E2FA and E2FB transcription factors coordinate cell proliferation with seed maturation

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Summary

The E2F transcription factors and the RETINOBLASTOMA RELATED (RBR) repressor protein are principal regulators coordinating cell proliferation with differentiation, but their role during seed development is little understood. We show that in the fully developed embryos, cell number was not affected either in single or double mutants for the activator-type *E2FA* and *E2FB*. Accordingly, these E2Fs are only partially required for the expression of cell cycle genes. In contrast, the expression of key seed maturation genes; *LEAFY COTYLEDON 1-2* (*LEC1-2*), *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *FUSCA 3* (*FUS3*) and *WRINKLED 1* (*WRI1*) are upregulated in the *e2fab* double mutant embryo. In accordance, E2FA directly regulates LEC2, and mutation at the consensus E2F-binding site in LEC2 promoter de-represses its activity during the proliferative stage of seed development. Additionally, the major seed storage reserve proteins, 12S globulin and 2S albumin became prematurely accumulated at the proliferating phase of seed development in the *e2fab* double mutant. Our findings reveal a repressor function of the activator E2Fs to restrict the seed maturation program until the cell proliferation phase is completed.

Introduction

In multicellular organisms development is regulated by coordinating cell proliferation with differentiation. In plants, due to their sessile lifestyle and largely post-embryonic development, this coordination operates lifelong from early embryogenesis to post-embryonic organ development. Plants develop through transitions, but how these passages are regulated at the molecular level is not fully understood. The developing seed consists of two major and sequential programs; the initial morphogenic phase is driven by oriented cell divisions, which is followed by the maturation phase, where embryonic cells stop proliferating and seed storage reserves accumulate (Holdsworth et al., 2008; Lau et al., 2012; Sun et al., 2010). During the final phase of embryogenesis, desiccation tolerance is acquired and dormancy is established (Devic and Roscoe, 2016). The embryo formation, the accumulation of storage reserves, and the establishment of dormancy are all important agronomic traits defining seed quality.

Morphogenesis during seed development is completed in the early heart stage embryo, when all elements of the body pattern are already laid down (Wendrich and Weijers, 2013). The embryo still continues to grow afterwards, but less so by cell proliferation, but rather by cell expansion (Raz et al., 2001). Seed storage reserves, including fatty acids and proteins accumulate when cell division is completed (Goldberg et al., 1994). The current view is that the key genetic factors controlling seed maturation are four regulatory genes, including three related B3 domain transcription factors, the ABSCISIC ACID INSENSITIVE 3 (ABI3), FUSCA 3 (FUS3) and LEAFY COTYLEDON 2 (LEC2), collectively named AFL, and the LEAFY COTYLEDON 1 (LEC1), a CCAAT-binding transcription factor (Braybrook and Harada, 2008; Carbonero et al., 2017). The exact mechanism how the maturation phase is initiated through the control of these genes however is still not entirely clear.

Cell proliferation is highly regulated during embryo development. In Arabidopsis, as in other eukaryotes, CYCLIN-DEPENDENT KINASES (CDKs) play essential role in the regulation of the cell cycle (Gutierrez, 2009). Contrary to animals, Arabidopsis embryos can develop in the absence of the evolutionary conserved CDKA;1, but contain much fewer cells. The primary target for CDKA;1 is the single RETINOBLASTOMA-RELATED (RBR) protein, which was experimentally demonstrated with the rescue of most defects in the *cdka;1* mutant by the *rbr1-2* hypomorph mutant allele (Nowack et al., 2012). As the main RBR-kinase is the CDKA;1, it forms complex with regulatory CYCLIN subunits including D-type CYCLINs (CYCDs).

CYCDs have both discrete and overlapping tissue specific expression patterns in the developing seeds and mutations of the CYCD3 subgroup delay embryo development (Collins et al., 2012). CYCDs bind to Rb/RBR through their LxCxE amino acid motif, which leads to the phosphorylation and inactivation of Rb/RBR (Morgan 2007; Boniotti and Gutierrez 2001). The canonical role of RBR is to control the cell cycle through the repression of E2F transcription factors (Harashima and Sugimoto, 2016). In Arabidopsis, three E2F proteins are capable to form complexes with RBR (Magyar et al., 2016). Ectopic expression of E2FA or E2FB causes hyper-proliferation, while overexpression of E2FC inhibits cell division during post-embryonic development, placing them as activator and repressor type E2Fs, respectively (De Veylder et al., 2002; del Pozo et al., 2006; Magyar et al., 2005; Magyar et al., 2012; Sozzani et al., 2006). These three E2Fs require the dimerization partner protein A or B (DPA or DPB) for DNA binding (del Pozo et al., 2002; del Pozo et al., 2006; Magyar et al., 2000). Only E2FB and E2FC but not E2FA were found in association with components of the evolutionary conserved multi-subunit DP-Rb-E2F And-MuvB complex (DREAM – Kobayashi et al., 2015; Fischer and DeCaprio, 2015; Sadasivam and DeCaprio, 2013), demonstrating that activator E2FA and E2FB could have different functions (Horvath et al., 2017). Accordingly, E2FA in complex with RBR was shown to maintain the proliferation competence by repressing genes controlling the switch from mitosis to endocycle and cell elongation (Magyar et al., 2012), while E2FB was shown to regulate cell cycle in a more canonical way, where RBR represses the activation of cell cycle genes through the inhibition of E2FB. The function of these E2Fs in the developing embryo have not been fully characterized yet. Mutant embryos with compromised RBR function develop normally, but consist of twice as many cells as the wild type (Gutzat et al., 2011). Cell number in this *rbr* mutant increased from the bent cotyledon embryo stage onward during maturation, suggesting that RBR repression is required for the exit from cell proliferation to set the final cell number in the embryo (Nowack et al., 2012). In addition, *rbr* mutant seedlings ectopically express embryonic genes such as LEC2 and ABI3, indicating that RBR, apart from cell cycle genes, could regulate the expression of seed maturation genes (Gutzat et al., 2012). Whether plant RBR regulates cell proliferation in the developing embryo in association with E2Fs and whether they together control the developmental transitions to seed maturation is not known.

Here, we analysed the function of activator-type E2FA and E2FB in the developing Arabidopsis seeds and embryos. We found that in the *e2fab* double mutant (*e2fa-2/e2fb-1* - Heyman et al., 2011) cell number was not significantly affected in the fully developed embryos.

Accordingly, the activator function of E2FA and E2FB is not critical for embryonic cell proliferation. In contrast, the expression of key seed maturation genes; LEC1-2, ABI3, FUS3 were found to be significantly upregulated in the *e2fab* double mutant embryos. Our findings reveal a repressor function of the so-called activator E2Fs to restrict the seed maturation program until the cell proliferation phase is completed.

Results

The expression patterns of E2FA and E2FB are distinct in developing siliques

To investigate the involvement of activator E2Fs in the coordination of cell proliferation with differentiation, we first studied the expression of *E2FA* and *E2FB* genes. For that purpose, we harvested siliques from Arabidopsis wild type Columbia 0 ecotype (WT) with four different sizes, representing distinct embryo developmental stages (S1-4; pictured in Fig S1). To monitor the proliferative phase in this experimental system, we studied the expression of *CYCLIN-DEPENDENT KINASE B1;1* (*CDKB1;1*), a G2-M phase specific cell cycle regulatory gene, a known target for activator E2Fs (Vandepoele et al., 2005). *CDKB1;1* was found to express at the highest level in the youngest siliques (S1), decreased in the second silique sample (S2) and sharply diminished afterwards in the last two silique samples S3-4 (Fig. 1A). To monitor the maturation phase, we followed the expression of *LEC2*, and one of its predicted target gene, the *WRINKLED 1* (*WRI1* – Focks and Benning, 1998, an APETALA-2/ ETHYLENE-RESPONSE FACTOR AP-2/ERF) type transcription factor involved in the regulation of fatty acid synthesis (Fig. 1A). As expected, these were barely detectable during the proliferative phase (S1) and they both showed the highest expressions in the third silique sample (S3), containing long fully grown but green siliques and both declined in the S4 sample (Fig. 1A). Taken together, cell proliferation was the most active in the youngest siliques (S1 and S2), while the maturation phase started when proliferation activity decreased in the transient developmental phase (S2) and peaked in the next sample (S3), and both the cell cycle and maturation genes were hardly detectable in the post-mature seed developmental phase (S4).

To start understanding the function of E2Fs and RBR during seed development, we followed the transcript levels of the three E2Fs (*E2FA, E2FB, E2FC*) as well as the *RBR*. The repressor type *E2FC* and *RBR* were expressed at nearly constant levels from the proliferation to maturation phase of seed development (Fig. 1B). The expression pattern of activator *E2FA* was similar to the cell cycle regulator *CDKB1;1* gene; it was the highest in proliferating seeds, and gradually decreased afterwards, although not as sharply as the expression of *CDKB1;1* in the post-mitotic S3-4 siliques and remained clearly detectable (Fig. 1A,B). *E2FB* was also expressed during the early developmental phases (S1-S2), but unlike E2FA, its expression level increased during the maturation phase and it peaked afterwards in the post-mature

developmental stage (Fig. 1B). These results are in agreement with the gene expression data in the Arabidopsis eFP browser (Fig. S2; Winter et al., 2007), supporting overlapping as well as potentially specific functions for the activator E2Fs, *E2FA* and *E2FB* during silique and seed development.

E2FA and RBR proteins are abundant in the proliferative phase, while E2FB protein is also present in post-mitotic and post-mature seeds and siliques

Next we analysed the accumulation of E2FA and E2FB proteins in the developing siliques by specific antibodies in immunoblot assays (Fig. 1C). The E2FA protein accumulation followed its transcript level, was the highest in the proliferation phase of siliques (S1), decreased towards maturation phase in the S2 and diminished in the latest developmental phases (S3-4 - Fig. 1C). RBR is known to be abundant in proliferating tissues during vegetative development (Borghi et al., 2010; Magyar et al., 2012), and indeed, RBR level was high in the young siliques (S1-2), but contrary to its transcript level, RBR protein was hardly detectable in maturing siliques (S3) and further diminished from the post-mature S4 stage, indicating that the mRNAs and not the RBR protein are stored in the dry seeds. In contrast to E2FA and RBR, E2FB accumulated at a constitutive high level throughout seed and silique development, present both in the mitotically active and maturing siliques and interestingly also in the post-mature stage (Fig. 1C). We could not detect DPA in the developing siliques, probably because of its generally low level, but DPB showed a constitutive expression pattern throughout the analysed developmental period, similarly to E2FB (Fig. 1C). In the post-mature silique stage (S4), DPB was detected with a slower mobility, indicating a post-translational modification on this protein. The diminished abundance of RBR, but not E2FB, at the post-maturation stage suggests that E2FB may have an RBR-independent function during the establishment of seed dormancy.

Spatial and temporal regulation of E2FA and E2FB accumulation during embryogenesis

To analyse the spatial and temporal patterns of E2FA, E2FB and RBR proteins specifically in the developing embryos, we utilized our transgenic Arabidopsis lines expressing fluorescent protein-tagged E2FA, E2FB or RBR under the control of their own promoters (pgE2FA-

3xvYFP, pgE2FB-3xvYFP, pgRBR-3xCFP). Immature embryos were dissected from transgenic Arabidopsis seeds at various developmental stages and fluorescence signals were analysed by confocal laser microscopy (Fig. 2, S3). Cell proliferation continues during the heart stage, but gradually decreasing until the walking-stick embryo stage, when it completely stops (Raz et al., 2001). Both E2FA and E2FB proteins were found to be nuclear, and ubiquitously expressed in every embryonic cells from the globular to the mitotically quiescent walking-stick embryo stage (Fig. 2). The E2FA-vYFP signal was the brightest till the heart stage, after which it gradually diminished, but remained detectable at all stages, except the post-mature phase in S4, while the E2FB-vYFP signal was most intense at the torpedo stage and also could be detected in the latest embryo developmental stages (Fig. 2). The RBR-3xCFP was detected from the heart to the walking-stick embryo stage, but it was not present in post-mature embryos (Fig. S3). E2FA-3xvYFP and E2FB-3xvYFP signals were also present in the integuments of young seeds containing proliferating cells (Fig. S4).

Altogether these results show that both E2FA, E2FB as well as RBR proteins are present in the developing embryo both in proliferating and in post-mitotic embryonic cells, though at different abundance. Accordingly, E2FA and E2FB have the potential to participate in the establishment of quiescence in association with RBR, till the embryo reaches its final size at S3 stage.

In the *e2fab* double mutant the expression of cell cycle genes is compromised during the early developmental stage, while it becomes de-repressed later during the maturation

To examine whether E2FA and E2FB are required for the expression of cell cycle genes, we collected siliques at three developmental phases of single *e2fa-2* (Berckmans et al., 2011b) and *e2fb-1* (Berckmans et al., 2011a; Horvath et al., 2017), as well as the *e2fa-2/e2fb-1* double mutant (abbreviated as *e2fab* - Heyman et al., 2011). Previously it was shown that these mutant lines do not express the corresponding full size transcripts and proteins (Berckmans et al., 2011a; Berckmans et al., 2011b; Horvath et al., 2017; Kobayashi et al., 2015, and Figs S9, S10, S11). We followed the expressions of the G1-to-S phase regulatory *CYCD3;1*, the S-phase linked *ORIGIN RECOGNITION COMPLEX 2* (*ORC2*), the *MINICHROMOSOME MAINTENANCE 3* (*MCM3*) and the G2-to-M phase specific *CDKB1;1* E2F target genes using Q-RT-PCR (Fig. 3). In the WT siliques, all these cell cycle genes showed a generally similar

pattern: highest expression in the first silique sample, representing the proliferation phase, declined levels in the following one, and the lowest during the maturation phase (Fig. 3). Surprisingly, the expression of these cell cycle genes during the proliferative S1 stage was hardly affected in the single and just lowered in the *e2fab* double mutant, but only marginally in the case of *MCM3* and *CYCD3;1*, suggesting that these activator E2Fs are only partially required for their expressions.

Cell cycle genes almost completely diminished in the maturing siliques of the WT. To evaluate the effect of *e2fa-2* and *e2fb-1* mutations on their expressions, we replotted the normalised data representing the S3 stage (Fig. 3B). All these cell cycle genes were up-regulated in the *e2fa-2* mutant, while only the expression of *CYCD3;1* and *MCM3* was elevated in *the e2fb-1* mutant. These two further increased in the *e2fab* double mutant, suggesting that activator E2Fs act independently as repressors on them. In contrast, *CDKB1;1* expression diminished in the *e2fb-1* mutant, while it became elevated in the *e2fab* double to the same level as in the *e2fa-2* single, suggesting that these E2Fs oppositely regulate *CDKB1;1* expression. These results show that the E2FA and E2FB activator-type transcription factors can act as repressors during the maturation phase of seed development.

E2FA and E2FB are dispensable for embryonic cell proliferation

Previous results confirmed that cell number in the developing Arabidopsis embryo is regulated at the level and activity of RBR, which act on E2Fs (Gutzat et al., 2011; Nowack et al., 2012). To analyse the role of activator E2Fs, we isolated embryos from fully mature seeds of WT, single and double loss of function *e2fa-2* and *e2fb-1* mutants and determined the size of embryonic cotyledons and hypocotyl and their constituent cells under confocal laser microscopy after propidium iodide (PI) staining (Fig. 4, S5). The *e2fa* mutant embryo looked normal, while the *e2fb* was slightly larger than the WT (increased by 1,2 fold), containing more but slightly smaller cells (Fig. 4). The double *e2fab* mutant embryos were significantly larger, with enlarged cotyledons and hypocotyl. However, the number of cells in these *e2fab* mutant was calculated to be comparable with the WT control, while the cell size was considerably increased in comparison to WT, both in the cotyledon and in the hypocotyl epidermal tissue (Fig. 4C, D). We also observed that *e2fab* mutant plants produced shorter siliques containing less, but bigger and heavier seeds than the WT (Table 1). The short silique was due to reduced

fertility, as indicated by missing rather than aborted seeds in the silique in the *e2fab* double mutant (Fig. S6). Accordingly, the yield of the double *e2fab* mutant plants was behind WT (decreased by around 30%), while seed weight increased by 36%, indicating a negative correlation between total seed yield and average seed weight (Table 1). The large seed and embryo phenotype in this mutant could be the consequence of the allocation of extra resources to the few seeds produced (Ohto et al., 2005). Nevertheless, *e2fab* mutant embryos, in the absence of activator E2FA and E2FB functions, are larger than the WT control, although the total number of cells is not modified, supporting the view that the activator function of these E2Fs is not essential for cell proliferation during embryogenesis.

The AFL class of maturation genes are repressed by E2FA and E2FB

Previously it was shown that seed maturation genes, LEC2 and ABI3, were up-regulated in Arabidopsis seedlings where the RBR level was reduced by co-silencing (csRBR – Gutzat et al., 2011). The LEC2 gene is a putative E2F target, as it contains a consensus E2F-binding site in its promoter, although RBR could not be shown to directly bind to the LEC2, but only to the ABI3 promoter (Gutzat et al., 2011). To investigate the role of activator E2Fs, we followed the expression of *LEC2*, *LEC1*, *FUS3* and *ABI3*, as well as *WRI1* in developing siliques of single and double *e2fa-2* and *e2fb-1* mutants (Fig. 5). As expected, in WT the maturation genes were hardly detectable in the proliferating siliques (S1), increased afterwards (S2) and peaked during maturation (S3 - Fig. 5). The expression of all these maturation genes were upregulated in the *e2fa-2* and partly in the *e2fb-1* mutants during S3 phase. For *LEC1*, *LEC2* this solely depended on *e2fa-1* while for FUS3, ABI3 and to some extent for *WRI1* on both *e2fa-2* and *e2fb-1* (Fig. 5). In contrast to S3 phase, the LEC1 and LEC2 transcripts became prematurely upregulated during the S2 phase only in the *e2fb-1* mutant (Fig. 5B). These data indicate that both E2FA and E2FB could repress the *LEC1-2* genes, but in different seed developmental stages.

The expression of *LEC2* and *WRI1* is regulated by E2Fs during silique development

The promoter regions of LEC2 and WRI1 have putative E2F-binding sites, suggesting that E2Fs may directly control their expressions (Fig. 6A). To test this, we performed chromatin

immunoprecipitation (ChIP) experiments with anti-GFP antibody on silique samples collected from the maturation phase (S3) of pgE2FA-GFP and pgE2FB-GFP lines (Magyar et al., 2012). We could detect significant enrichment of E2FA-GFP but not E2FB-GFP protein to the promoter of *LEC2* and neither of these at the *WRI1* genes (Fig. 6B, S7). E2FA-GFP enrichment on the LEC2 promoter was located specifically to the region where consensus E2F-binding element was predicted (Fig. 6A, B). This result suggested that E2FA could directly regulate the expression of *LEC2* during the maturation phase. This experiment cannot rule out whether E2FC has a role during S3 stage or there are E2F association during earlier seed developmental phases, which is not amenable for ChIP. To gain further evidence for the E2F-mediated regulation of genes during seed maturation, we mutated the putative E2F-binding site identified in the promoter regions of LEC2 and WRI1. We generated reporter lines expressing the cyan fluorescent protein (CFP) either under the control of the native or the E2F-binding-site mutant LEC2 and WRI1 promoters. Representative lines were selected and siliques were harvested at different developmental stages as before (S1-4). The intact LEC2 promoter-reporter line (pLEC2-CFP) showed similar expression pattern as the endogenous *LEC2* transcript (Fig. 1A); almost no *LEC2* expression in the earliest seed developmental phase (S1), but it increased in the transition S2 phase, reached the maximum level in the maturation phase (S3) and diminished afterwards in post-mature seeds (S4 - Fig. 6C). In contrast, the E2F-site mutant LEC2 promoter-reporter line (p^{mutE2F}LEC2-CFP) showed an elevated and nearly constitutive transcript level throughout the silique development stages (Fig. 6C). The WRI1 promoter-reporter line (pWRI1-CFP) also closely followed the endogenous *WRI1* expression, peaking during the maturation (S3) phase (Fig. 6D, 1A). We analysed two independent E2F-binding site mutant WRI1 promoter-reporter lines (p^{mutE2F}WRI1-CFP, Line 22 and 24). Both of these reporter lines were expressed prematurely in the early developmental phases of S1-2, line 24 to a larger extent than line 22 (Fig. 6D). To back up these results we also monitored the CFP protein levels in these pWRI1 reporter-CFP lines during silique development (Fig. 6E). In the intact pWRI1-CFP line, CFP was exclusively accumulated at high level during the maturation phase (S3), while CFP protein could be detected in the earlier developmental silique stages in both p^{mutE2F}WRI1-CFP lines (Fig. 6E). These data further support that the timing of expression for these maturation genes are regulated by E2Fs.

Contrary to the reporter pLEC2-CFP lines, the pWRI1-CFP signal was high enough to allow confocal microscopy detection in the developing embryos. Confirming previous findings, in the pWRI1-CFP line the fluorescence signal was hardly detectable in the heart stage embryo, being

the brightest at the beginning of maturation phase in the early torpedo embryo stage, and gradually declined afterwards during maturation and diminished in the fully mature embryo (Fig. 7A – Baud et al., 2007). In contrast, both p^{mutE2F}WRI1-CFP line 22 (Fig. 7B), and line 24 (Fig. S8) showed a strong CFP signal in the heart stage embryo, which was maintained at high level until the mid and late torpedo embryo stages (Fig. S8). While the CFP signal was stronger for longer period of time in the p^{mutE2F}WRI1-CFP lines, the signal was missing in the root tip region of the immature embryos in comparison to the pWRI1-CFP line (Fig. 7C, S8B), suggesting that E2Fs both temporally and spatially regulate the expression of WRI1 during embryogenesis.

Seed reserve accumulation is prematurely activated in the *e2fab* double mutant

The results presented so far indicated that E2FA and E2FB repress key maturation genes during seed and silique development, which prompted us to test whether these activator E2Fs could regulate the seed maturation program. The two major seed storage proteins (SSPs) are the globulin (12S) and the albumin (2S) that represent up to one third of the dry weight in Arabidopsis seeds (Baud et al., 2002). To study the role of activator E2Fs, we determined the 2S albumin and 12S globulin levels during silique and seed development in single and double *e2fa* and *e2fb* mutants (Fig. 8). As known, these SSPs exclusively accumulate during the maturation phase (S3) of the control WT siliques (Fig. 8A; Vicente-Carbajosa and Carbonero, 2005), but became considerably more abundant in the S1 stage in *e2fa-2* and to a lower degree in the *e2fb-1* mutants, while the upregulation in double *e2fab* mutant was comparable with that of *e2fa-2* at this S1 stage (Fig. 8A-C). The position of the T-DNA insertion in the *e2fa-2* allele is just after the MARKED-Box (MB), while for *e2fb*-1 it is after the dimerization domain (DD, Fig. S9A). The MB domain strengthen the interaction with DPs directed by dimerization domain, a requirement to bind the target promoters (Black et al., 2005; Rubin et al., 2005). To address whether the upregulation of seed storage proteins are differently affected by *e2fa* or *e2fb* mutations, or correlates with the site of T-DNA insertion and possible production of truncated proteins with different properties, we analysed our mutant collection of *e2fa* and *e2fb* alleles (Fig. S9A). First we confirmed by Q-RT-PCR using insertion surrounding primers that T-DNA insertion is present in these mutants (Fig. S9B, E), while with 5`-specific primers we could detect both *E2FA* and *E2FB* transcripts in the mutants (Fig. S9F, G). By RT-PCR with

primer pairs spanning till the T-DNA, we further confirmed that these *e2f* mutants produce transcripts down to the insertion sites (Fig. S10A, B), while using primers downstream of the insertion could not amplify any fragments (Fig. S10C, D). Using an E2FB antibody targeted to the C-terminus, we established that there is neither full length nor truncated E2FB proteins containing part of the C-terminus in the *e2fb-1* and *e2fb-2* mutants (Fig. S11A). To test for the existence of a truncated E2FB protein, we used an N-terminal-specific E2FB antibody. This antibody is specific to recognise the overexpressed E2FB-GFP, but too weak to detect the endogenous E2FB, unless it was enriched through DPA co-immunoprecipitation (Fig. S11C), and in this way we could confirm the existence of a truncated E2FB protein in the *e2fab* double mutant (Fig. S11D). By using an N-terminal-specific E2FA antibody, the full length protein was recognised in WT, *e2fb-1* and *e2fb-2* mutants, while a smaller sized protein was detected in the *e2fa-2* single and *e2fab* double mutants (Fig. S11B). This protein could not be observed in *e2fa-1* supporting that this is a truncated E2FA specific for *e2fa-2* mutation. These results support that the *e2fa* and *e2fb* T-DNA insertion mutants can produce truncated proteins that expect to affect RBR recruitment and transactivation. In addition, these truncated proteins may have different ability to bind to DNA; the MB domain is intact in *e2fa-2* that should allow strong DNA binding, the T-DNA insertion disrupt the MB region in *e2fa-1* and *e2fb-1* at comparable position, which is expected to weaken their DNA binding activities, while the dimerization domain is disrupted in the *e2fb-2*, which should prevent DNA binding. With this in mind, we went on to determine how these different *e2fa* and *e2fb* mutant alleles affect the accumulation of 12S globulin and 2S albumin protein at different stages of silique development. These storage proteins are only present at the mature S3 stage in WT. In contrast, they were prematurely accumulated in these *e2f* mutants except in *e2fb-2* (Fig. 8B, C). Interestingly, the extent of premature expression of these storage proteins followed the predicted binding of truncated E2FA or E2FB to DNA as it was the strongest in *e2fa-2* as well as in the double *e2fab*, weaker in *e2fb-1* and *e2fa-1*, and no effect in *e2fb-2*. This suggests that the binding of these E2F mutant proteins to target DNA sequences without the ability to recruit the repressor RBR protein is what leads to the premature expression of seed storage proteins.

Because SSPs started to accumulate earlier during seed development in the *e2f* mutants, we wondered whether they reached higher levels in the fully developed post-mature dry seeds than in WT. We found that in the single *e2fa-1* and *e2fb-1* mutants both 2S albumin and 12S globulin accumulated to comparable levels than in the WT, while the 12S globulin became more abundant in the *e2fab* double mutant seeds (Fig. 8D). We also determined the total protein

content in mature seeds, and as shown in Table 2, it was significantly higher than the wild-type in the *e2fab* mutant. Thus, the embryo of the *e2fab* double mutant might become larger than WT because it contains more seed storage reserves.

In summary, we uncovered an important regulatory function for the activator E2Fs during the early morphogenic seed developmental phase to restrict the maturation program until the proliferation is active (Fig. 9).

Discussion

Here we showed that the two activator E2Fs, E2FA and E2FB coordinate cell proliferation with differentiation during seed and embryo development by multiple mechanisms: (i) both are contributing to the expression of cell cycle genes in the early phases of embryo development, but they are not essential for cell proliferation, (ii) they have distinct roles to repress S- and M-phase genes during seed maturation, when embryo quiescence is established, (iii) these activator E2Fs also have distinct roles to repress embryonic-differentiation genes including LEC2 and WRI1, (iv) these E2F transcription factors are critical for the timing and extent of seed storage protein accumulation (Fig. 9).

The cell number is not affected in developing embryo when E2FA and E2FB are mutated

The expression of S-phase specific genes were not affected in the single *e2fa-2* and *e2fb-1*, but in the double *e2fab* mutant, indicating that they act redundantly on S-phase regulatory genes. In contrast the mitotic *CDKB1;1* was exclusively regulated by E2FB but not by E2FA. In agreement, *E2FB* but not *E2FA* is expressed during the G2/M phases of the cell cycle (Mariconti et al., 2002; Magyar et al., 2005). The moderate overexpression of E2FA up-regulates S-phase specific genes, while the ectopic expression of CYCD3;1 hyper-activates both S- and M-phase regulatory genes, similarly to E2FB (de Jager et al., 2009). Moreover, it was suggested that E2FB is the canonical cell cycle activator E2F in Arabidopsis, based on the finding that it is released from RBR repression in the CYCD3;1 overexpressor line, while the E2FA-RBR complex was found to be regulated differently (Magyar et al., 2012).

In spite of the partial requirement for these activator E2Fs to fully promote cell cycle genes, the double *e2fab* mutant embryos consist of comparable number of cells to the control wild type. These findings demonstrate that (i) E2FA and E2FB are partially required but not essential for the expression of cell cycle target genes during embryonic cell divisions and (ii) the reduced expression of these cell cycle genes does not manifest in reduced cell proliferation. This is in agreement with other results showing that the regulatory roles for activator E2Fs are not critical for meristematic cell proliferation during post-embryonic development (Wang et al., 2014). Together with findings in animal systems, a universal model is emerging, where activator E2F functions are not required for normal cell proliferation either in embryonic or in post-embryonic development, which holds both for animals and for plants (Chen et al., 2009b; Magyar et al., 2016; Zappia and Frolov, 2016).

We could not detect developmental abnormalities in the *e2f* mutant embryos, except the significantly enlarged seed and embryo size in the double *e2fab* mutant. Interestingly, the double *e2fab* mutant develops shorter siliques containing fewer seeds than the control and we found that the double *e2fab* mutant have compromised fertility. It was shown that fertility problems might account for 33% of the increase in average seed weight (Ohto et al., 2005). This value matches the increase we observed with the *e2fab* double mutant. In agreement with the lack of cell proliferation defects, the plant stature of the *e2fab* double mutant does not differ from the WT during post-embryonic development. Recently it was suggested that the three Arabidopsis E2Fs regulate germline development in a redundant manner and affect fertility both through pollen development and megaspore mother cells (Yao et al., 2018). Indeed, the triple *e2fabc* mutant plants hardly produce seeds, but the plant stature is seemingly unaffected (Wang et al., 2014; Yao et al., 2018). Thus, none of the three canonical plant E2Fs are essential for cell proliferation at least during the sporophyte development. There are indications that some of the non-canonical E2Fs, i.e. E2FD may have positive roles in cell proliferation (Sozzani et al., 2010), possibly by competing with repressor complexes at E2F sites when the canonical E2Fs are missing.

E2FA and E2FB function as repressors in post-mitotic embryonic cells during maturation

Cell cycle genes are turned off during the transition phase from proliferation to maturation in the developing embryo, but the molecular mechanism is not yet clear. We show here that cell

cycle genes remained partially on even after the completion of the proliferation phase in the double *e2fab* mutant. This shows that these two E2Fs function as repressors on cell cycle genes as seed development progresses into the maturation phase. It is likely that E2Fs form complex with the transcriptional repressor RBR protein at this phase of seed development to establish quiescence as it was shown before during seedling and leaf development (Kobayashi et al., 2015). In agreement, E2FA and E2FB as well as their up-stream regulator RBR proteins are present in post-mitotic embryonic cells. The cell number in *rbr* mutant embryos increases during the maturation phase (Gutzat et al., 2011). However, in the *e2fab* mutant embryo we did not see 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.

These data support that RBR is central to determine the cell number in developing embryos and other plant organs in close association with E2Fs through the formation of repressor complexes. Interestingly, when RBR level or activity are reduced in plants the result is hyper-proliferation and tumorous growth, just like when *Rb* is eliminated in animals (Borghi et al, 2010; Gutzat et al., 2011; Chen et al., 2009b). The simultaneous inactivation of activator E2F1-3 in *rb* mutant animals restore normal development, indicating that animal E2F activator function is essential for tumour development, but dispensable for normal proliferation (Chen et al., 2009b). In plants it remains to be demonstrated whether the elimination of E2Fs in lines where *rbr* is compromised could restore the normal proliferation rate. Because RBR was also shown to be the primary target of CDKA;1 (Nowack et al., 2012), it would be also interesting to examine whether the elimination of E2Fs in *cdka;1* mutant could restore the embryo defect

Activator type E2Fs function as repressors to regulate the timing of embryo maturation program in developing seeds

Loss of function mutations in the LEC genes have defect in reserve accumulation (Braybrook and Harada, 2008). We found that both LEC genes were prematurely up-regulated in the *e2fab* 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 regulate LEC2, but earlier than E2FA. In agreement, expression of LEC2 became de-regulated when the E2F site in the

promoter was mutated, and showed a nearly maximum level of expression already during the morphogenic developmental phase. We also studied another putative E2F target gene, WRI1, and show that it is prematurely activated when the E2F-binding site was mutated. These data points to E2Fs as negative regulators of maturation genes and not just limiting their expression while cell proliferation is ongoing, but also fine tune their expressions during the maturation phase. In young Arabidopsis seedlings of *rbr* co-suppression line the maturation genes, including *LEC2* and *ABI3*, remain active, indicating that RBR controls these genes during post-embryonic development (Gutzat et al., 2011). It is also known that these maturation genes are under the control of the Polycomb Group (PcG) (Yang et al., 2013). Whether E2Fs together with RBR are also involved in this repressor complex remains to be seen.

We found that the major seed storage proteins 12S globulin and 2S albumin prematurely accumulate already at the morphogenic developmental phase in seeds of *e2fa-2*, *e2fb-1* and in the *e2fab* double mutants. Interestingly, at this early time point none of the regulatory AFL genes of seed maturation were upregulated in these *e2fa* and *e2fb* mutants, suggesting that these are not involved in the observed advance in the accumulation of seed storage proteins. Previously, E2FA was found to repress the switch from mitosis to endocycle during leaf development by forming repressor complex with RBR (Magyar et al., 2012). Simultaneous overexpression of E2FA with its dimerization partner, DPA, delays differentiation during early seedling development (De Veylder et al., 2002; Kosugi and Ohashi, 2003). The presented data here shows that E2FA is a potential repressor of the developmental transition program of seed maturation, suggesting that this developmental role of E2FA is more general. Whether E2FA performs this repressive role in complex with RBR is not yet known. Both E2FA and RBR proteins clearly accumulate at the highest level in the morphogenic seed developmental phase, supporting the hypothesis that they can form complex at this early seed developmental stage. Interestingly, the accumulation of SSP proteins was less pronounced in the *e2fa-1* and *e2fb-1* mutants in comparison to the *e2fa-2*, and was not observed in the *e2fb-2*. We confirmed that truncated proteins can be produced till the T-DNA insertion. Since all these truncated proteins are predicted to lack the ability to bind RBR or to transactivate, the difference between these alleles could be their ability to bind DNA, however these need to be experimentally verified. It is possible that the truncated E2FA mutant product occupies the binding sites and thus prevent the formation of other repressor complexes. Accordingly, all the three E2Fs including E2FC and possibly also the non-canonical E2Fs, the DELs might regulate the timing of seed maturation.

In conclusion, 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.

Materials and Methods

Plant material, growth conditions and silique collection

Arabidopsis (*Arabidopsis thaliana*) Col-0 ecotype was the wild type and background of every transgenic lines used in this study. *In vitro* cultured plants were grown on half strength germination medium under continuous light at 22°C. Soil grown plants were cultivated in greenhouse at 22°C in long-day condition (16h light/ 8h dark photoperiod). All the T-DNA insertion mutant lines used in the experiments are previously published; *e2fb-1* - SALK_103138, *e2fb-2* - SALK_120959, *e2fa-1* – MPIZ-244, *e2fa-2* - GABI-348E09 (Berckmans et al., 2011a; Berckmans et al., 2011b; Horvath et al., 2017), the double *e2fa-2/e2fb-1* was reported by (Heyman et al., 2011). Total seed weight, seed size, number of siliques on the main inflorescence, seed number per siliques and silique size were measured by using ten plants per genotype according to (Van Daele et al., 2012). Seed size was calculated from 100 seeds imaged by stereo microscope and analysed by Image J software (Schneider et al., 2012).;

Siliques were collected from soil grown plants at four different developmental stages. These are S1: young siliques 2-3 days after pollination (DAP) with 0.2-0.3 cm length; S2: siliques 4-7 DAP with 0.4-0.6 cm size; S3: full size siliques 8-12 DAP; S4: full size yellow siliques, 13-18 DAP.

Generation of reporter lines and transgenic Arabidopsis plants

To construct the transcriptional reporters pLEC2-CFP and pWRI1-CFP, promoter regions of *LEC2* and *WRI1* genes were PCR amplified (3162bp and 1864 bp upstream of the translational start codon, respectively; cloning primer combinations described in Supplemental Table S1). Multisite Gateway cloning strategy was used to make promoter-reporter gene fusions following the protocols in the Gateway Cloning Technology booklet (Invitrogen, USA). The LEC2 and WRI1 promoter regions were cloned into pGEM-based plasmids and together with the CFP reporter in pGEM 221 plasmid introduced to pGreenII-based pGII0229 destination vector. Site-

directed mutagenesis is carried out using the Quick-change mutagenesis (Papworth et al., 1996). The E2F-binding site TTTCCCCC on WRI1 promoter at -359bp position was mutated to TTTCC*AA*C, the CGGGAAAA motif on LEC2 promoter at -2bp position was mutated to *TT*GGAAAA. Primers used for the mutagenesis described in Supplemental Table S1. Transgenic *Arabidopsis* plants were generated by using the floral-dip method for *Agrobacterium*-mediated transformation and primary transformants were selected on soil by spraying BASTA (glufosinate-ammonium). 24 pWRI1-CFP, 31 p^{mutE2F}WRI1-CFP, 26 pLEC2-CFP, and 24 p^{mutE2F}LEC2-CFP primary transgenic lines were identified and genotyped on PPT selection. Single insertion lines were identified and used for further analysis (5 in each case).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from siliques and developing seeds using the CTAB-LiCl method (Jaakola et al., 2001). Isolated RNA samples were treated with DNaseI (Thermo Fisher Scientific). 1 µg of RNA was used to prepare cDNA from each sample using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) and the Applied Biosystems 7900-HT Fast Real-Time detection system. For amplification, a standard two-step thermal cycling profile was used (15 s at 95 °C and 1 min at 60 °C) during 40 cycles, after a 10-min preheating step at 95 °C. Samples were run in triplicates, and UBC18 was used as internal reference gene. Data analysis was carried out by the 2-ΔCT or the 2-ΔΔCT method. Student's t-test was performed to determine the significance of differences between groups. Data are presented as mean±s.d.

Protein analysis, protein extraction, antibody preparation, and immunoblot assay

Siliques were collected from different developmental stages (40-50 siliques per each line) and snap-frozen in liquid nitrogen. For detecting E2F-DP and cell cycle proteins in immunoblot assay, total proteins were extracted from developing immature siliques (stage S1-3) in extraction buffer (25mM TRIS-HCl, 15mM MgCl$_2$, 15mM EGTA, 15mM p-nitrophenylphosphate, 100 mM NaCl, 60 mM ß-glycerophosphate, 1mM DTT, 0,1% Igepal, 5mM NaF, protease inhibitor cocktail for plant tissue (Sigma) and 1mM phenylmethyl sulphonylfluoride –(Magyar et al., 1993), while total proteins from post-maturing siliques (S4) or 100 dry seeds were extracted in extraction buffer in mortar cooled in liquid nitrogen (100

mM Tris-HC1, pH 8.0, 0.5% (w/v) SDS, 10% (v/v) glycerol and 2% (v/v) 2- mercaptoethanol – Hou et al., 2005), followed by boiling for 3 min, and centrifugation for 10 min at 4 ^{0}C. The latter extraction method was used for detecting seed storage proteins in siliques of S1 to S4 stages. The precipitated material (20-40 μg) was separated on SDS-PAGE (10, 12 or 15%) and either stained by Coomassie-Brilliant Blue R250 or blotted to PVDF membrane. Antibodies used in immunoblotting experiments: chicken polyclonal anti-RBR (in dilution 1:2000; Agrisera), rat polyclonal antibody anti-E2FA (in dilution 1:300 - see below), rabbit polyclonal antibody anti-E2FB, (in dilution 1:500; Magyar et al., 2005), and N-terminal specific chicken polyclonal anti-E2FB (in dilution 1:300 – see below), rabbit polyclonal anti-DPB (in dilution 1:500; Lopez-Juez et al., 2008), rabbit polyclonal antibody anti-2S albumin, and anti-12S globulin (in dilution 1:10000; Shimada et al., 2003).

To produce E2FA antibody, a 270 bp fragment encoding the N-terminal 90 aa of Arabidopsis E2FA (E2FA-N-90) was amplified using the following primers: BamHI-FWD: 5'- ATA GGA TCC ATG TCC GGT GTC GTA CGA TC -3'; SalI-REV: 5'- ATA GTC GAC CTA TCTAAC AAC GAC AGC ATC TTC CT -3' (restriction sites underlined). The BamH I-Sal I digested E2FA-N-90 fragment was subcloned into pET-28a(+) vector (Novagen) to obtain 6xHis-E2FA-N-90 and this construct was transformed into BL21(DE3) Rosetta cells. Protein production was induced with 0.5 mM IPTG (3h, 37 °C, 250 rpm shaking), cells were lysed in 6 M GuHCl lysis buffer and the cleared lysate was loaded onto HIS-Select Nickel Affinity Gel (Sigma-Aldrich, P6611). The 6xHis-E2FA-N-90 protein was purified according to the manufacturer's instructions and used to immunize rats. Immunoglobulin fraction of crude rat sera was obtained by ammonium-sulfate precipitation, anti-E2FA antibody was further purified on nitrocellulose bound recombinant protein following the protocol of (Kurien, 2009).

To produce the N-terminal specific E2FB antibody, a 267 bp fragment encoding the N-terminal 89 amino acids was amplified using the following pimers: BamHI-FWD: 5'- AC GGA TCC ATG TCT GAA GAA GTA CCT -3'; Sal1-REV: 5' ATA GTC GAC TGA TAC AGG TGT TTG AAG -3' (restriction sits underlined). The PCR E2FB-N-89 fragment was cloned into pGEX-4T-1 vector (GE Healthcare Life Sciences) into the BamH1-Sal1 sites, and the recombinant GST-tagged E2FB-N-89 protein was purified after IPTG induction according to the manufacturer's instructions and immunize chicken (Agrisera, Sweden). The antibody was further purified like the anti-E2FA antibody (see above).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out according to Saleh et al., 2008. Two grams of siliques from developmental stage S3 of E2FA-GFP (Berckmans et al., 2011b) or E2FB-GFP expressing plants were crosslinked with 1% formaldehyde solution at ZT2 - 6DAG. Chromatin was precipitated using anti-GFP polyclonal rabbit antibody (Invitrogen) and were collected with salmon sperm DNA/protein A-agarose (Sigma). The purified DNA was used in qRT-PCR reactions to amplify promoter regions with specific primers listed in Supplemental Table S1. Relative DNA enrichment was calculated by dividing the antibody immunoprecipitation signals with the no-antibody signals.

Dissecting of embryos, and microscopy

Immature embryos of transgenic lines expressing the fluorescent tagged E2FA, E2FB or RBR proteins under the control of their own promoters (pgE2FA-3xvYFP or pgE2FB-3xvYFP or pgRBR-3xCFP) were dissected under stereo-microscope, and observations were made with Leica Confocal Laser-Microscope. Mature dried seeds were imbibed for 1 h and dissected under the stereo-microscope and isolated embryos were stained with propidium iodide (PI) and photographed, and organ and epidermal cell sizes were measured by using Image J software (Schneider et al., 2012).

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The authors have made the following declarations about their contributions:

Z.M. conceived the idea and designed the study to analyse the function of E2FA and E2FB during seed development, discussed these ideas with L.B and J.P. T.L., E.M. and E.Ő developed the staging protocol for siliques and performed the expressional studies. T.L. made the promoter constructs and together with Z.M. analysed the transgenic lines by confocal microscopy. T.L., V.N. and Z.M did the imaging and analysis of embryos. E.M. and Z.M. characterised the insertion mutants. ChIP was performed by Cs.P. Anti-E2FA specific antibody was generated by G.V.H and Cs.V. The manuscript was written by Z.M, and L.B. seen and commented by all authors.

References

Baud, S., Boutin, J. P., Miquel, M., Lepiniec, L. and Rochat, C. (2002). An integrated overview of seed development in Arabidopsis thaliana ecotype WS. *Plant Physiol Bioch* **40**, 151-160.

Baud, S., Dubreucq, B., Miquel, M., Rochat, C. and Lepiniec, L. (2008). Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. *Arabidopsis Book* **6**, e0113.

Baud, S., Mendoza, M. S., To, A., Harscoet, E., Lepiniec, L. and Dubreucq, B. (2007). WRINKLED1 specifies the regulatory action of LEAFY COTYLEDON2 towards fatty acid metabolism during seed maturation in Arabidopsis. *Plant J* **50**, 825-838.

Berckmans, B., Lammens, T., Van Den Daele, H., Magyar, Z., Bogre, L. and De Veylder, L. (2011a). Light-dependent regulation of DEL1 is determined by the antagonistic action of E2Fb and E2Fc. *Plant Physiol* **157**, 1440-1451.

Berckmans, B., Vassileva, V., Schmid, S. P., Maes, S., Parizot, B., Naramoto, S., Magyar, Z., Alvim Kamei, C. L., Koncz, C., Bogre, L., et al. (2011b). Auxin-dependent cell cycle reactivation through transcriptional regulation of Arabidopsis E2Fa by lateral organ boundary proteins. *Plant Cell* **23**, 3671-3683.

Black, E. P., Hallstrom, T., Dressman, H. K., West, M. and Nevins, J. R. (2005). Distinctions in the specificity of E2F function revealed by gene expression signatures. *Proc Natl Acad Sci U S A* **102**, 15948-15953.

Borghi, L., Gutzat, R., Futterer, J., Laizet, Y., Hennig, L. and Gruissem, W. (2010). Arabidopsis RETINOBLASTOMA-RELATED Is Required for Stem Cell Maintenance, Cell Differentiation, and Lateral Organ Production. *Plant Cell* **22**, 1792-1811.

Braybrook, S. A. and Harada, J. J. (2008). LECs go crazy in embryo development. *Trends Plant Sci* **13**, 624-630.

Carbonero, P., Iglesias-Fernandez, R. and Vicente-Carbajosa, J. (2017). The AFL subfamily of B3 transcription factors: evolution and function in angiosperm seeds. *J Exp Bot* **68**, 871-880.

Chen, D., Pacal, M., Wenzel, P., Knoepfler, P. S., Leone, G. and Bremner, R. (2009a). Division and apoptosis of E2f-deficient retinal progenitors. *Nature* **462**, 925-929.

Chen, H. Z., Tsai, S. Y. and Leone, G. (2009b). Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nat Rev Cancer* **9**, 785-797.

Chong, J. L., Wenzel, P. L., Saenz-Robles, M. T., Nair, V., Ferrey, A., Hagan, J. P., Gomez, Y. M., Sharma, N., Chen, H. Z., Ouseph, M., et al. (2009). E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. *Nature* **462**, 930-934.

Collins, C., Dewitte, W. and Murray, J. A. (2012). D-type cyclins control cell division and developmental rate during Arabidopsis seed development. *J Exp Bot* **63**, 3571-3586.

de Jager, S. M., Scofield, S., Huntley, R. P., Robinson, A. S., den Boer, B. G. and Murray, J. A. (2009). Dissecting regulatory pathways of G1/S control in Arabidopsis: common and distinct targets of CYCD3;1, E2Fa and E2Fc. *Plant Mol Biol* **71**, 345-365.

De Veylder, L., Beeckman, T., Beemster, G. T., de Almeida Engler, J., Ormenese, S., Maes, S., Naudts, M., Van Der Schueren, E., Jacqmard, A., Engler, G., et al. (2002). Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. *Embo J* **21**, 1360-1368.

De Veylder, L., Beeckman, T. and Inze, D. (2007). The ins and outs of the plant cell cycle. *Nat Rev Mol Cell Biol* **8**, 655-665.

del Pozo, J. C., Boniotti, M. B. and Gutierrez, C. (2002). Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF(AtSKP2) pathway in response to light. *Plant Cell* **14**, 3057-3071.

del Pozo, J. C., Diaz-Trivino, S., Cisneros, N. and Gutierrez, C. (2006). The balance between cell division and endoreplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCFSKP2A pathway in Arabidopsis. *Plant Cell* **18**, 2224-2235.

Devic, M. and Roscoe, T. (2016). Seed maturation: Simplification of control networks in plants. *Plant Sci* **252**, 335-346.

Dimova, D. K., Stevaux, O., Frolov, M. V. and Dyson, N. J. (2003). Cell cycle-dependent and cell cycle-independent control of transcription by the Drosophila E2F/RB pathway. *Genes Dev* **17**, 2308-2320.

Duronio, R. J., Ofarrell, P. H., Xie, J. E., Brook, A. and Dyson, N. (1995). The Transcription Factor E2f Is Required for S-Phase during Drosophila Embryogenesis. *Gene Dev* **9**, 1445-1455.

Fischer, M. and DeCaprio, J. A. (2015). Does Arabidopsis thaliana DREAM of cell cycle control? *Embo J* **34**, 1987-1989.

Focks, N. and Benning, C. (1998). wrinkled1: A novel, low-seed-oil mutant of Arabidopsis with a deficiency in the seed-specific regulation of carbohydrate metabolism. *Plant Physiol* **118**, 91-101.

Frolov, M. V., Huen, D. S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M. and Dyson, N. J. (2001). Functional antagonism between E2F family members. *Gene Dev* **15**, 2146-2160.

Goldberg, R. B., de Paiva, G. and Yadegari, R. (1994). Plant embryogenesis: zygote to seed. *Science* **266**, 605-614.

Gutierrez, C. (2009). The Arabidopsis cell division cycle. *Arabidopsis Book* **7**, e0120.

Gutzat, R., Borghi, L., Futterer, J., Bischof, S., Laizet, Y., Hennig, L., Feil, R., Lunn, J. and Gruissem, W. (2011). RETINOBLASTOMA-RELATED PROTEIN controls the transition to autotrophic plant development. *Development* **138**, 2977-2986.

Gutzat, R., Borghi, L. and Gruissem, W. (2012). Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. *Trends Plant Sci* **17**, 139-148.

Harashima, H. and Sugimoto, K. (2016). Integration of developmental and environmental signals into cell proliferation and differentiation through RETINOBLASTOMA-RELATED 1. *Curr Opin Plant Biol* **29**, 95-103.

Heyman, J., Van den Daele, H., De Wit, K., Boudolf, V., Berckmans, B., Verkest, A., Alvim Kamei, C. L., De Jaeger, G., Koncz, C. and De Veylder, L. (2011). Arabidopsis ULTRAVIOLET-B-INSENSITIVE4 maintains cell division activity by temporal inhibition of the anaphase-promoting complex/cyclosome. *Plant Cell* **23**, 4394-4410.

Holdsworth, M. J., Bentsink, L. and Soppe, W. J. (2008). Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytol* **179**, 33-54.

Horvath, B. M., Kourova, H., Nagy, S., Nemeth, E., Magyar, Z., Papdi, C., Ahmad, Z., Sanchez-Perez, G. F., Perilli, S., Blilou, I., et al. (2017). Arabidopsis RETINOBLASTOMA RELATED directly regulates DNA damage responses through functions beyond cell cycle control. *Embo J* **36**, 1261-1278.

Jaakola, L., Pirttila, A. M., Halonen, M. and Hohtola, A. (2001). Isolation of high quality RNA from bilberry (Vaccinium myrtillus L.) fruit. *Mol Biotechnol* **19**, 201-203.

Kobayashi, K., Suzuki, T., Iwata, E., Nakamichi, N., Chen, P., Ohtani, M., Ishida, T., Hosoya, H., Muller, S., Leviczky, T., et al. (2015). Transcriptional repression by MYB3R proteins regulates plant organ growth. *Embo J* **34**, 1992-2007.

Kosugi, S. and Ohashi, Y. (2002). Interaction of the Arabidopsis E2F and DP proteins confers their concomitant nuclear translocation and transactivation. *Plant Physiology* **128**, 833-843.

Kosugi, S. and Ohashi, Y. (2003). Constitutive E2F expression in tobacco plants exhibits altered cell cycle control and morphological change in a cell type-specific manner. *Plant Physiol* **132**, 2012-2022.

Kurien, B. T. (2009). Affinity purification of autoantibodies from an antigen strip excised from a nitrocellulose protein blot. *Methods Mol Biol* **536**, 201-211.

Lau, S., Slane, D., Herud, O., Kong, J. and Jurgens, G. (2012). Early embryogenesis in flowering plants: setting up the basic body pattern. *Annu Rev Plant Biol* **63**, 483-506.

Li, X., Cai, W., Liu, Y., Li, H., Fu, L., Liu, Z., Xu, L., Liu, H., Xu, T. and Xiong, Y. (2017). Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes. *Proc Natl Acad Sci U S A* **114**, 2765-2770.

Lopez-Juez, E., Dillon, E., Magyar, Z., Khan, S., Hazeldine, S., de Jager, S. M., Murray, J. A., Beemster, G. T., Bogre, L. and Shanahan, H. (2008). Distinct light-initiated gene expression and cell cycle programs in the shoot apex and cotyledons of Arabidopsis. *Plant Cell* **20**, 947-968.

Magyar, Z., Atanassova, A., De Veylder, L., Rombauts, S. and Inze, D. (2000). Characterization of two distinct DP-related genes from Arabidopsis thaliana. *FEBS Lett* **486**, 79-87.

Magyar, Z., Bako, L., Bogre, L., Dedeoglu, D., Kapros, T. and Dudits, D. (1993). Active Cdc2-Genes and Cell-Cycle Phase-Specific Cdc2-Related Kinase Complexes in Hormone-Stimulated Alfalfa Cells. *Plant Journal* **4**, 151-161.

Magyar, Z., Bogre, L. and Ito, M. (2016). DREAMs make plant cells to cycle or to become quiescent. *Curr Opin Plant Biol* **34**, 100-106.

Magyar, Z., De Veylder, L., Atanassova, A., Bako, L., Inze, D. and Bogre, L. (2005). The role of the Arabidopsis E2FB transcription factor in regulating auxin-dependent cell division. *Plant Cell* **17**, 2527-2541.

Magyar, Z., Horvath, B., Khan, S., Mohammed, B., Henriques, R., De Veylder, L., Bako, L., Scheres, B. and Bogre, L. (2012). Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes. *Embo J* **31**, 1480-1493.

Mariconti, L., Pellegrini, B., Cantoni, R., Stevens, R., Bergounioux, C., Cella, R. and Albani, D. (2002). The E2F family of transcription factors from Arabidopsis thaliana. Novel and conserved components of the retinoblastoma/E2F pathway in plants. *J Biol Chem* **277**, 9911-9919.

Morgan, D. O. (2007). *The Cell Cycle: Principles of Control*: Oxford University Press.

Nowack, M. K., Harashima, H., Dissmeyer, N., Zhao, X., Bouyer, D., Weimer, A. K., De Winter, F., Yang, F. and Schnittger, A. (2012). Genetic framework of cyclin-dependent kinase function in Arabidopsis. *Dev Cell* **22**, 1030-1040.

Ohto, M. A., Fischer, R. L., Goldberg, R. B., Nakamura, K. and Harada, J. J. (2005). Control of seed mass by APETALA2. *Proc Natl Acad Sci U S A* **102**, 3123-3128.

Papworth, C., Bauer, J. C., Braman, J. and Wright, D. A. (1996). QuikChange site-directed mutagenesis. *Strategies.* **9**, 3-4.

Raz, V., Bergervoet, J. H. and Koornneef, M. (2001). Sequential steps for developmental arrest in Arabidopsis seeds. *Development* **128**, 243-252.

Rowland, B. D. and Bernards, R. (2006). Re-evaluating cell-cycle regulation by E2Fs. *Cell* **127**, 871-874.

Royzman, I., Whittaker, A. J. and Orr-Weaver, T. L. (1997). Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes Dev* **11**, 1999-2011.

Rubin, S. M., Gall, A. L., Zheng, N. and Pavletich, N. P. (2005). Structure of the Rb C-terminal domain bound to E2F1-DP1: a mechanism for phosphorylation-induced E2F release. *Cell* **123**, 1093-1106.

Sadasivam, S. and DeCaprio, J. A. (2013). The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nat Rev Cancer* **13**, 585-595.

Santos-Mendoza, M., Dubreucq, B., Baud, S., Parcy, F., Caboche, M. and Lepiniec, L. (2008). Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. *Plant Journal* **54**, 608-620.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675.

Shimada, T., Fuji, K., Tamura, K., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (2003). Vacuolar sorting receptor for seed storage proteins in Arabidopsis thaliana. *P Natl Acad Sci USA* **100**, 16095-16100.

Sozzani, R., Maggio, C., Giordo, R., Umana, E., Ascencio-Ibanez, J. T., Hanley-Bowdoin, L., Bergounioux, C., Cella, R. and Albani, D. (2010). The E2FD/DEL2 factor is a component of a regulatory network controlling cell proliferation and development in Arabidopsis. *Plant Mol Biol* **72**, 381-395.

Sozzani, R., Maggio, C., Varotto, S., Canova, S., Bergounioux, C., Albani, D. and Cella, R. (2006). Interplay between Arabidopsis activating factors E2Fb and E2Fa in cell cycle progression and development. *Plant Physiology* **140**, 1355-1366.

Sun, X. D., Shantharaj, D., Kang, X. J. and Ni, M. (2010). Transcriptional and hormonal signaling control of Arabidopsis seed development. *Curr Opin Plant Biol* **13**, 611-620.

Tsai, S. Y., Opavsky, R., Sharma, N., Wu, L., Naidu, S., Nolan, E., Feria-Arias, E., Timmers, C., Opavska, J., de Bruin, A., et al. (2008). Mouse development with a single E2F activator. *Nature* **454**, 1137-1141.

Van Daele, I., Gonzalez, N., Vercauteren, I., de Smet, L., Inze, D., Roldan-Ruiz, I. and Vuylsteke, M. (2012). A comparative study of seed yield parameters in Arabidopsis thaliana mutants and transgenics. *Plant Biotechnol J* **10**, 488-500.

Vandepoele, K., Vlieghe, K., Florquin, K., Hennig, L., Beemster, G. T., Gruissem, W., Van de Peer, Y., Inze, D. and De Veylder, L. (2005). Genome-wide identification of potential plant E2F target genes. *Plant Physiol* **139**, 316-328.

Venable, D. L. (1992). Size-Number Trade-Offs and the Variation of Seed Size with Plant Resource Status. *Am Nat* **140**, 287-304.

Vicente-Carbajosa, J. and Carbonero, P. (2005). Seed maturation: developing an intrusive phase to accomplish a quiescent state. *Int J Dev Biol* **49**, 645-651.

Wang, S., Gu, Y., Zebell, S. G., Anderson, L. K., Wang, W., Mohan, R. and Dong, X. (2014). A noncanonical role for the CKI-RB-E2F cell-cycle signaling pathway in plant effector-triggered immunity. *Cell Host Microbe* **16**, 787-794.

Wendrich, J. R. and Weijers, D. (2013). The Arabidopsis embryo as a miniature morphogenesis model. *New Phytologist* **199**, 14-25.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V. and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *Plos One* **2**.

Wu, L., Timmers, C., Maiti, B., Saavedra, H. I., Sang, L., Chong, G. T., Nuckolls, F., Giangrande, P., Wright, F. A., Field, S. J., et al. (2001). The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**, 457-462.

Wu, Y., Shi, L., Li, L., Fu, L., Liu, Y., Xiong, Y. and Sheen, J. (2019). Integration of nutrient, energy, light and hormone signalling via TOR in plants. *J Exp Bot*.

Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C. and Sheen, J. (2013). Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* **496**, 181-186.

Yang, C., Bratzel, F., Hohmann, N., Koch, M., Turck, F. and Calonje, M. (2013). VAL- and AtBMI1-mediated H2Aub initiate the switch from embryonic to postgerminative growth in Arabidopsis. *Curr Biol* **23**, 1324-1329.

Yao, X., Yang, H., Zhu, Y., Xue, J., Wang, T., Song, T., Yang, Z. and Wang, S. (2018). The Canonical E2Fs Are Required for Germline Development in Arabidopsis. *Front Plant Sci* **9**, 638.

Zappia, M. P. and Frolov, M. V. (2016). E2F function in muscle growth is necessary and sufficient for viability in Drosophila. *Nat Commun* **7**.
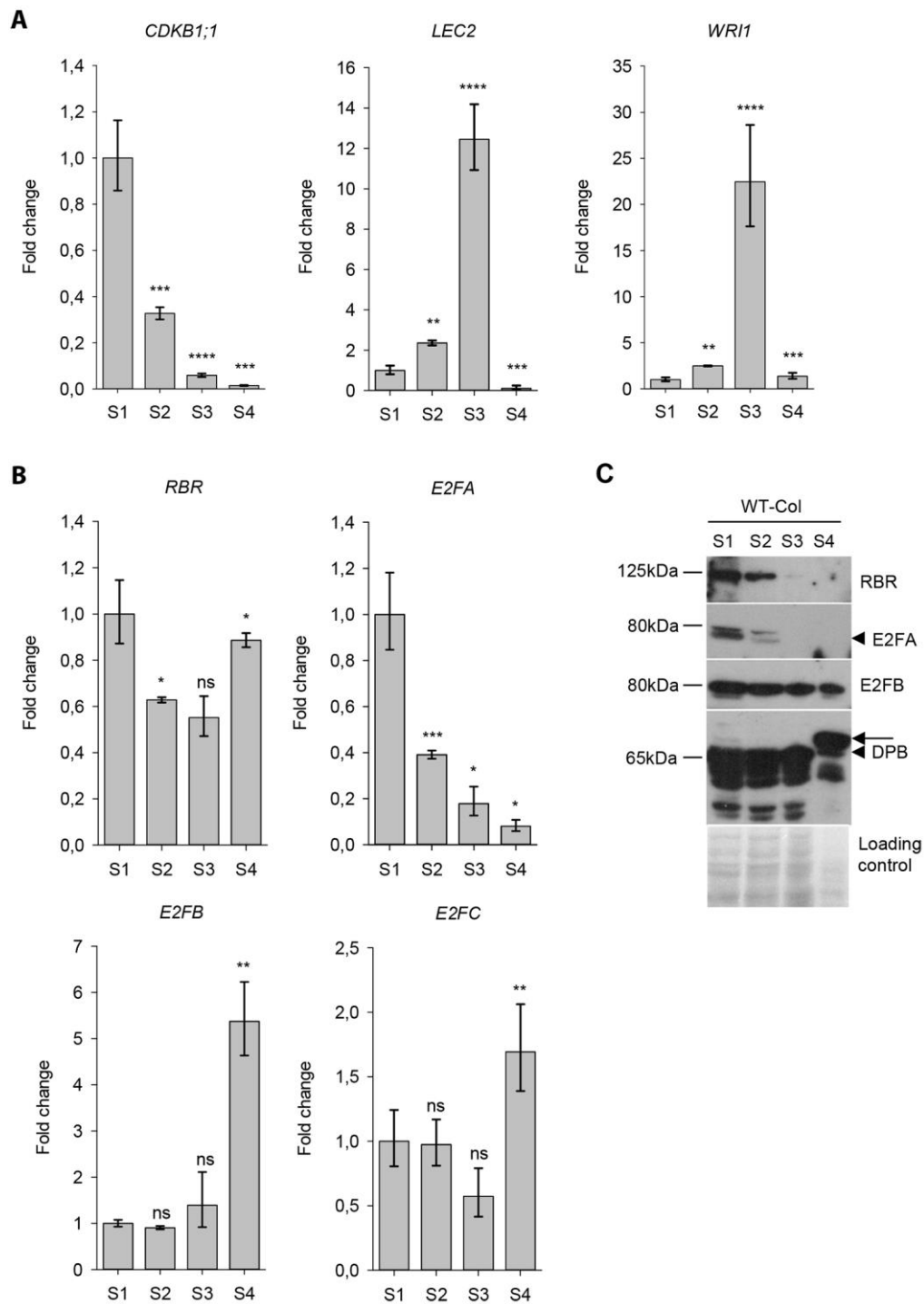
Figures

Figure 1. The expression profiles of E2FA and E2FB are distinct in the developing siliques, but overlap in the proliferation phase. (A) Q-RT-PCR analyses of the G2- and M-phase specific CDKB1;1, the seed maturation LEC2 and WRI1 genes in the developing siliques of

the wild-type (WT) at four silique developmental stages (S1 to S4, pictured in Suppl. Fig. S1). (**B**) The transcript levels of the three E2Fs, namely E2FA, E2FB and E2FC and the single RBR genes were also analysed in these silique samples by qRT-PCR. Values represent fold changes normalised to the value of the S1 silique stage (set arbitrarily at 1). Data are combined from n = 3 biological repeats, and bars represent means with standard deviations. $P \leq 0,05$ (*), 0,01 (**), 0,001 (***), 0,0001 (****) were considered significant between consecutive silique stages. ns means non-significant difference. Abbreviations of the genes and the primer sequences are listed in Supplemental Table S1. (**C**) To follow the accumulation levels of RBR, E2FA, E2FB, and DPB proteins in developing siliques (S1-4) specific antibodies were used in immunoblot assays as indicated. The Ponceau stained proteins were used as loading control. Arrowheads indicate the corresponding E2FA and DPB proteins, and arrow marks a slower migrating form of DPB in S4 silique stage. Molecular weights of the specific proteins are shown on the left side.
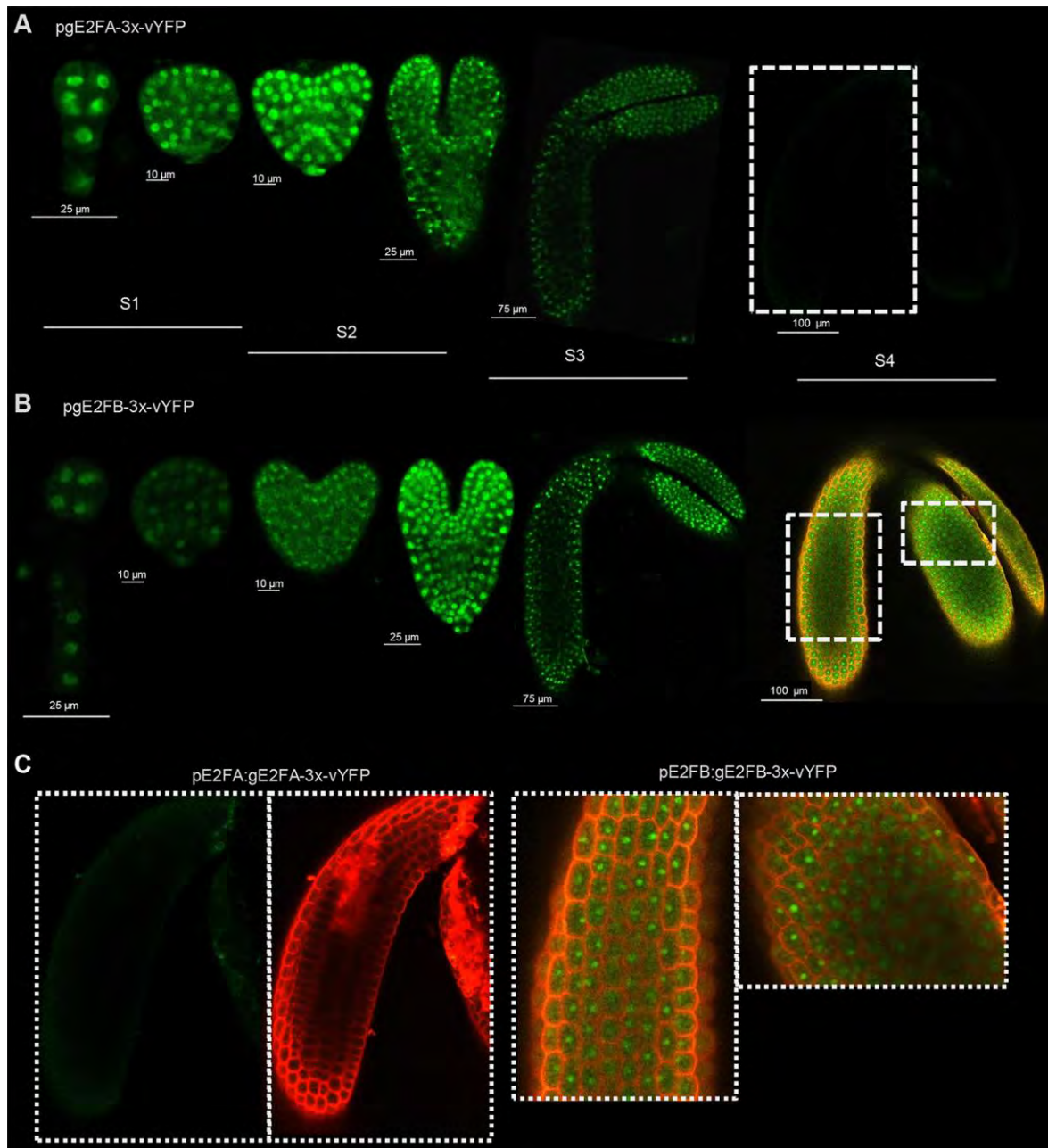
Figure 2. Spatial and temporal regulation of E2FA and E2FB accumulation during embryogenesis. (A-B) Representative CM images of developing embryos dissected from immature seeds of pgE2FA-3xvYFP (**A**), and pgE2FB-3xvYFP (**B**) transgenic lines. White open boxes with dashed line outline the epidermal regions of hypocotyls and cotyledon in the post-mature E2FA-3xvYFP (left side) and E2FB-3xvYFP (right side) embryos as indicated in (**C**). The vYFP signal is green, the cell wall is counterstained with propidium iodide (PI-red). In the case of post-mature E2FB-3xvYFP embryo the merged images of the YFP and the PI signals are shown. Silique stages (S1-4) correspond to the different embryo developmental phases are indicated. Scale bars are included at the bottom side of embryo images.
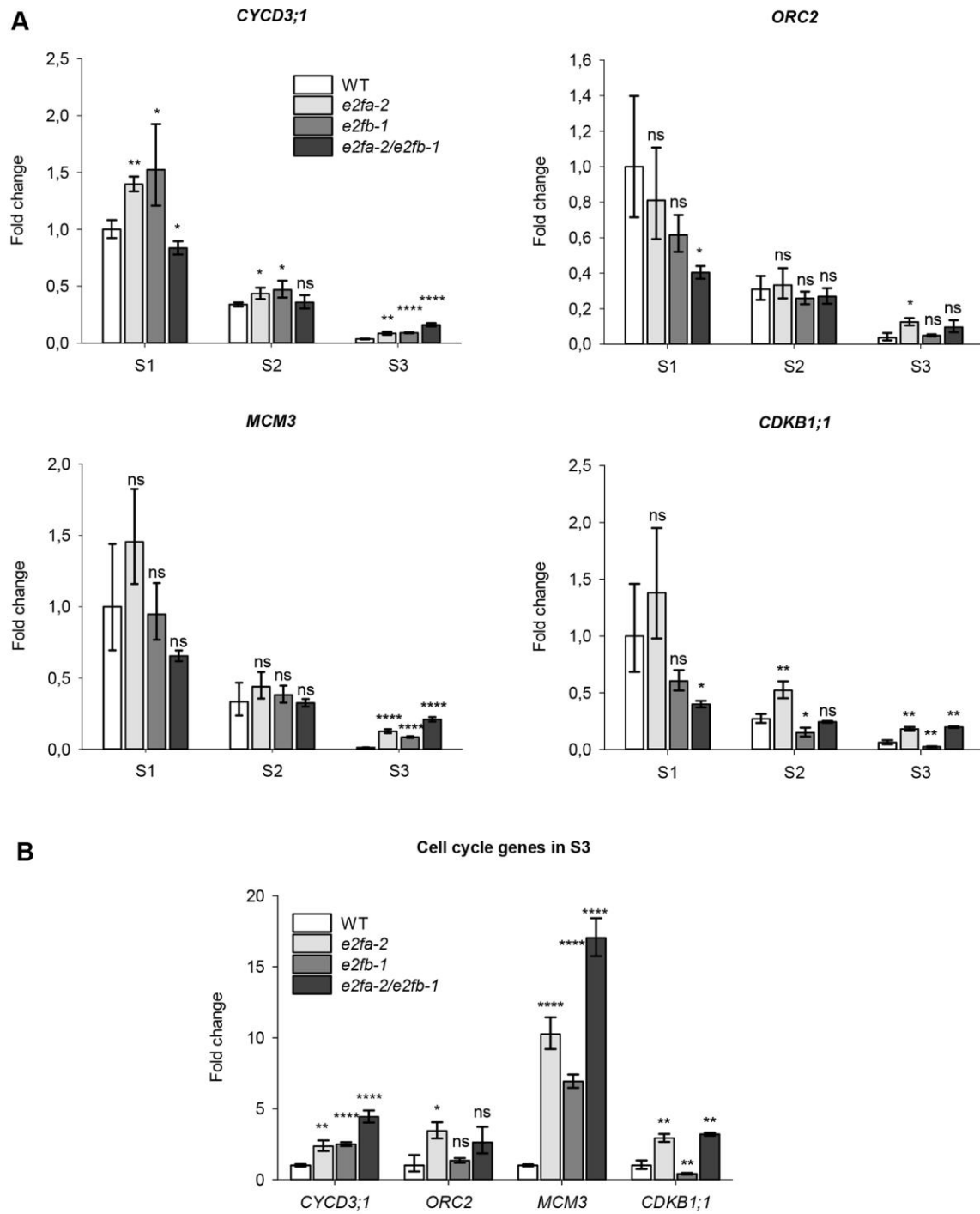
Figure 3. E2FA and E2FB could function either as activator or repressor on cell cycle genes depending on the developmental stage of siliques and seeds. (A) Comparison of the CYCD3;1, ORC2, MCM3, and CDKB1;1 transcript levels in developing siliques of the *e2fa-2, e2fb-1,* and *e2fa-2/e2fb-1* single and double mutants, respectively with the control (WT) at

three silique developmental stages from S1 to S3 (pictured in Suppl. Fig. S1). Values represent fold changes normalised to the value of the WT at the S1 silique stage (set arbitrarily at 1). (**B**) The expression levels of cell cycle genes in the S3 maturation phase are compared between the *e2f* mutants and the control WT. The values represent fold change normalised to the value of the relevant gene from WT at the S3 silique stage (set arbitrarily at 1). Data are combined from n = 3 biological repeats, and bars represent means with standard deviations. P≤0,05 (*), 0,01 (**), 0,001 (***), 0,0001 (****) were considered significant between the corresponding mutant and the WT, at a given silique stage. ns means non-significant difference. Abbreviations of the genes and the primer sequences are listed in Supplemental Table S1.
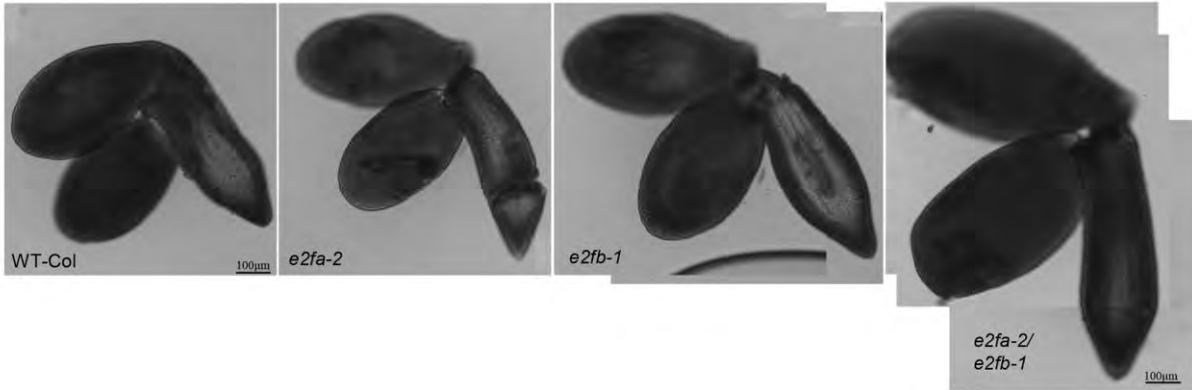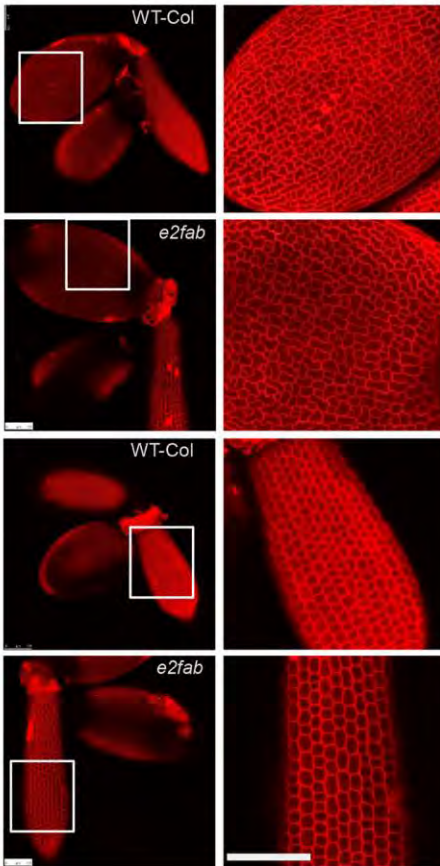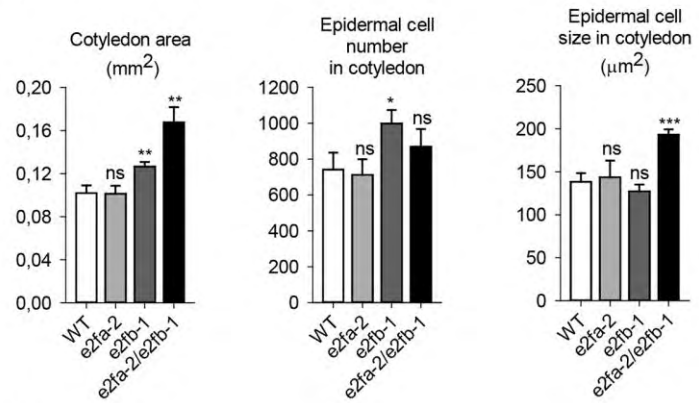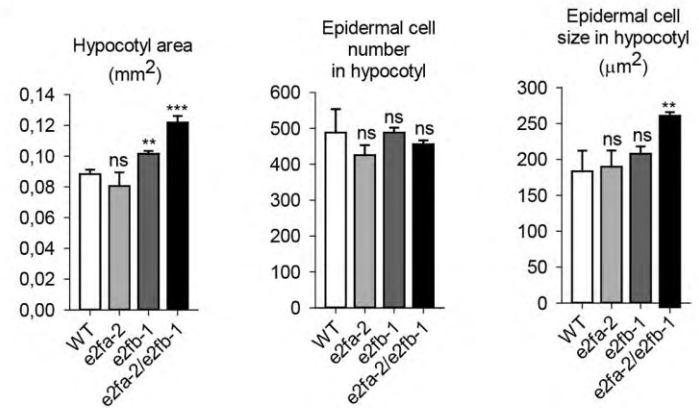
Figure 4. E2FA and E2FB are dispensable for embryonic cell proliferation. (**A**) Representative pictures of mature embryos from wild type (WT) and *e2fa-2, e2fb-1* single and *e2fa-2/e2fb-1* double mutants dissected from mature dry seeds, and photographed in CM. Bars: 100 μm. (**B**) Propidium idodide stained WT-Col and *e2fa-2/e2fb-1* double mutant embryos were photographed in CM (additional images are shown in Fig. S S10). White open boxes outline

epidermal regions in cotyledons and hypocotyls of WT and *e2fa-2/e2fb-1* double mutant and enlarged on the right side to show epidermal cell sizes. Bars: 100 µm. (**C-D**) Entire cotyledon (**C**) and hypocotyl (**D**) area was measured of mature dried embryos for determining their areas. Data are combined from n = 3 biological repeats, N = 10 samples in each. Cell size and cell number was calculated by using image analyses (Image J). Sample size N≥200 cells/image (n=4). Error bars represent standard deviation. P≤0,05 (*); 0,01(**), 0,001(***) were considered significant between the corresponding mutant and the WT. ns means non significant difference.
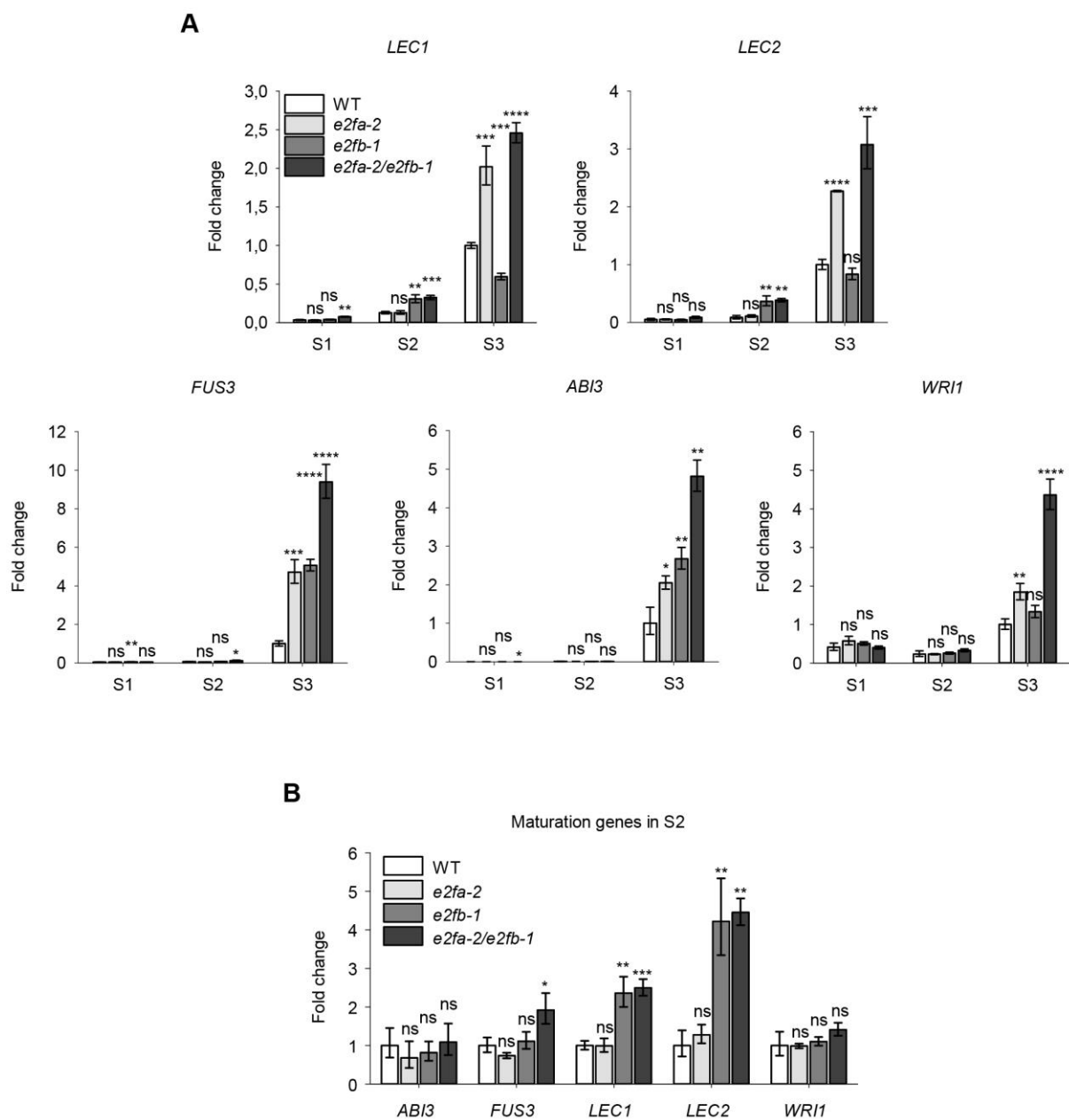
Figure 5. Activator E2Fs repress key maturation genes in developing siliques and seeds.
(A) Comparison of the LEC1, LEC2, FUS3, ABI3, and WRI1 transcript levels in developing
siliques of the *e2fa-2*, *e2fb-1*, and *e2fa-2/e2fb-1* single and double mutants, respectively with
the control (WT) at three silique developmental stages from S1 to S3 (pictured in Suppl. Fig.
S1). Values represent fold changes normalised to the value of the WT at the S3 silique stage
(set arbitrarily at 1). **(B)** The expression levels of maturation genes in the transition S2 phase in
the *e2f* mutants and the control WT; values represent fold change normalised to the value of the

relevant gene from WT at S2 (set arbitrarily at 1). Data are combined from n = 3 biological repeats, and bars represent means with standard deviations. P≤0,05 (*), 0,01 (**), 0,001 (***), 0,0001 (****) were considered significant between the corresponding mutant and the WT, at a given silique stage. ns means non-significant difference. Abbreviations of the genes and the primer sequences are listed in Supplemental Table S1.
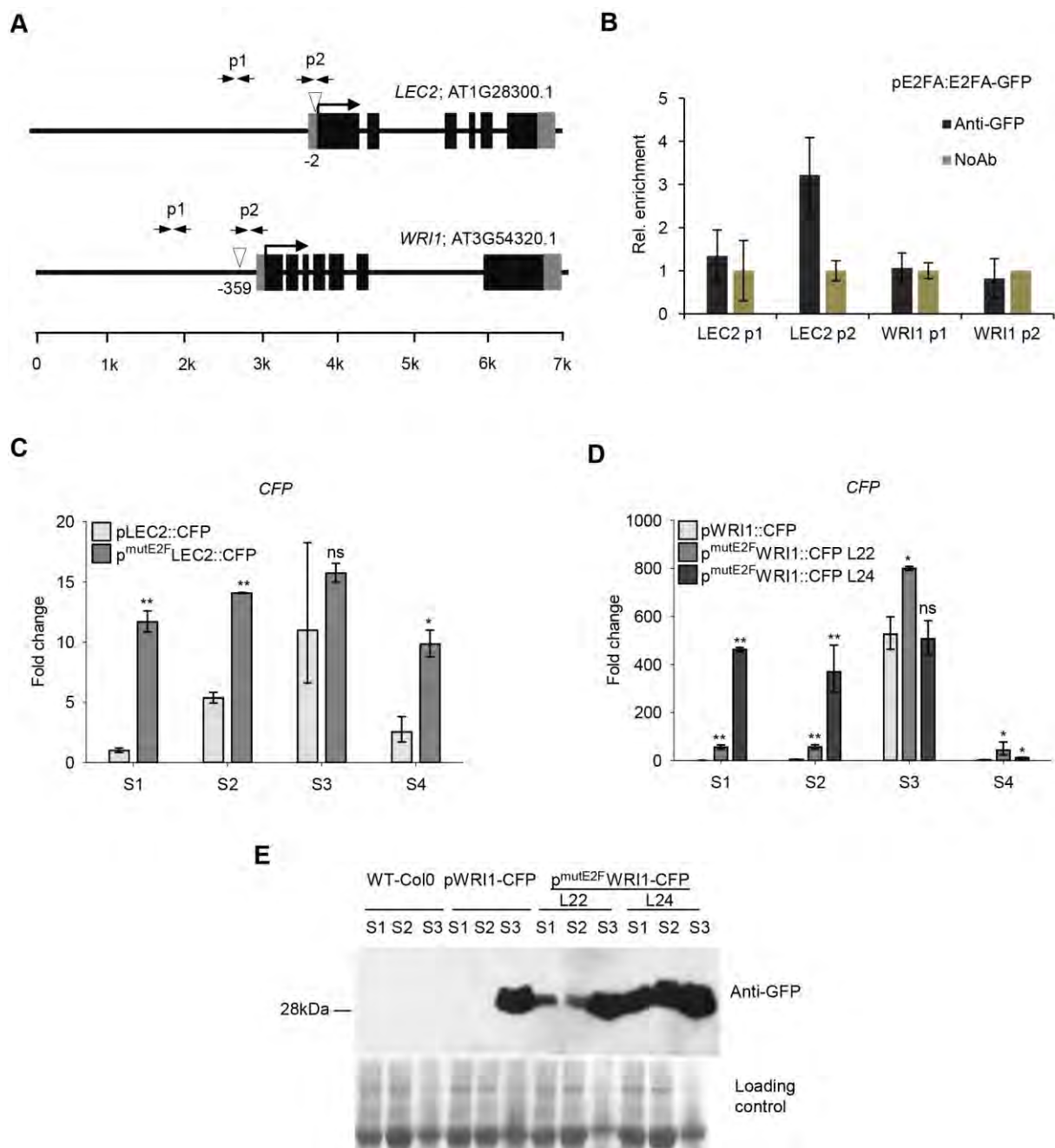
Figure 6. E2Fs could regulate the temporal control of LEC2 and WRI1 genes during silique development. (**A**) Schematic representation of the LEC2 and WRI1 promoters; arrows labelled by p1, p2 indicate the position of the primer pairs used for qPCR analysis. The position of the canonical E2F elements (white arrowheads) and their distance from the start codon (ATG) are depicted. (**B**) Chromatin immunoprecipitation (ChIP) followed by qRT-PCR was carried out on chromatin isolated from developing green siliques (6-10 days after pollination -

DAP) of pgE2FA-GFP transgenic line using polyclonal anti-rabbit GFP antibody. The graph shows the results of a representative experiment with three biological replicates. Nonparametric MannWhitney U test was used for statistical analysis between values of Ab and NoAb samples (* P < 0,05). The labels p1, p2 on x axis refer to the regions indicated in (**A**). (**C-D**) The expression levels of reporter LEC2 (**C**) and WRI1 (**D**) constructs either under the control of the intact (pLEC2::CFP, pWRI1::CFP, respectively) or the E2F-binding-site-mutant promoter version (p^{mutE2F}LEC2::CFP, p^{mutE2F}WRI::CFP, respectively) were determined by qRT-PCR in the developing siliques from S1 to S4 (pictured in Fig. S S1). L22 and L24 represent two independent E2F-binding-site-mutant promoter lines (**D**). Values represent fold changes normalised to the value of the intact promoter construct at the S1 silique stage (set arbitrarily at 1). Data are combined from n = 3 biological repeats, and bars represent means with standard deviations. P≤0,05 (*), 0,01 (**) were considered significant between the corresponding mutant and the intact promoter construct, at a given silique stage. ns means non-significant difference. (**E**) By using anti-GFP antibody in immunoblot assay CFP protein level was followed in developing siliques from S1 to S3 of the same transgenic lines shown in **D** as indicated. Molecular weight of the CFP protein (28kDa) is indicated on the left side. The coomassie stained proteins were used as loading control. Abbreviations of the genes and the primer sequences are listed in Supplemental Table S1.
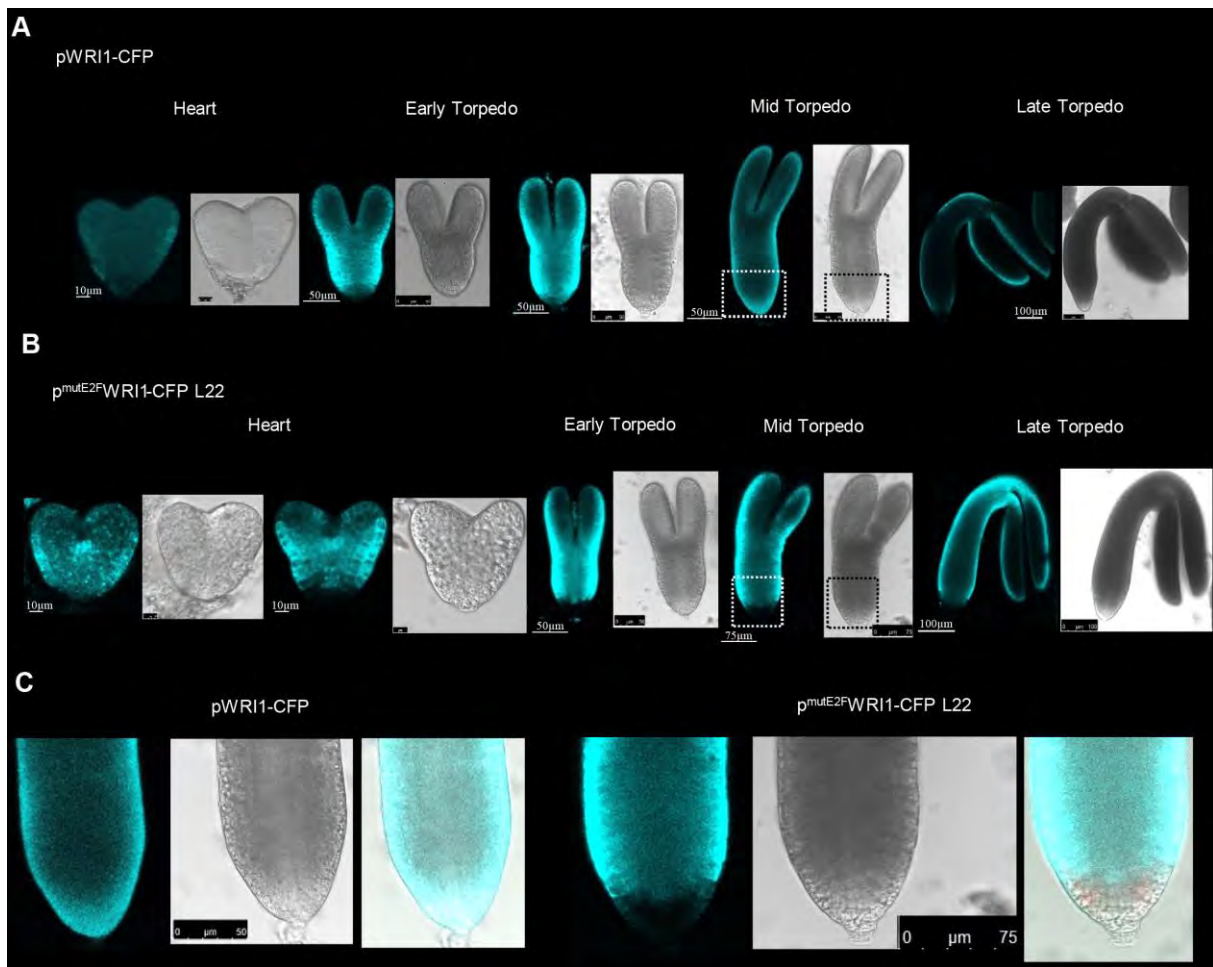
Figure 7. The temporal and spatial expression of WRI1 in the developing embryo depend on its regulatory E2F-binding site. (**A-B**) Representative CM images of developing embryos from the intact (**A**) and E2F-binding site mutant (**B**) WRI1 promoter (pWRI1::CFP and p^mutE2FWRI1::CFP reporter lines - upper and lower embryo images, respectively) dissected from immature seeds. (**C**) The hypocotyl-root tip regions of mid-torpedo stage embryos outlined with open white boxes in (**A**) are enlarged in (**B**). CFP signal (blue), bright field, and the merged images are shown. Scale bars are included at the bottom side of embryo images (μm).
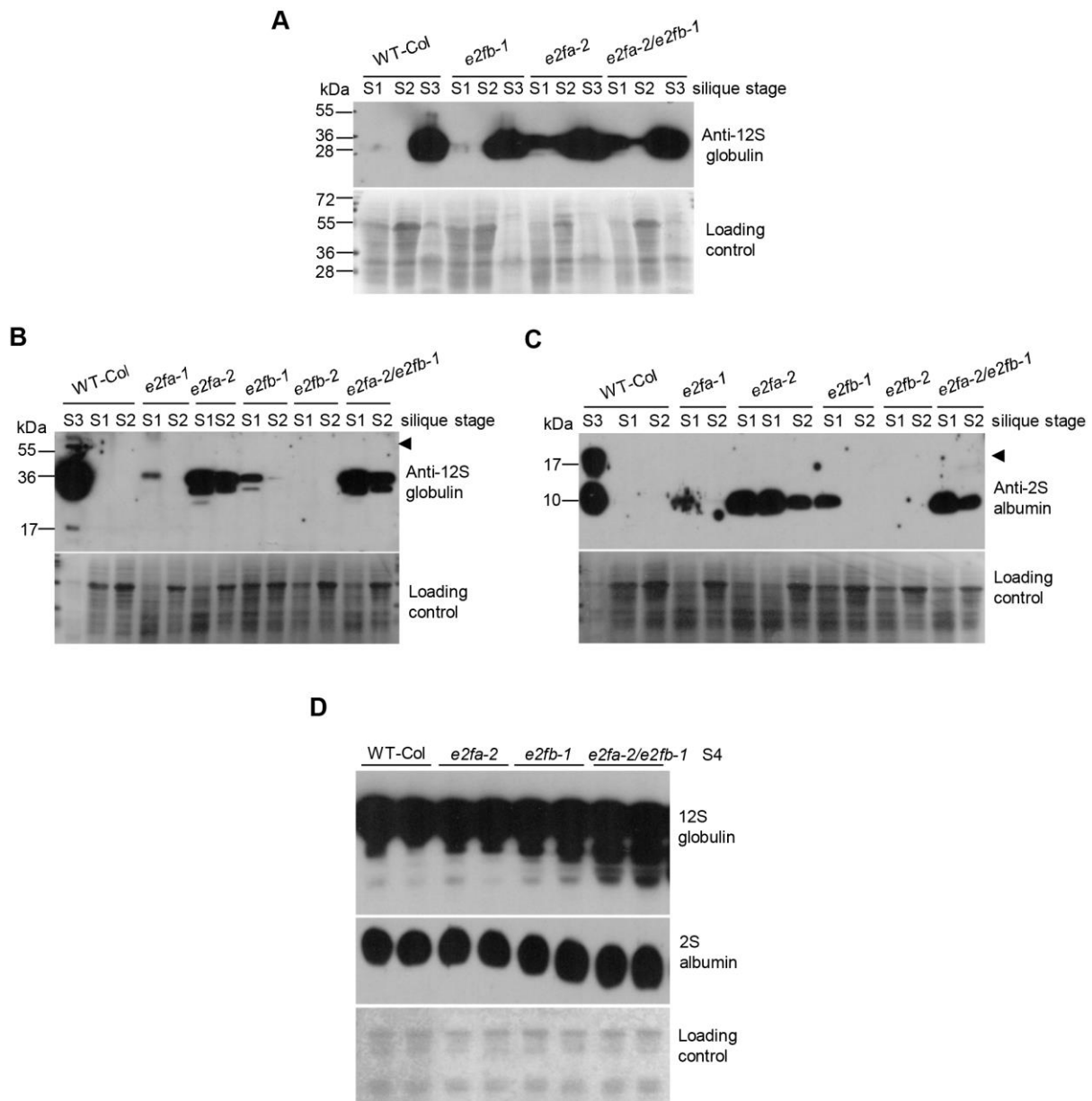
Figure 8. Seed storage proteins 2S albumin and 12S globulin show premature accumulation in the *e2f* mutant siliques and seeds. (**A**) Accumulation levels of 12S globulin in the single *e2fa-2*, *e2fb-1* and in the double *e2fa-2/e2fb-1* mutants at three silique and seed developmental stages (S1-3) were compared to the control WT in immunoblot assay by using specific antibody. (**B-C**) Seed storage proteins were detected in the early developing siliques and seeds of *e2fa-1*, *e2fa-2*, *e2fb-1* and *e2fb-2* single, as well as *e2fa-2/e2fb-1* double mutants by using anti-12S (**B**) or anti-2S (**C**) antibodies in immunoblot assays. The WT maturation-phase silique sample (S3) was used as positive control, and 1/4th of the S1-2 samples were

analysed (5μg). Arrowhead indicates precursors of 12S or 2S proteins. (**D**) The amount of globulin (12S) and albumin (2S) in post-mature seeds (silique stage 4 - S4) of single and double activator *e2f* mutants as well as the WT were compared by using specific anti-12S and anti-2S antibodies in immunoblot assays. Coomassie-stained proteins were used as loading controls.
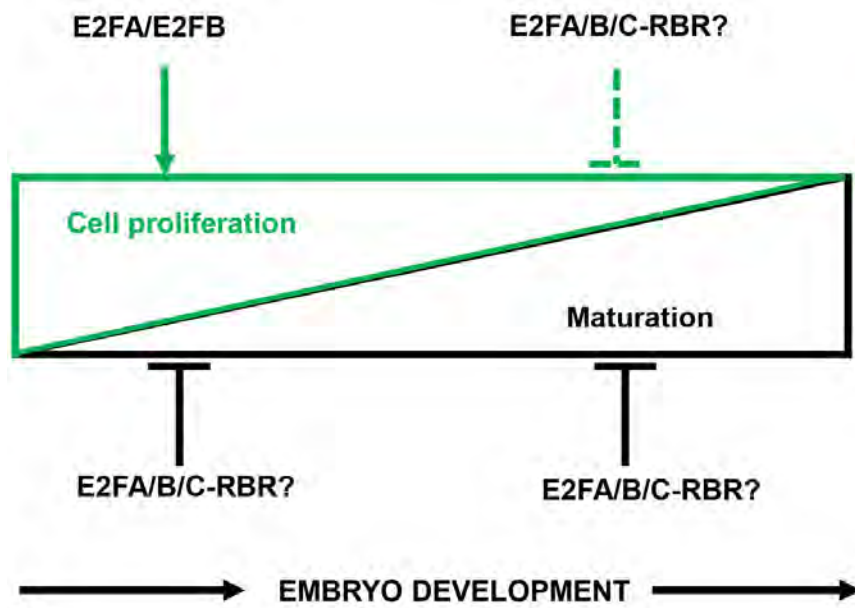
Figure 9. Model explaining the functions of activator E2Fs during seed and embryo development. The proliferative morphogenic and the differentiation-related maturation phases are the two major and oppositely regulated phases of seed and embryo development (green and black triangle). Activator E2Fs are required for the full activation of cell cycle genes in the morphogenic developmental phase, while 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. Maturation program is inhibited in the proliferative phase by activator E2Fs through either repressing the expression of maturation genes like LEC2 or inhibiting the accumulation of seed storage proteins, 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.

Tables

Table 1. Double *e2fa-2/e2fb-1* mutant plant produces fewer but bigger and heavier seeds than the control WT.

Genotype	Seed weight*	Total seed weight (mg)**	Silique length (cm)***	Silique no.****	Seeds per silique	Seeds size (mm²)
Wild type	2,2 ± 0,1	370 ± 60	1,6 ± 0,1	47 ± 2,5	57 ± 5,7	0,182 ± 0,015
e2fa-2	2,17 ± 0,05	332 ±59	1,5 ± 0,1	48 ± 4,2	53 ± 3,9	0,187 ± 0,015
e2fb-1	2,36 ± 0,11	383 ± 61	1,7 ± 0,1	51 ± 4,1^{A}	56 ± 8,9	0,205 ±0,017^{C}
e2fa-2/e2fb-1	3,0 ± 0,11^{B}	267 ± 48^{B}	1,1 ± 0,2^{C}	56 ± 4,4^{C}	22 ± 3,1^{D}	0,221 ± 0,017^{D}

Plants were grown under identical conditions. Repetition in different period of the year gave similar results. Means±SD are shown.

* Weight of seeds is given in mg per 100 seeds (n=10/line). ** Total seed weight was measured by weighting the harvested seeds (n=10/line) and is given in mg. Silique length (***) and number (****) was determined on the primary inflorescence (n=10/line; N=50-70). P≤0,05 (^{A}), 0,01 (^{B}), 0,001 (^{C}), 0,0001 (^{D}) were considered significant between the corresponding mutant and the WT. Non-labelled values considered non-significant differences.

Table 2. *e2fa-2/e2fb-1* double mutant seeds have higher protein levels than do wild-type seeds.

Genotype	µg of protein/seed*	Ratio[†]
Wild type	2,2 ± 0,1	370 ± 60
e2fa-2	2,17 ± 0,05	332 ± 59
e2fb-1	2,36 ± 0,11	383 ± 61
e2fa-2/e2fb-1	3,0 ± 0,11[C]	267 ± 48

*Means SD are shown.
†Ratio of values from *e2f* mutants to wild type. P≤0,001 ([C]) was considered significant between the *e2fa-2/e2fb-1* mutant and the WT.

Table S1. List of primers and their sequences used for mutagenesis, and qRT-PCR, and in ChIP assay. Italic letters represent the E2F-binding site in the primer pairs used for mutagenesis of LEC2 and WRI1 promoters. The nucleotid position of primer pairs from distance of the ATG start codon of the open reading frame (ORF) of E2FA and E2FB as well as the size of the PCR products are indicated.

Gene name	Forward primer	Reverse primer
Cloning and mutagenesis		
WRI1 (AT3G54320)	CTCTGAAACGAATATATGATACTA	TAAACTCTGAGAAAGTTTAGATTT
LEC2 (AT1G28300)	TGATTTAAACTTTTCGCTTGGGCA	TTTTCCCGGAGAGAGAGAGAGAGA
pWRI1-E2F-MUT	CATTCCAACT*TTTTCCAAC*AAAAAATTAGAG	TCTAATTTTTTG*TTGG*AAAAGTTGGAATG
pLEC2-E2F-MUT	CTCTCTCTCTCTCTCTC*TTGGAAAAG*CAAGTTTGTAC	TACAAACTTGC*TTTTCC*AAGAGAGAGAGAGAGAG
qRT-PCR		
ORC2 (AT2G37560)	TCCCGAATCACAACAAACTC	CCACAATAATGGAGGTTGA
MCM3 (AT5G46280)	TGGGCAGCACATGAGGAC	CACTTTGTTATCATCTTGCAGTTT
CYCD3;1 (AT4G34160)	GCAGCATAAGTTCAAGTGTGTAGC	AAACCGTAAGAGGCAGCTCTGG
CDKB1;1 (AT3G54180)	TCTGTTGGTTGTATCTTTGCTGA	CATTGCTGCTCAGTTGGTGT
RBR (AT3G12280)	CGCTTCCATTTTGGTTTTGA	TGAACAACAGCAGCAGCAAC
E2FA (AT2G36010)	CAACCCAGAAACTGCTATTGTT	GTCCGACTCATCATTTTCAAC
E2FC (AT1G47870)	TGCCGTTATGACAGTTCTTTAGGG	AGTGTTCCATCCTCAGCTTCCT
E2FB (AT5G22220)	GGACCGAGCGACAACAAA	AGGTGATCTCGTAGCAGTGGA
WRI1 (AT3G54320)	AAGTACTTGTACCTCGGCACCT	CAATCGCAGCCATGTCATA
LEC2 (AT1G28300)	GGTCCAATAACAAGAGCAGAATG	CAGCTCCATTTTGCTTCACA
LEC1 (AT1G21970)	GTTATGGTATGTTGGACCAATCC	TTCATCTTGACCCGACGAC
FUS3 (AT3G26790)	TGATACTCCCGAAGAAAGCC	CTATACTTGAAGGTCCAAACGTG
ABI3 (AT3G24650)	GGCAGGGATGGAAACCAGAAAGA	GGCAAAACGATCCTTCCGAGGTTA
UBC18 (AT5G42990)	ACAGCAATGGACATATTTGTTTAGA	TGATGCAGACTGAACTCACTGTC
CFP	TGAAGTTCATCTGCACCA	CTTGTACAAGCTTCGTC
Chromatin Immunoprecipitation (ChIP)		
WRI1prom1(-1036)	ACTCGTGTGTCTGCTTAAATCA	AGCACGTGTCAATCCGAAAC
WRIprom2(-243)	GACAGCGTGGAGAGTAAAGC	GGAGGAAAGGGCTAATTGGG
LEC2prom1(-1342)	TCATGGTTAGAAATTTGGTGACAGT	TCGAAATCATAACCCATAGAACACT
LEC2prom2(-110)	CATCTGCAACATTTTGACTCGTT	CAGAGTTTGCGTTAGAAGAGGG

Table S2. Primers and their sequences used for the RT-PCR analysis. The size of the PCR products are indicated (bp means base pair). The left or right position of the primers refer to their location according to the T-DNA insertion.

Gene name	Forward primer	Reverse primer
RT-PCR		
E2FA Start-*e2fa-1* left (816bp)	ATGTCCGGTGTCGTACGATCT	TCCTCTGTTTGTCTGATTTGGTTG
E2FA Start-*e2fa-2* left (980bp)	ATGTCCGGTGTCGTACGATCT	GGTCAGCCGCTTCATCTGGA
E2FB Start-*e2fb-1* left (702bp)	ATGTCTGAAGAAGTACCTCAA	CTTTCTTGTGATTCTCTGATTTGGT
E2FB Start-*e2fb-2* left (645bp)	ATGTCTGAAGAAGTACCTCAA	TGTACTTCATCCTGTAGGTTAGC
E2FA Start-*e2fa-1* right (938bp)	ATGTCCGGTGTCGTACGATCT	CATGAGGAGCTTTGACGGCT
E2FA Start-*e2fa-2* right (1090bp)	ATGTCCGGTGTCGTACGATCT	TGCACCACTCCCATTTGTGT
E2FB Start-*e2fb-1* right (970bp)	ATGTCTGAAGAAGTACCTCAA	TTCATCAGCCTGAGGAATGTC
E2FB N-term-*e2fb-2* right (518bp)	TCTGCGAAGTCTAATAAGTCTGGA	GTGCCTTTACAGCTATCAGCG

Silique stage1: 2-3 DAP
Silique stage2: 4-7 DAP
Silique stage3: 8-12 DAP
Silique stage4: 13-17 DAP
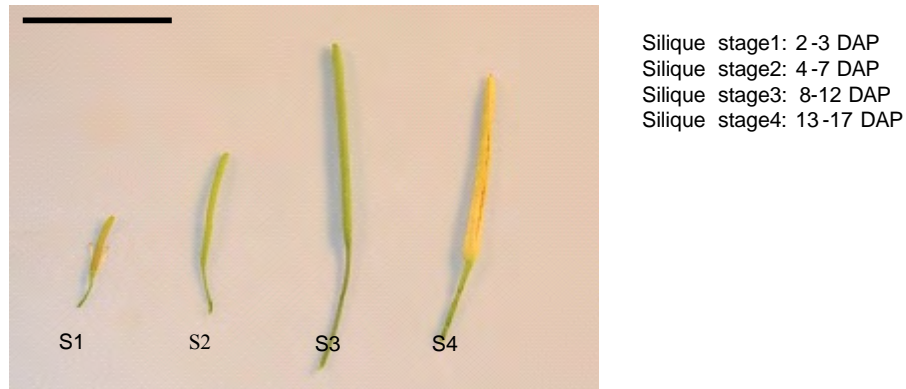
Figure S1. Silique samples were collected with different sizes representing distinct seed developmental stages. Representative pictures of the developing silique samples in four sizes; the few mm long S1, the 0,4-0,6 cm long S2, the full size siliques with green seeds (S3) and the yellow silique containing brown dry seeds referred as S4. Scale bar is 1 cm. Timing of seed development as days after pollination (DAP) is indicated on the right side.
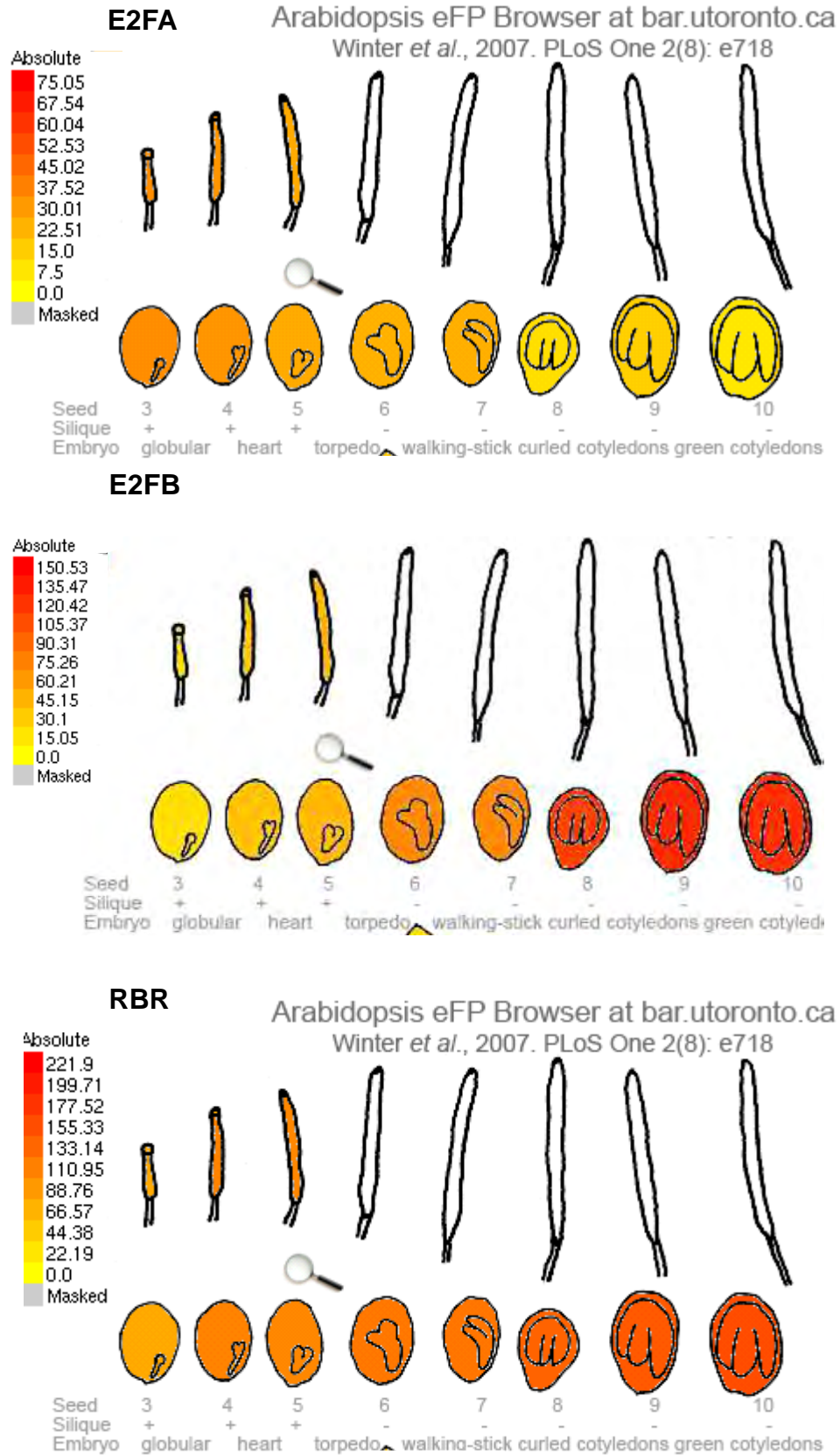
Figure S2. **Seed specific expression pattern for *E2FA* (At2g36010), *E2FB* (At5g22220) and *RBR* (At3g12280) using the Arabidopsis eFP-browser (Winter et al., 2007).** Values indicated correspond to Affymetrix signal values.

A

B
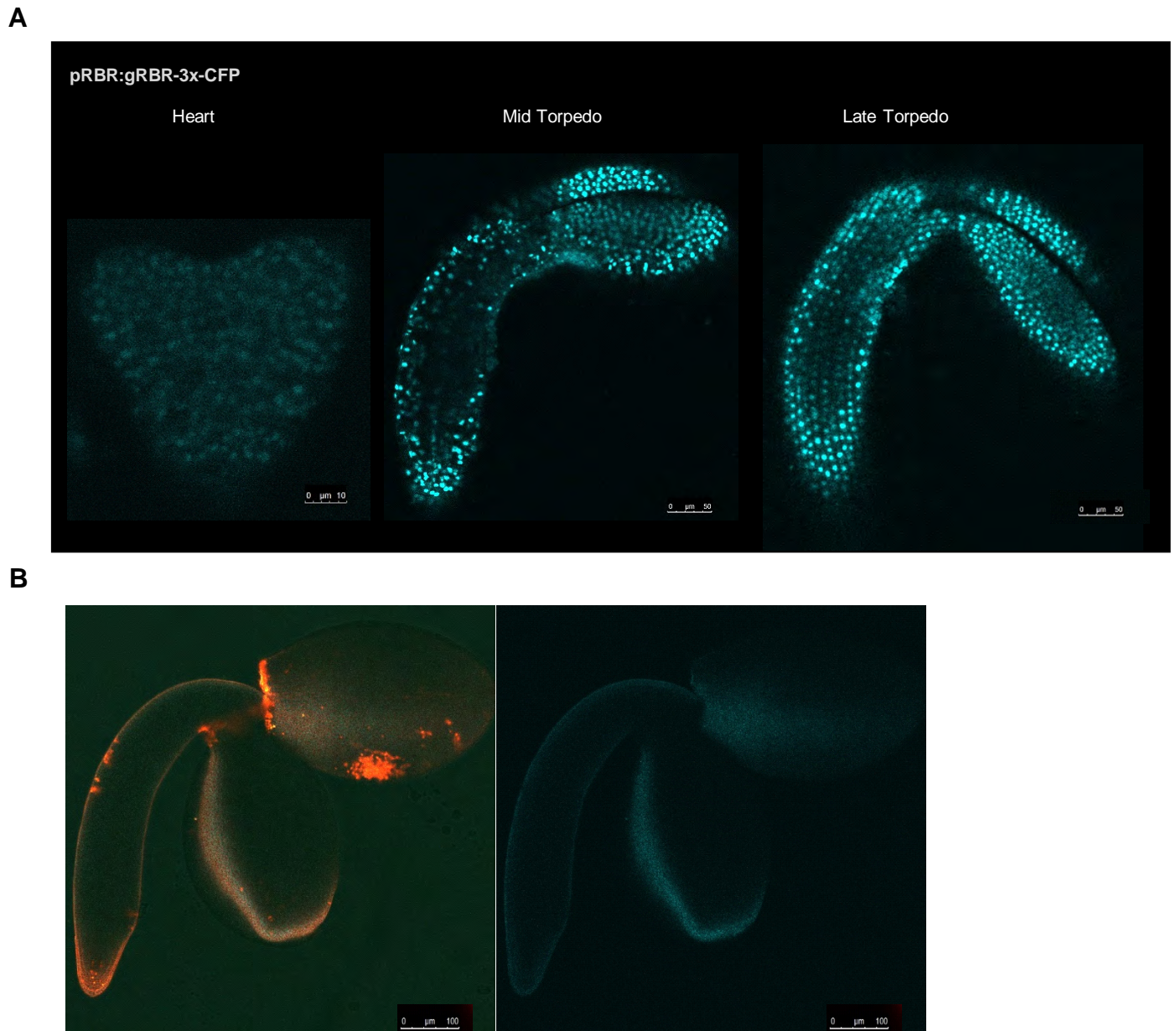
Figure S3. Accumulation of RBR protein during embryo development.

(**A**) Representative CM images of heart, late torpedo, and walking stick stage embryos of pgRBR-3xCFP line. (**B**) Representative CM image of a full-size embryo of the RBR-CFP expressing line. Red signal is propidium iodide, blue signal is CFP. Scale bars are included at the bottom side of embryo images (µm).
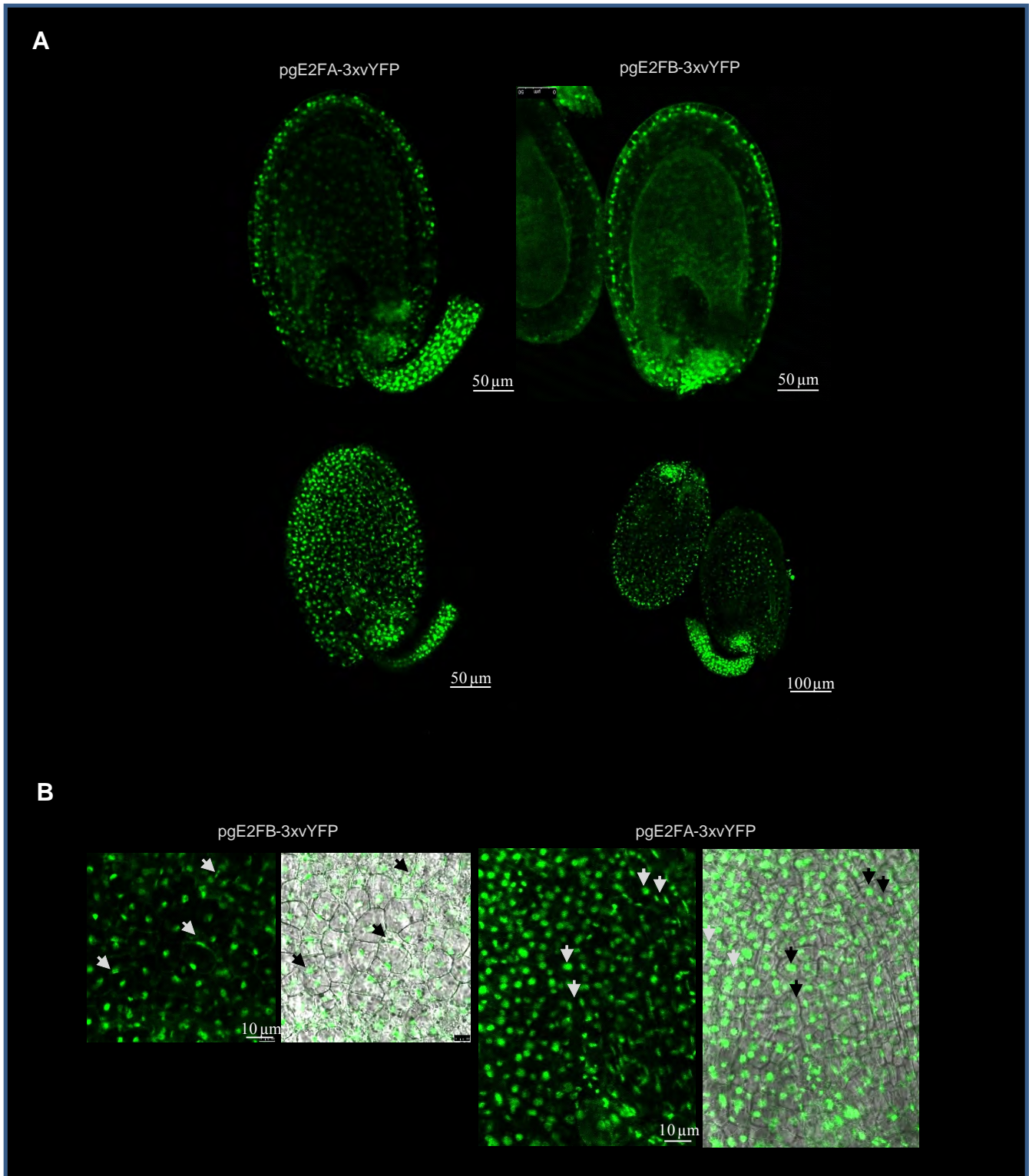
Figure S4. **Activator E2FA and E2FB proteins accumulate in the nucleus of the integument's epidermal cells.** (**A**) Representative CM images of the E2FA-GFP and E2FB-GFP expressing transgenic seeds during the morphogenic developmental phase. (**B**) Proliferating epidermal cells in the integuments of E2FA-GFP and E2FB-GFP seeds are shown by arrows. GFP signal is green. Merged GFP and bright field images are shown. Scale bars are included at the bottom side of seed images.
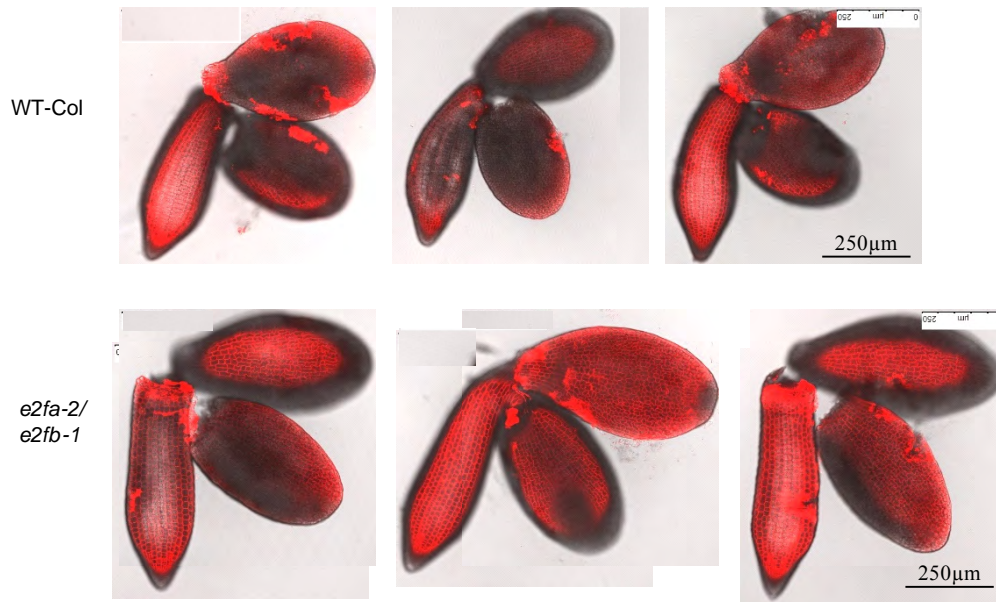
Figure S5. **The double *e2fa-2/e2fb-1* mutant embryo is larger than the control WT.** Representative CM images of propidium iodide (PI) stained embryos isolated from dry seeds of the double *e2fa-2/e2fb-1* mutants and the control WT. Merged PI-stained and bright field images. Scale bar is 250 µm.

Development: doi:10.1242/dev.179333: Supplementary information

WT-Col0

e2fa-2/e2fb-1

Figure S6. The double *e2fa-2/e2fb-1* mutant is partially sterile. Opened siliques of the WT Col control and double *e2fa-2/e2fb-1* mutant showing missing seeds in the later. Scale bar: 0,5 cm.
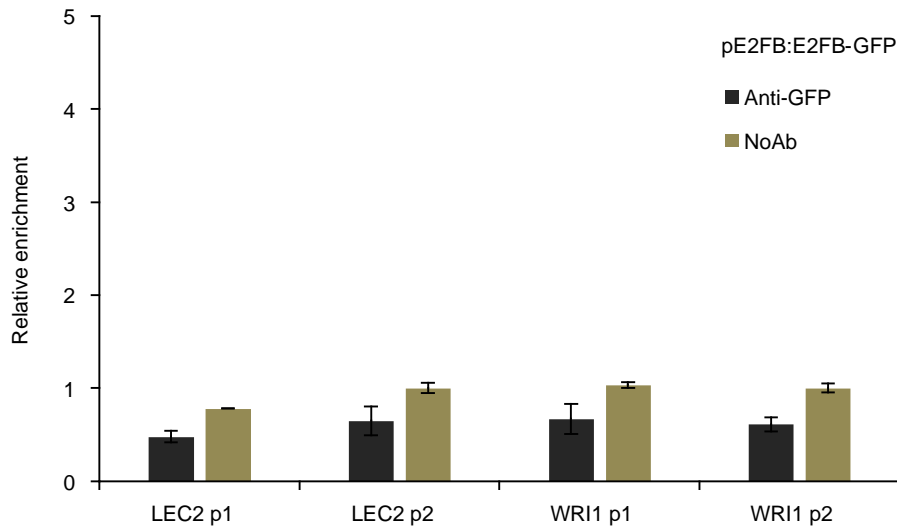
Figure S7. E2FB do not bind to the promoter region of LEC2 and WRI1 gene in maturing siliques. Chromatin immunoprecipitation (ChIP) followed by qRT-PCR using primer pairs specific for LEC2 and WRI1 promoters was carried out on chromatin isolated from developing green siliques (6-10 days after pollination - DAP) of pgE2FB-GFP transgenic line using polyclonal anti-rabbit GFP antibody. The graph shows the results of a representative experiment with three biological replicates. Nonparametric MannWhitney U test was used for statistical analysis between values of Ab and NoAb samples (* P < 0,05). The labels p1, p2 on x axis refer to the regions in the promoter region of LEC2 and WRI1 as indicated in Figure 6A.
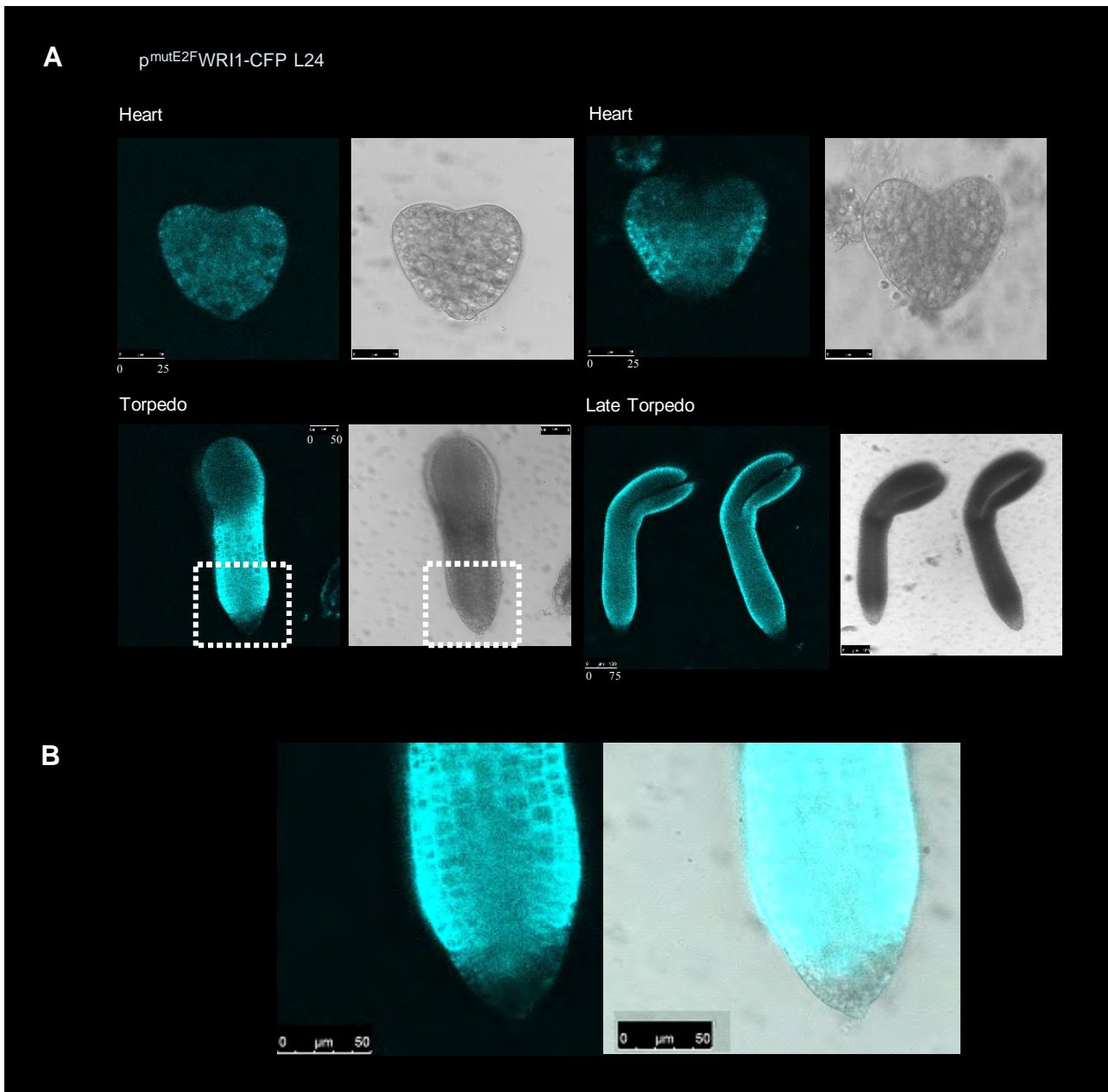
Figure S8. E2F-binding site mutant WRI1 promoter shows premature activity during embryo development. (A) Representative CM images of developing embryos from an independent E2F-binding site mutant WRI1 promoter reporter line (p^{mutE2F}WRI1::CFP L24) dissected from immature seeds. Strong CFP signal has already been detected in the heart stage embryos just like in the other E2F-binding site mutant WRI1 reporter line (p^{mutE2F}WRI1::CFP L22 - Figure 7). **(B)** The root tip regions of torpedo stage embryo outlined with open white boxes in (**A**) are enlarged in (**B**). CFP signal (blue), and a merged image with bright field are shown. Scale bars are included at the bottom side of embryo images (µm).
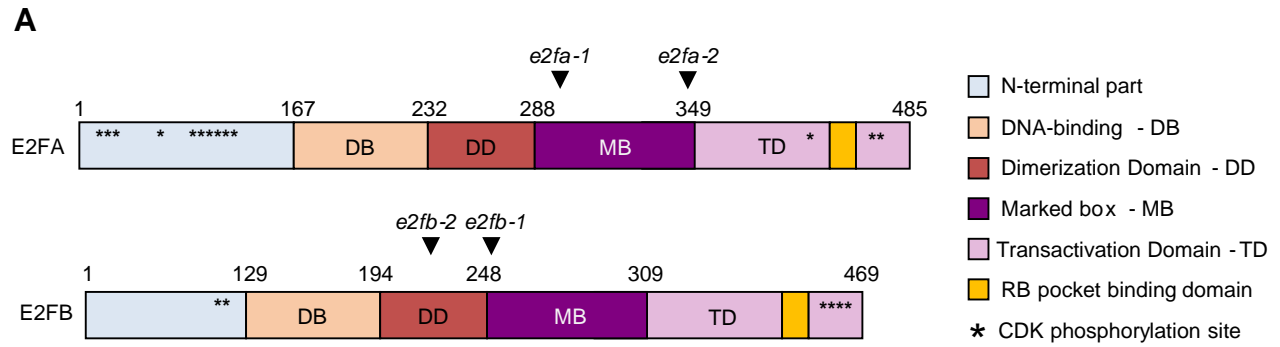
A

B

E2FA-Insertion
specific (e2fa-2)

C

E2FA-Insertion
specific (e2fa-1)

D

E2FB-Insertion
specific (e2fb-1)

E

E2FB-Insertion
specific (e2fb-2)

F

E2FA-Nterm
specific

G

E2FB-Nterm
specific
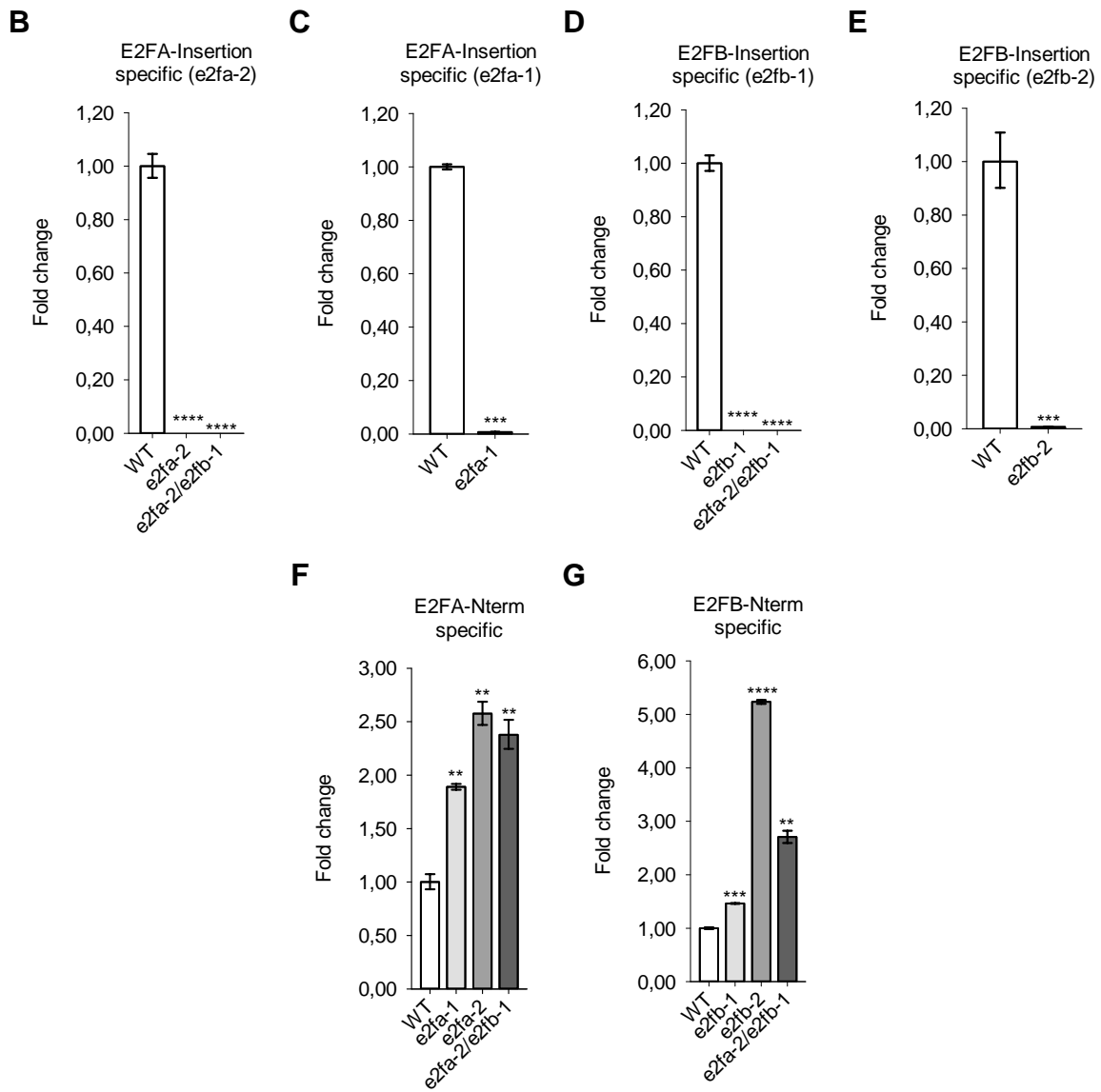
Figure S9. T-DNA insertion mutants for E2FA and E2FB do not express the full transcripts. (**A**) Structural organization of E2FA and E2FB proteins. Colours represent different domains as indicated. Arrowheads point to the position of the different T-DNA insertions. (**B-G**) Transcript levels of E2FA (**B, C, F**) and E2FB (**D, E, G**) in WT and T-DNA insertion mutants (*e2fa-2*, *e2fb-1*, and/or *e2fa-2/e2fb-1*). Levels of each transcript were determined by qRT-PCR using primer pairs specific for the insertion site of e2fa-2 (**B**) or *e2fa-1* (**C**), or *e2fb-1* (**D**), or *e2fb-2* (**E**), or specific for the N-terminal part of E2FA (**F**) or E2FB (**G**) preceding the insertion site. Values represent fold changes normalised to the value of the WT (set arbitrarily at 1). Data are combined from n = 3 biological repeats, and bars represent means with standard deviations. P≤0,01 (**), 0,001 (***), 0,0001 (****) were considered significant between the corresponding mutant and the WT. Abbreviations of the genes and the primer sequences are listed in Supplemental Table S1.
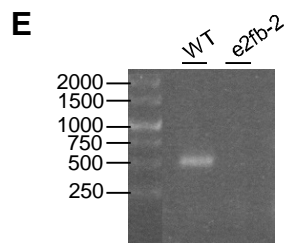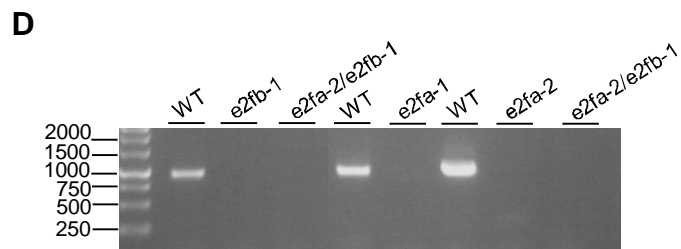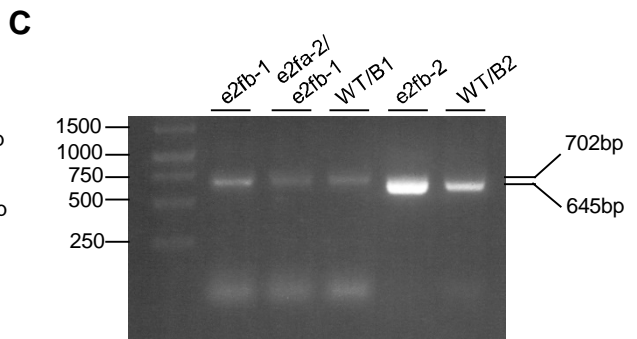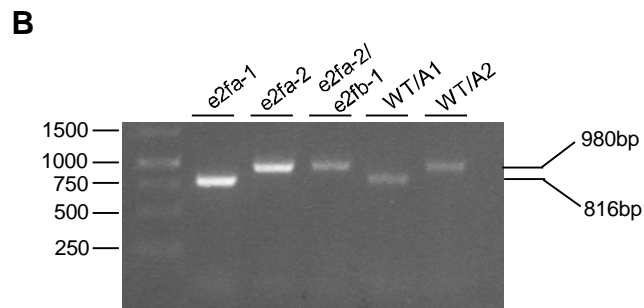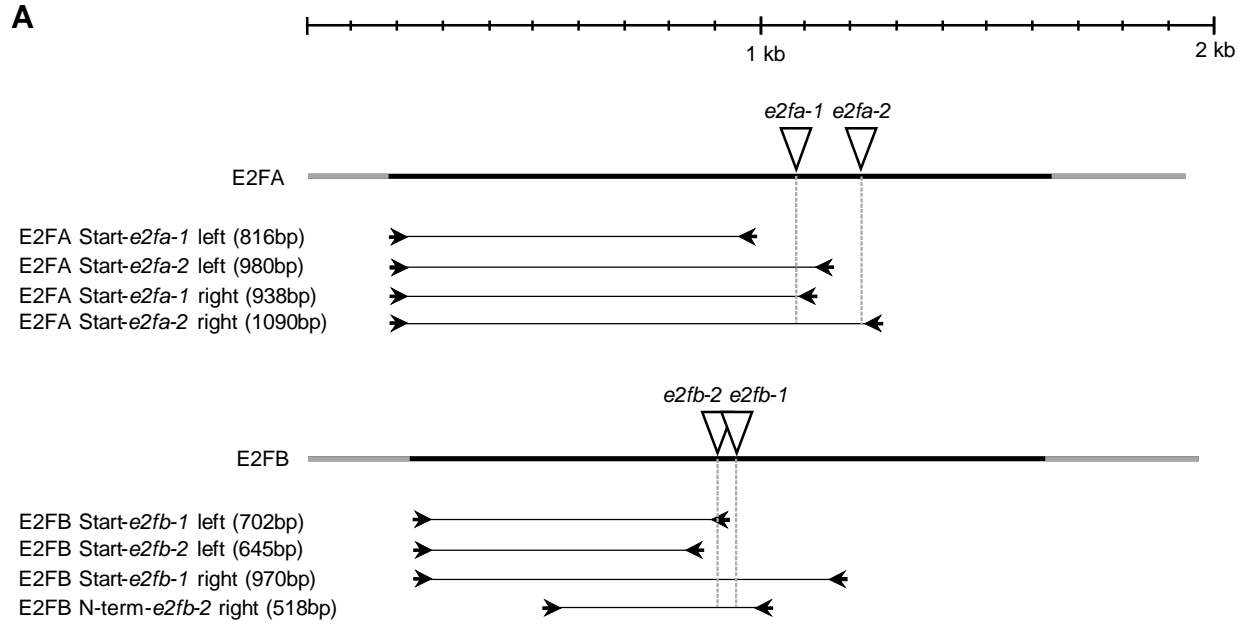
Figure S10. T-DNA insertion mutants for E2FA and E2FB express transcripts down to the insertion site. (A) Positions of the T-DNA insertions (white triangles) are shown on E2FA and E2FB mRNA, respectively. Gray and black sections indicate untranslated and translated regions. Arrows connected by black lines mark amplicons generated by specific primer pairs. Above a scale bar shows sizes in kilobases (kb). **(B-E)** cDNA prepared from WT and *e2fa* and *e2fb* single and *e2fa-2/e2fb-1* double mutant seedlings was subjected to RT-PCR analysis by using the specific primer pairs covering the entire regions in advance of the insertion (**B-C**), or beyond the insertion (**D-E**) and compared their size to the corresponding control WT products as indicated. (**B-C**) Transcripts of E2FA (**B**) and E2FB (**C**) could be transcribed in the single e2fa (*e2fa-1, e2fa-2*), e2fb (*e2fb-1 and e2fb-2*) and in the double *e2fa-2/e2fb-1* insertion mutants down to the insertion site in identical size with the control WT. (**D-E**) Transcripts were not synthesized beyond the insertion sites neither in the e2fb nor in the e2fa mutants when the reverse primers were designed next to the insertions. Primer sequences are shown in Supplemental Table S2.
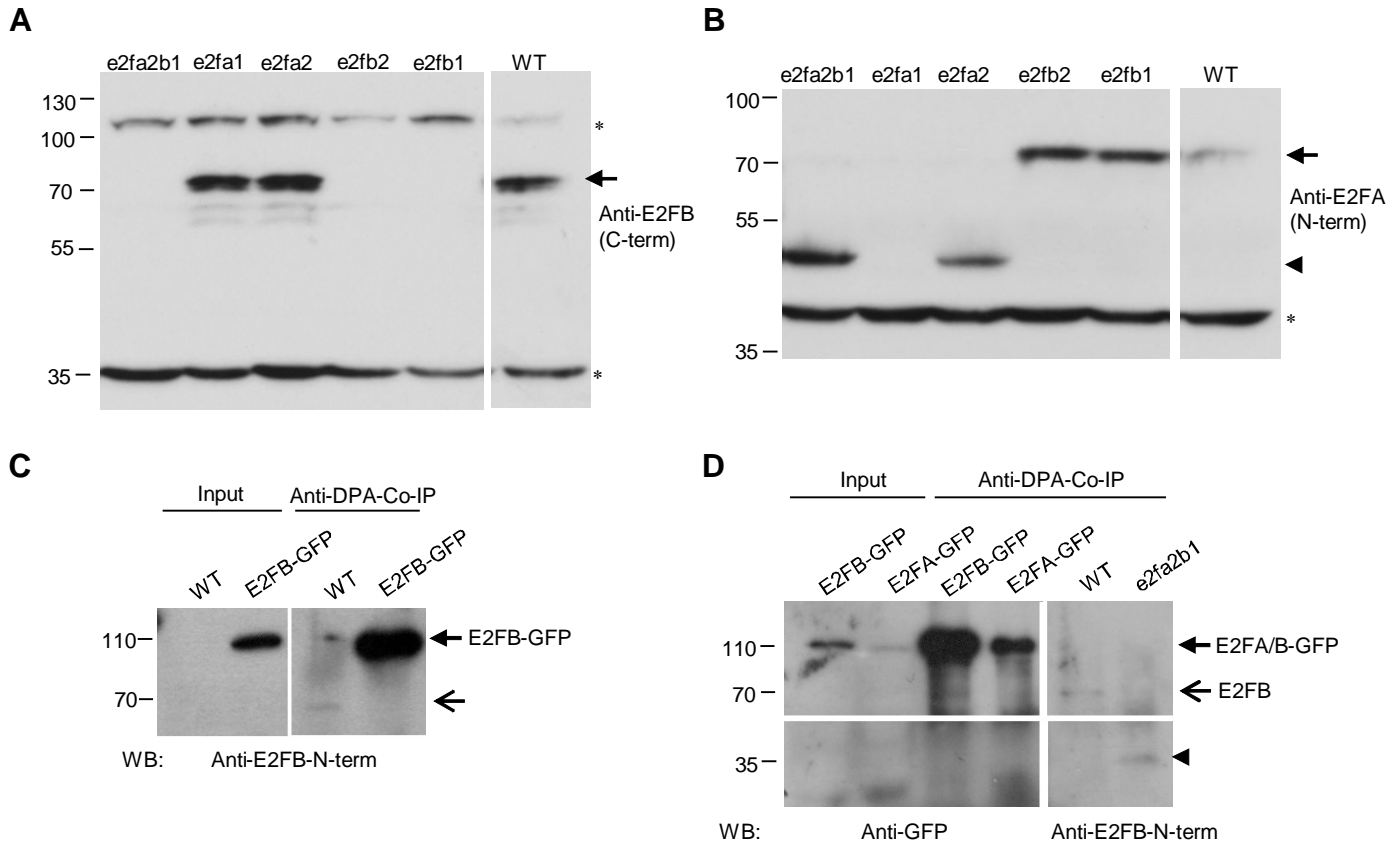
Figure S11. T-DNA insertion mutants for E2FA and E2FB synthesize proteins with smaller size than the full length E2F proteins. (**A-B**) Western blot analysis of seedlings of single and double e2fa and e2fb mutants in comparison to the WT control by using a C-terminal specific anti-E2FB (**A**), or an N-terminal specific anti-E2FA (**B**) antibody. Arrow indicates the full size E2FA or E2FB proteins, arrowhead shows a smaller size possible truncated E2FA protein (45-50kDa), while asteriks mark aspecific cross-reacting protein bands. (**C**) An N-terminal specific anti-E2FB antibody could recognize the full length endogenous E2FB in the WT control but only in the anti-DPA immunoprecipitated sample (open arrow). (**D**) By using the same antibody an E2FB protein with smaller size (about 35kDa) was recognized in the e2fa-2/e2fb-1 double mutant seedlings immunoprecipitated by anti-DPA antibody (arrowhead). As positive control anti-DPA antibody could readily immunoprecipitate the E2FA-GFP and the E2FB-GFP proteins. Arrow shows the GFP-tagged E2FA and E2FB, open arrow marks endogenous E2FB, and arrowhead shows the potentially truncated E2FB mutant form.