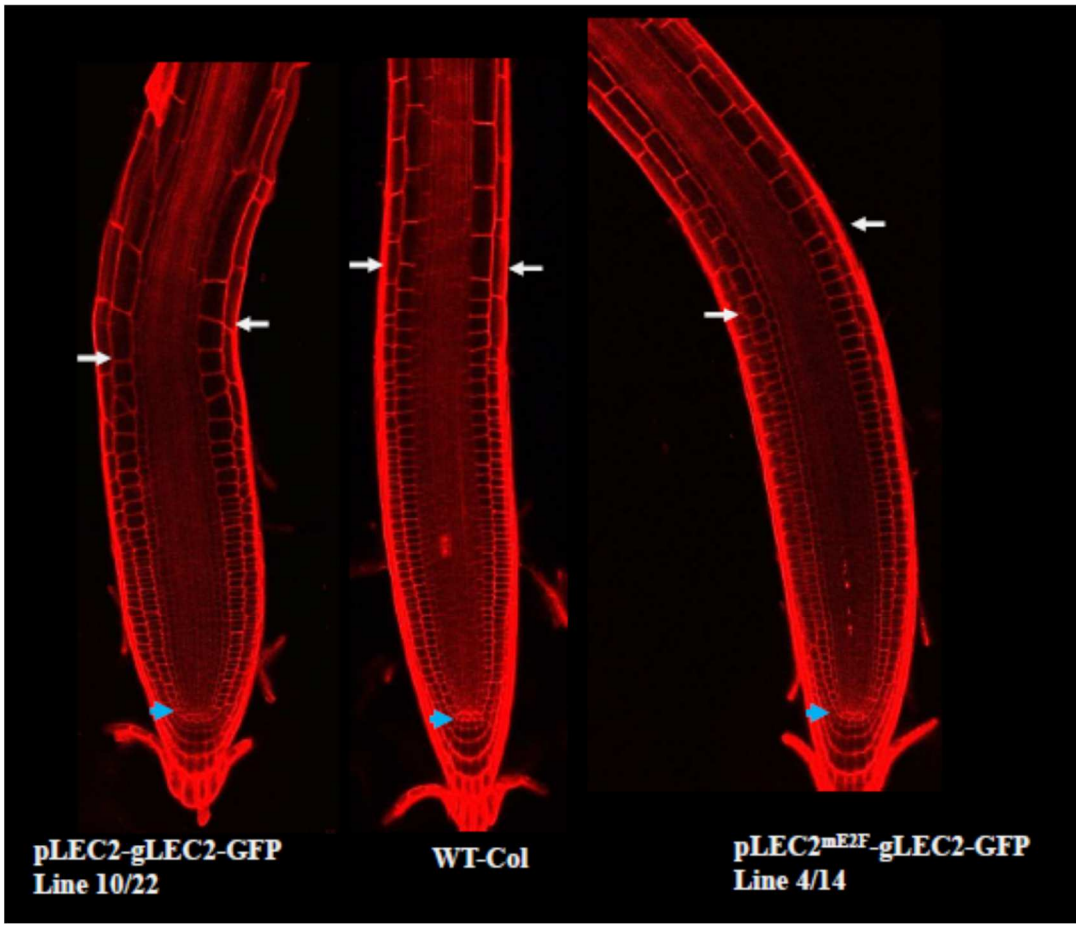


Figure 10. LEC2 shortened root meristem size in E2F-dependent manner. Transgenic roots expressing LEC2 under the control of its native and E2F-site mutant promoter were analysed under confocal laser microscopy, and compared to WT-Col0 as indicated. Roots were stained with propidium iodide (red colour) before microscopy.

Transgenic plants expressing high level of WRI1-GFP or LEC2-GFP under their own promoter show developmental abnormalities (Fig. 11).

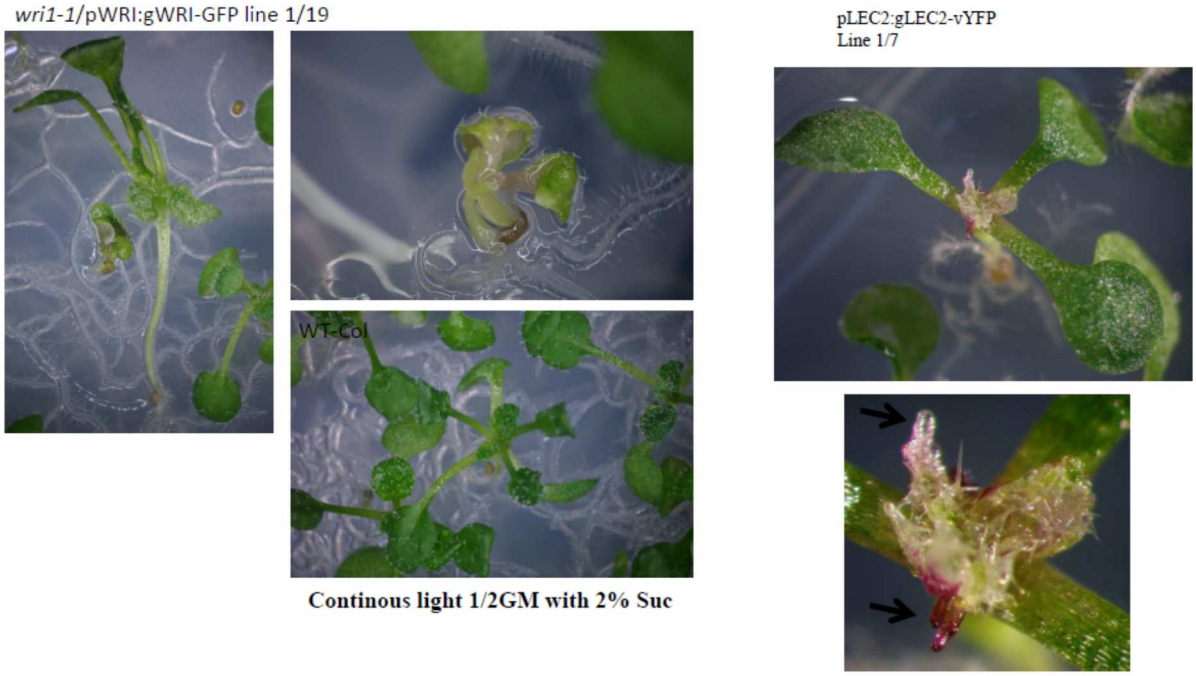


Figure 11. Elevating WRI1 or LEC2 transcription factors could interfere with development. Transgenic seedlings expressing high level of WRI1-GFP in the wri1-1 mutant growing in continuous light and on medium supplemented with 2% sucrose show symptoms resemble to plants grow in limited light condition (left picture) including long hypocotyl, small hardly expanding cotyledons, and long petioles. Another mutant WRI-GFP expressing seedling shows defect in seedling establishment and delayed development (above the WT-Col). LEC2-vYFP expressing seedlings show de-differentiation signs on the margins of the developing new leaves. Arrows show embryo like protrusions.

4. Test the function of E2FB, WRI1 and LEC2 as dose dependent regulator of biomass, yield and oil content in rapeseed

To test whether elevating the expression of E2FB, WRI1 or LEC2 in *Brassica* could have a similar impact on growth, and yield as well as on seed oil content like in *Arabidopsis* first we have identified and cloned the genomic clones of these genes from *Brassica napus*. Since the *Arabidopsis* E2FB and the *Brassica* E2FB show considerable level of identity on sequence level first we have optimized that *Brassica* transformation method by using Agrobacterium strains carrying the *Arabidopsis* E2FB construct. The transformation protocol we have used here relies on the regeneration of viable plants from cotyledon or hypocotyl explants isolated from germinated seedlings (Bhalla and Singh, 2007). We have used the model variety, Westar of *B. napus* as it has been reported to be the highest transformation efficiency (Bhalla and Singh, 2007). Since the selection marker gene in the original destination vector (pGreen-based) was

Norflurazon (NF - a chemical inhibitor of chloroplast differentiation), first we had to change destination vector containing different selection marker gene (we choosed the phosphinothricin herbicide) since the *Brassica* transformation starts by generating transformed callus cells and NF was impractical there as these cells were maintained later in the presence of sucrose. We could identify positive callus cells and propagated them further on regeneration medium to get *Brassica* transgenic plants. In our practice the required time was 20-24 weeks significantly longer than described in the original protocol (12-14 weeks) to get transgenic plantlets of rapeseed ready for establishment under glasshouse. Generally we needed longer time for shoot initiation and regeneration as well as for getting properly developed roots of the positive antibiotic resistant shoot explants. All together to get seeds from the transformant rapeseeds we needed close to one year. The primary transformant lines first were tested by PCR for the presence of the resistance gene (BASTA).

We have generated independent *Brassica* lines expressing various levels of *Arabidopsis* E2FB (AtE2FB) proteins (Figure 12). Altogether we have 13 T1 lines, and the positive lines were propagated further. To see whether the *Arabidopsis* E2FB shows its characteristic distribution pattern in the root meristem we have analysed rapeseed roots under the confocal laser microscopy. The *Arabidopsis* E2FB protein was found in the nucleus of *Brassica* root cells, and as expected it showed the highest level in post-mitotic root cells such as we could observe the E2FB protein in the differentiated columella cells (Figure 12).

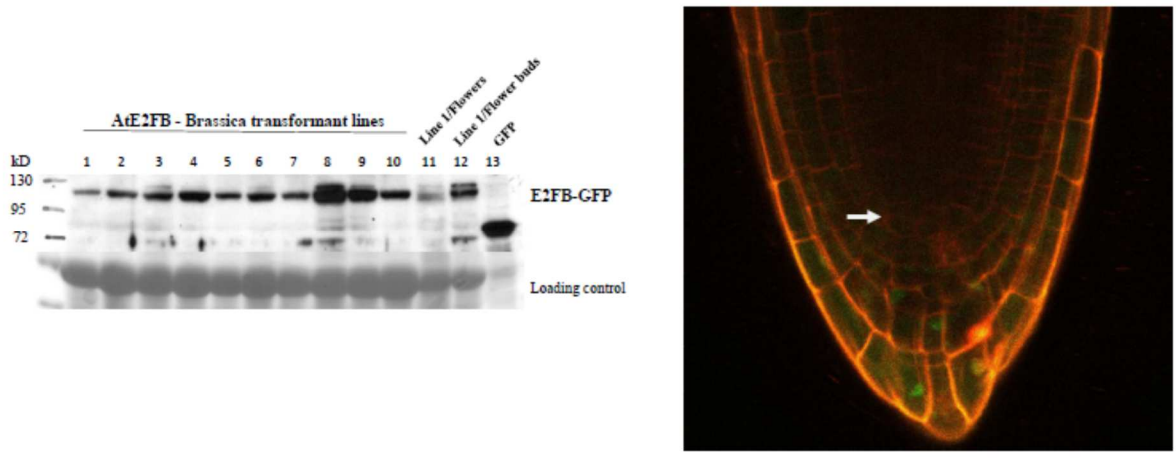


Figure 12. *Brassica* transgenic lines expressing different level of *Arabidopsis* E2FB proteins. Ten independent T1 lines were identified and the presence of E2FB-GFP fused protein was monitored by using immunoblot assay. Ten protein samples were extracted from leaves (1-10th samples on the blot), the 11 and 12 samples were derived from flowers or flower buds as indicated. We have also used protein extracts from p35S:GFP transgenic

Arabidopsis seedlings (lane 13 as indicated). Ponceau-S stained proteins on the membrane were used as loading control (left picture). Transgenic *Brassica* seedlings were grown on vertical plate and propidium iodide (PI) stained root was analysed under confocal laser microscopy. Arrow marks the position of quiescent cells in a lateral root.

Next we have generated transgenic *Brassica* lines with the *Arabidopsis* WRI1 fused with GFP and under the control of its own regulatory sequence. We have identified six independent transgenic T1 lines, and propagated further to get the T2 seeds. E2FB and WRI1 transformant T2 seeds were measured (100 seeds) and their mass was compared to the seed mass of the control rapeseeds (Table). As the preliminary data shows there are considerable variations in seed mass, and we have seen some improvement in few cases. Recently, seed specific overexpression of WRI1 enhanced seed mass of the transgenic rapeseeds (Wu et al., 2014). We are currently analysing the expression levels of E2FB and WRI1 in these T2 lines to couple the expression levels of the transgene with the observed changes in the seed mass. In addition we have started the analyses of seed oil contents of these seeds (in progress). Phenotypic analyses of these lines are also in progress. Transgenic *Brassica* lines containing extra copy of BraE2FB and BraLEC2 were also generated and their characterization as well as their further propagations are in progress.

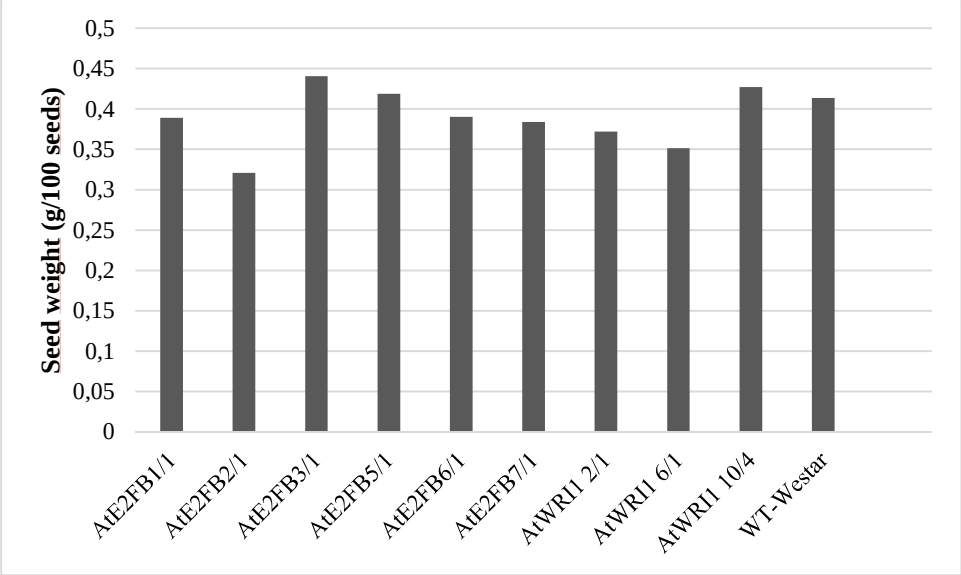


Table. Elevating the expression of E2FB and WRI1 in *Brassica* could enhance seed mass.

Arabidopsis E2FB and WRI1 genomic clones in fusion with GFP and under their native promoter were introduced into *Brassica napus* Westar variety. One hundred of T2 matured seed weights were determined.

Conclusions

Arabidopsis is the most advanced model to find genes and regulatory networks controlling biomass production, which in turn can be applied for further growth improvement in other species. *Brassica* and *Arabidopsis* are closely related, and thus has the highest potential for knowledge transfer. Growth relies on the production of cells, which in plants is restricted to meristems. Cell production in plants is regulated by an evolutionary conserved transcriptional master switch the E2F-RBR pathway. We have discovered here that plant E2F and RBR proteins function in evolutionary conserved complexes called DREAM to regulate cell cycle entry, control the transition from G2 to M phase and maintain quiescence in cells committed to differentiate. Interestingly, plants have more DREAM complexes than other eukaryotes, and E2F and RBR could present in the same complexes but surprisingly inside the cell cycle. We revealed that E2FB from rapeseed under the control of its own promoter could also function in similar DREAM complexes in *Arabidopsis* than the endogenous *Arabidopsis* E2FB. We demonstrated that E2FB transcription factor could either function as activator or repressor depending on cell type or developmental stage. We established regulatory links between E2F and the seed specific WRI1 and LEC2 genes. Parallel we have discovered that these plant specific transcription factors have functions outside of seed development, and they could regulate growth and development. In *Arabidopsis*, we confirmed that these genes function as dose-dependent growth regulators. We have generated transgenic *Brassica* lines by introducing extra genomic copy of E2FB, WRI1 and LEC2 originated both from rapeseed and *Arabidopsis*. The *Arabidopsis* E2FB shows similar expression patterns in the roots of both rapeseed and *Arabidopsis*. Altogether our data clearly supports that gene regulatory pathways can be transferred from *Arabidopsis* to rapeseed, and has the potential to improve growth in rapeseed.

Publication list

Support of the OTKA grant was acknowledged in the following scientific papers:

1. Beatrix M. Horvath, Hana Kourova, Szilvia Nagy, Edit Nemeth, **Zoltan Magyar**, Csaba Papdi, Zaki Ahmad, Gabino F. Sanchez-Perez, Serena Perilli, Ikram Blilou, **Aladár Pettkó-Szandtner**, Zsuzsanna Darula, Tamas Meszaros, Pavla Binarova, Laszlo Bogre and Ben Scheres. Arabidopsis RETINOBLASTOMA RELATED directly regulates DNA damage responses through functions beyond cell cycle control. EMBO Journal 2017 (accepted for publication).

2. **Magyar Z**, Bögre L, Ito M. DREAMs make plant cells to cycle or to become quiescent. Curr Opin Plant Biol. 2016 Dec; 34:100-106.

3. Kosuke Kobayashi, Toshiya Suzuki, Eriko Iwata1, Norihito Nakamichi, Takamasa Suzuki, Poyu Chen, Misato Ohtani, Takashi Ishida, Hanako Hosoya, Sabine Müller, **Tünde Leviczky**, **Aladár Pettkó-Szandtner**, Zsuzsanna Darula, Akitoshi Iwamoto, Mika Nomoto, Yasuomi Tada, Tetsuya Higashiyama, Taku Demura, John H Doonan, Marie-Theres Hauser, Keiko Sugimoto, Masaaki Umeda, **Zoltán Magyar**, László Bögre & Masaki Ito. Transcriptional repression by MYB3R proteins regulates plant organ growth. Transcriptional repression by MYB3R proteins regulates plant organ growth. EMBO J. 2015 Aug 4;34(15):1992-2007.

4. Bögre L, Henriques R, **Magyar Z**. TOR tour to auxin.
EMBO J. 2013 Apr 17;32(8):1069-71.

Congress presentations with acknowledgement of the OTKA financial support

1, Anikó Varga, Anita Kovács and Zoltán Magyar: Kinetin switch E2FB from activator to repressor in differentiating root cells. 11th Congress of the Hungarian Society of Plant Biology, Szeged 2014.

2, Tünde Leviczky, Binish Mohammed, Aladár Pettkó-Szandtner, Beatrix Horvath, Safina Khan, Anita Kovács, Ben Scheres, László Bögre and Zoltán Magyar. Developmental regulation of cell division by the RBR complex with E2FB in leaf pavement cells and meristemoids, 11th Congress of the Hungarian Society of Plant Biology, Szeged 2014.

3, Márta Deli, Anikó Varga, Anita Kovács and Zoltán Magyar Kinetins switch of E2FB from activator to repressor in the Arabidopsis root meristem. Signalling in plant development. EMBO Conference. 2015Brno 20-24 Sept 2015.

4, Vaskó-Tünde Leviczky, Binish Mohammed, Márta Deli, Aladár Pettkó-Szandtner, Anita Kovács, László Bögre and Zoltán Magyar. Dual functions of E2FB transcription factor during leaf development. Signalling in plant development. EMBO Conference 2015 Brno 20-24. Sept 2015.

5, Aladár Pettkó-Szandtner, Zsuzsa Darula, K Kobayashi, T Suzuki, Tünde Leviczky, Anita Kovács, László Bögre, M Ito, Zoltán Magyar. Arabidopsis DREAM complexes: variations on a theme. Signalling in plant development. EMBO Conference 2015 Brno 20-24. Sept 2015.

6, Tünde Leviczky, Binish Mohammed, Aladár Pettkó-Szandtner, Beatrix Horvath, Anita Kovács, Márta Deli, Csaba Papdi, László Bögre and Zoltán Magyar. Developmental regulation of cell division by E2FB in leaf pavement cells and meristemoids. Plant organ symposium – Abstract Book, 10-12 March 2015, Ghent, 2015.