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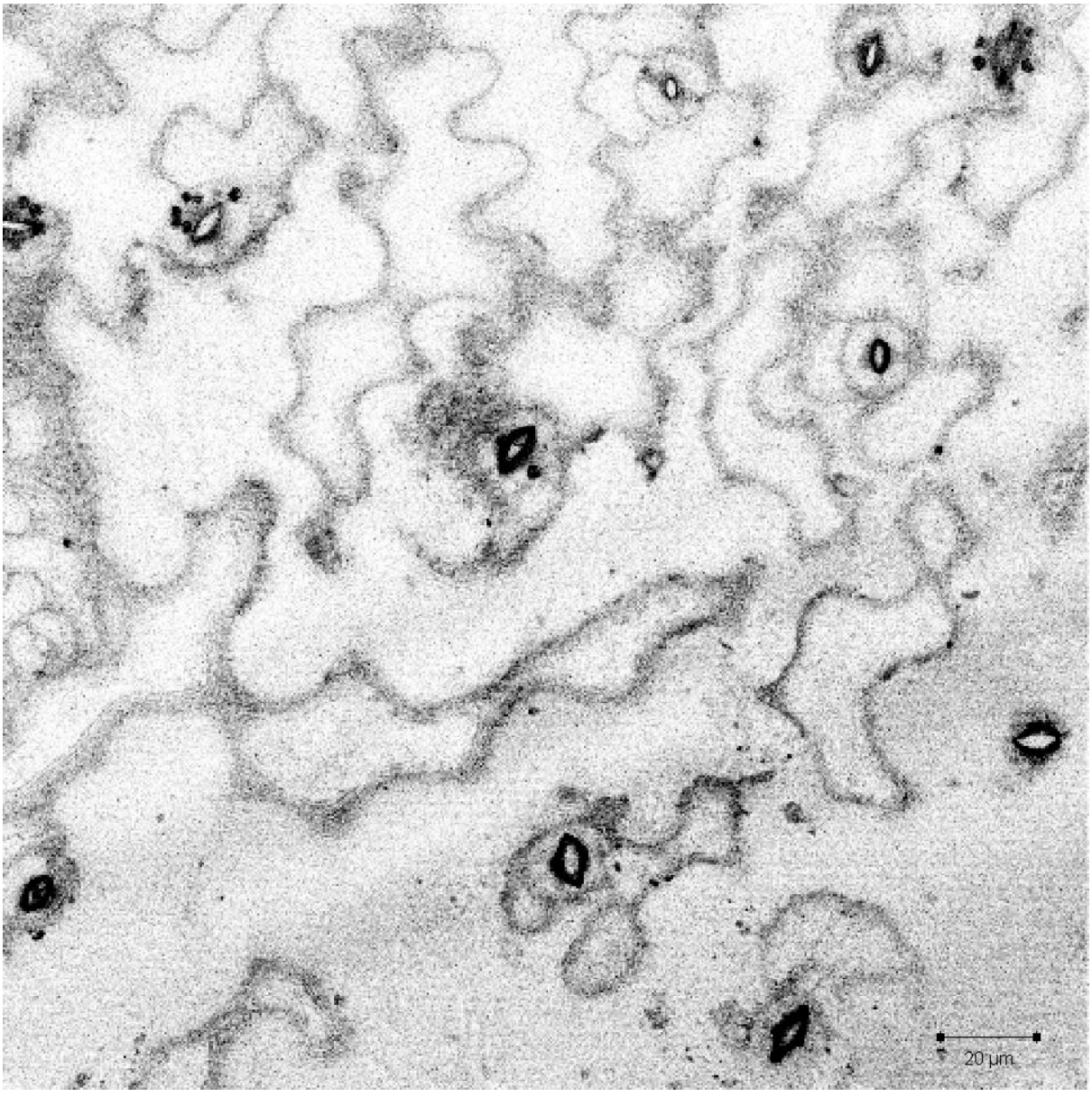

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The role of actin cross-linking protein Fimbrin1 in Arabidopsis seed dormancy

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Abstract

Seed dormancy can increase the storage time of seeds, but can also result in unequal germination. Therefore, understanding the underlying processes are of major interest. This report combines research on the actin cytoskeleton with seed physiology, which has not been done before. Gene expression data indicated high levels of the actin filament bundling protein fimbrin1 (FIM1) in maturating and dry seeds. Shortly after imbibition, the expression of fimbrin1 rapidly decreases and the expression of actin depolymerisation factor 6 (ADF6) increases at the same time. This led to the hypothesis that the bundling activity of FIM1 is an important factor for seed dormancy. Here, we performed germination assays with FIM1 mutant seeds and with Actin Interacting Protein 1 (AIP1) RNAi seeds of A*rabidopsis thaliana*. Moreover, we transformed *A. thaliana* plants with a fluorescent protein (GFP) fused to a Fimbrin Actin Binding Domain 2 (FABD2) under the control of an embryonic active promoter. The objective was to confirm that actin filament bundling plays a role in dormancy and germination. We found that seeds of the *fim1* mutant show a decreased dormancy index compared to the control (col-0) seeds. The germination assays done with AIP1 RNAi seeds indicated that the actin organization has influence on the germination characteristics, as well as that the balance of actin binding proteins is probably of major influence. Future studies can be done with the transgenic seeds we have produced. Especially microscopic observations in these seeds will increased our understanding of both actin dynamics as seed dormancy and germination.

Contents

Introduction

Seeds are the most used starting material for plant propagation, breeding and food production. They are very important for human food production and supply. The quality of seed is a significant factor in producing high quality end products. Dormancy is an important agronomical and ecological trait by preventing germination under temporally favourable conditions. In nature, dormancy increases the seeds fitness (Lee et al., 2010). In an agronomical view, dormancy can also be seen as a negative trait, in which it affects parameters such as total percentage, rate and uniformity of germination. Even a short delay of germination can have a big impact regarding competition for light and nutrients (Gilliland et al., 2003). By modifying dormancy mechanisms, the seed quality can be enhanced. However, for efficient modification, understanding of underlying mechanisms is required. It is known that phytohormones have a strong regulating effect on seed germination (Gilliland et al., 2003).

Phytohormones are organic compounds that regulate plant growth at very low concentrations (Zahir et al., 2001). Plant growth is often regulated by multiple hormones that have antagonistic effects (Ogawa, 2003). The ratio between hormone concentrations determine the developmental plan that is being followed. The major known phytohormones are auxins, gibberellins, ethylene, cytokinins, abscisic acid, strigolactones, brassinosteroids, jasmonic acid and salicylic acid. From these, abscisic acid (ABA) and gibberellic acid (GA) are of major importance in seed dormancy and germination and are known to have opposing effects (Gilliland et al., 2003; Bentsink et al., 2006).

Phytohormones influence seed germination

Completion of germination can be defined as the protrusion of the radicle through the endosperm and seed coat (Joosen et al., 2010). The onset of germination is the elongation of a small group of cells in the hypocotyl-radicle transition zone (Sliwinska et al., 2009). Phytohormone mutants can be used to study cellular organisation during dormancy or germination. GA stimulates germination and weakening of the barrier tissues (Finkelstein et al., 2008). The GA concentration increases after imbibition, just before radical protrusion (Ogawa, 2003). In addition, GA deficient mutants have been shown unable to germinate (Debeaujon and Koornneef, 2000). Lee, Piskurewicz et al. (2010) have shown that ABA is

actively produced in dormant seed coats during imbibition, and this represses the germination. The different concentrations of phytohormones result in differential regulation of gene expression. However, the precise downstream effects are less well known (Bentsink et al., 2006). The expression levels of several hundreds of genes have been found to be influenced by ABA. Promoter sequences can contain regions that regulate the promoter activity when a certain compound is bound. Two of such regions are the ABA responsive element (ABRE) and the GA-downregulated (GADOWNAT) motif (Huang et al., 2007). ABA responsive elements have been found to be significantly overrepresented in a set of 442 dormant related genes (Cadman et al., 2006), as well as in 24 Arabidopsis genes that are GA down regulated during germination (Ogawa, 2003; Bentsink et al., 2006). The ABRE and GADOWNAT regions have been found in the promoter regions of the actin (*ACT7*) and fimbrin (*FIM1*) genes (Ogawa, 2003). ABA and GA may, by this mechanism, influence the organisation of the actin cytoskeleton. Gene expression levels can be measured by using DNA micro arrays. Gene expression levels of *Arabidopsis thaliana* tissues have been measured and published (Winter et al., 2007; Bassel et al., 2008). In this data, we found a number of actin related proteins with changed expression levels during imbibition. This suggests that the actin organisation is changed during germination.

The role of actin during germination

Actin is present in all plant cells and occurs in a monomeric (globular or G-actin) and filamentous form (F-actin). The actin monomers can polymerize into filaments, which form the actin cytoskeleton. The *A. thaliana* actin gene family consists of 10 members (McDowell et al., 1996). The actin isoforms differ in their biochemical properties and expression levels. The actin7 gene (*ACT7*) is abundantly expressed at all seed and vegetative stages (Winter et al., 2007; Bassel et al., 2008). When the *ACT7* gene is mutated, the total amount of F-actin is decreased, causing delayed and less efficient germination. The actin organization has previously been studied in easily observable cells, such as root hairs, trichomes (Dong et al., 2001; Smith and Oppenheimer, 2005; Hussey et al., 2006) and pollen tubes (Wu et al., 2010). The actin cytoskeleton has not been studied in seeds. The actin cytoskeleton has a role in organelle trafficking and signal transduction (Allwood, 2002; Smith and Oppenheimer, 2005). F-actin is essential for cell elongation in higher plants, and only to a minor extend for cell morphogenesis (Baluska et al., 2001; Smith and Oppenheimer, 2005).

Cell membrane and cell wall matrix materials are transported by Golgi-derived vesicles to the location of cell growth. Filamentous actin has an critical role in guiding these vesicles to their destination (Hussey et al., 2006). Arabidopsis plants treated with an actin depolymerizing drug (such as latrunculin B) during germination have shown to be morphologically normal, but growth was stunted (Baluska et al., 2001). Since actin is involved in all these cases, we expect it has also an role during germination.

Regulation of actin

The actin cytoskeleton structure and dynamics are modified by several classes of Actin Binding Proteins (ABPs). Functions of ABPs can be actin filament bundling, nucleation, capping, fragmentation, transport and monomer sequestering (Hussey et al., 2006). Among others, the fimbrin protein family is involved in actin filament bundling. In relation to fimbrin, the term 'cross-linking' is more commonly used, although there is not an obvious physiological reason for this naming distinction. In Arabidopsis, the fimbrin family consist of five members (Wu et al., 2010). Fimbrin5 (FIM5) has been studied in Arabidopsis pollen. There, FIM5 was proposed to stabilize actin filaments and aid in the creation of higher order structures (Fig. 1 and 2)(Kovar et al., 2000; Wu et al., 2010). Actin stabilising is counteracted by actin severing proteins, such as ADFs. The expression of *FIM1* is high in dry seeds, but decreases within three hours after imbibition. At the same time, the expression of Actin Depolymerisation Factor 6 (*ADF6*) increases (Baxter et al., 2007; Bassel et al., 2008). ADFs sever actin filaments and increase the depolymerisation from the pointed end, this contributes to the actin dynamics (Hussey et al., 2006). We examined the effect of ADF inactivation on germination parameters indirectly by using alcohol inducible AIP1 RNAi seeds, because ADF mutant seeds were not available. AIP1 is needed as co-factor to activate ADF (Ketelaar et al., 2004). Because of the high expression levels of *FIM1* in dry seeds and the gene expression switch during imbibition from actin filament stabilising to severing proteins, we hypothesise that the cross-linking activity of FIM1 is an important factor for seed dormancy. Dense actin aggregates, caused *in vitro* by fimbrin, might be present in seeds too and function as some sort of actin storage mechanism needed for seed longevity. In this study, Arabidopsis seed germination assays are performed to study the effect of phytohormones, fimbrin1 mutation and decreased ADF activity via AIP1 RNAi on seed dormancy. In addition, plants are transformed with a GFP:FABD2 construct under an

embryonic promoter, to visualize the actin cytoskeleton in seeds of *fim1* and hormone mutant lines.

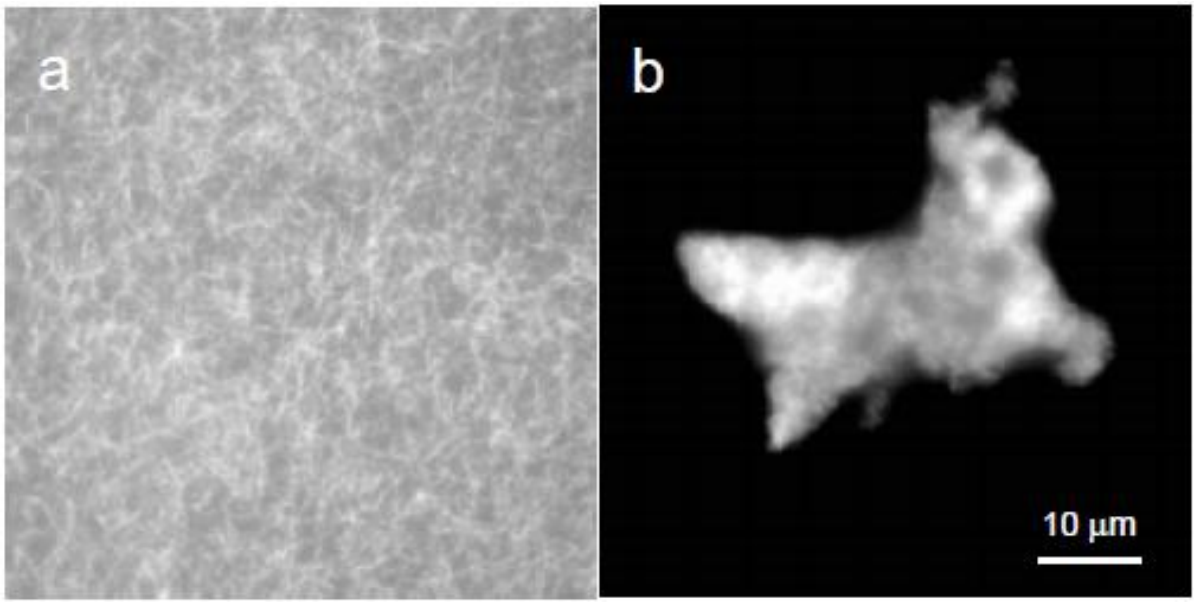


Fig. 1. Cross-linking of (pollen) F-actin by *AtFIM1* (rhodamine-phalloidin staining). Without (a) or with (b) *AtFIM1* (Kovar et al., 2000).

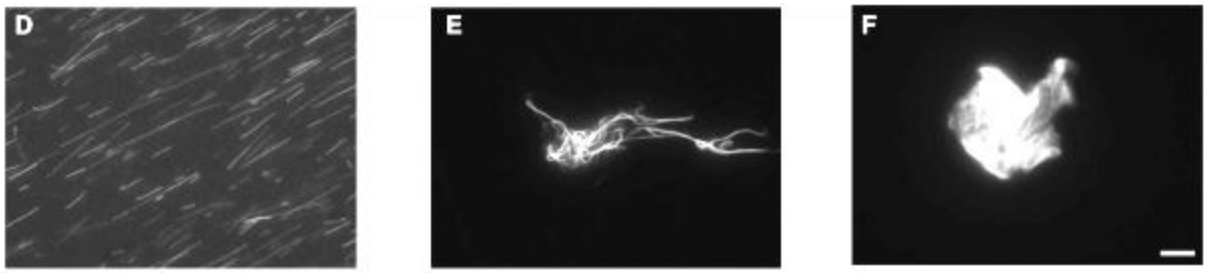


Fig. 2. Fluorescence microscopy of *in vitro* actin (D), actin + 1 µM *FIM5* (E) and actin + 1 µM *FIM1* (F). Bar = 5 µm (Wu et al., 2010).

Material & Methods

Production of an embryo active GFP-FABD2 construct and plant transformation

Plasmids containing the genes of interest were provided by the cell biology and molecular biology laboratories of Wageningen University. All promoter sequences were obtained by designing primers and performing the corresponding PCRs. See tables 1 and 2 for the PCR conditions. The PCR product was purified with NucleoSpin® Extract II (Bioke, http://www.bioke.com) according to the manufacturer's instructions. The promoters were chosen based on expression data of (Winter et al., 2007; Bassel et al., 2008). The data was sorted on the expression level in the embryo during seed development and germination. Two promoters were used: At5g54770 (pTHI) and At2g02760 (pUBC)(Table 3). A third promoter (pEMB At1g80070) was amplified but not used.

Preparation of FABD2 entry clones

The *FABD2* and *FIM1* sequences were available in expression clones (pMDC43 and pET28 respectively) and were transferred into an entry clone (pDonr221) by a BP reaction (Invitrogen, http://www.invitrogen.com). This procedure was later repeated with FABD2 obtained by a PCR (Tables 1-3, Fig. 3) and used for the final Multisite Gateway reaction.

Ligation of the promoter sequence into GW421 and GW388

Plasmid GW421 (containing GFP sequence) and plasmid 388 (containing mCherry sequence) were digested with 4 µl AscI in 4 µl fast digest buffer in 22 µl dH20 for 15 minutes at 37 °C. The plasmid ends were dephosphorylated to prevent self-ligation with Calf Intestine Alkaline Proteinase (CIAP, Fermentas, http://www.fermentas.com) for 45 minutes at 37 °C. CIAP was deactivated by incubating at 65 °C for 15 minutes. The plasmids were loaded on gel and the non-circular (open) slow migrating fraction was isolated from the gel using the gel extraction protocol from the NucleoSpin® Extract II kit (Bioke). The promoter sequences of pTHI and pUBC were ligated into the digested, dephosphorylated and purified plasmids GW421 and GW388 with 10x ligation buffer and T4 ligase (Promega, http://www.promega.com).

Multisite Gateway reaction

Two Eppendorf tubes with 10 fmol plasmid DNA per entry clone (pTHI:GFP or pUBC:GFP, FABD2 and terminator sequence) and 20 fmol of destination plasmid GW238. dH2O was added to a total volume of 8 µl. 5x LR-clonase PLUS enzyme mix (Invitrogen) was briefly vortexed and 2 µl was added to both tubes. After an 16 hour incubation at RT, 1 µl proteinase K (Fermentas) was added and samples were incubated at 37 °C for 10 minutes.

Transformation of *E. coli*

1 µl of plasmid recombination mixture was added to 50 µl electrocompetent DH5α cells. Cells were pipetted into a cold cuvette and a 2500V pulse was applied. Directly after electroporation, 700 µl cold LB medium was directly added in the cuvette. The suspension was transferred into an Eppendorf tube and incubated for 1 hour at 37 °C. Cells were plated in two bacteria concentrations on 1.5% agar LB medium with kanamycin (25 µg/ml). Low concentration: 50 µl of total suspension volume, high concentration: cells pelleted and resuspended in 50 µl LB. Plates were incubated upside down overnight at 37 °C.

Transformation of Agrobacterium C58C1

Overnight culture of *Agrobacterium tumefaciens* strain C58C1 was centrifuged at 3000 rpm for 5 minutes. Washed with 5 ml dH_2O and spun again and resuspended in 100 µl LB medium. 5 µl of the multisite gateway binary plasmid was added, mixed and incubated at room temperature for 5 minutes. Hereafter, it was put in liquid nitrogen for 5 minutes and then at 37 °C for 30 minutes. 700 µl LB was added and tubes were put at 180 rpm for 4 hours at 28 °C. The cells were plated with rifampicine (25 µg/ml), gentamicin (25 µg/ml) and spectinomycin (20 µg/ml). Plates were incubated for 3 days at 30 °C. One colony for both lines was grown for 3 days in 200 ml LB with the same antibiotic concentrations as before. These two Agrobacterium cultures were used for floral dipping.

Transformation of *A. Thaliana*

The *A. thaliana* plants were transformed by the floral dipping method (Clough and Bent, 1998). First, the Agrobacterium cultures (see previous paragraph) were centrifuged at 4000 rpm for 15 minutes and resuspended in 1L of 5% sucrose solution with 0.05% Silwett L-77. The plants were evenly divided into two groups and dipped in either the Agrobacterium

culture suspension containing the thiamine or ubiquitin promoter sequence. The two trays with plants were put in a plastic bag for one day.

Seed sterilising and sowing

Seeds were sterilized for 1 minute with 70% ethanol, followed by a 5 minute treatment with 15-20% household bleach (4% hypochlorite) and 0.05% triton X-100. After sterilization, the seeds were washed 2-3 times with dH2O, and stratified at 4 °C for 3 days. Seeds were germinated on 1/2 MS (Duchefa, http://www.duchefa.com) medium plates (pH 6) containing 0.7% agarose (no sugar). After approximate one week, transformed seedlings (broad and dark green cotyledons) were transplanted to 4x4x4 cm rockwool blocks in a climate chamber at 20 °C day, 18 °C night with 16 h of light (35 Wm^{-2}) at a relative humidity of 70%.

PCR conditions

For promoter amplification, Phusion DNA polymerase was used. Dreamtaq was used for testing of insert presence.

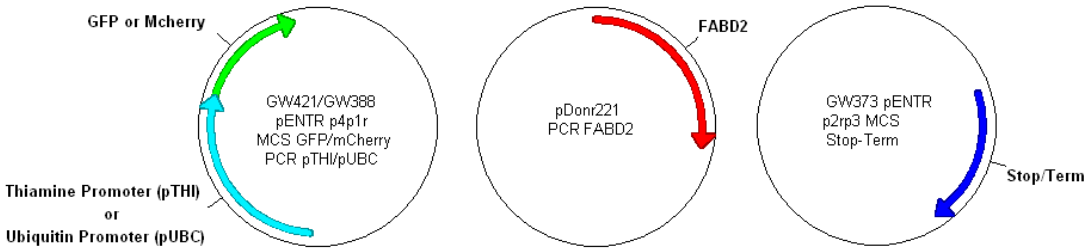
Phusion		Dreamtaq	
1 µl	*A. thaliana* genomic DNA		*E. coli* cells
10 µl	HF buffer	5 µl	10x Dreamtaq buffer
1 µl	dNTPs	1 µl	dNTPs
3 µl	Foreward primer	3 µl	Foreward primer
3 µl	Reverse Primer	3 µl	Reverse Primer
31.5 µl	dH2O	37.6 µl	dH2O
0.5 µl	Phusion	0.4 µl	Dreamtaq

Table 1. PCR mixture composition

Phusion		Dreamtaq	
98 °C	30 sec.	95 °C	5 min.
98 °C	10 sec.	95 °C	3 min.
57 °C	30 sec.	57 °C	30 sec.
72 °C	90 sec. (35x)	72 °C	90 sec. (35x)
72 °C	10 min.	72 °C	15 min.

Table 2. PCR time and temperature settings.

Overview of the used constructs



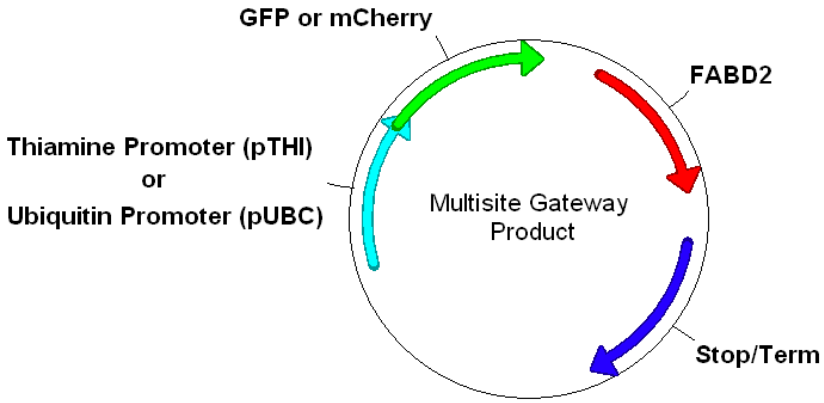


Fig. 3. Schematic view of plasmids with genes of interest, before and after the multisite gateway reaction (backbone plasmid GW238 not shown).

Fimbrin1 At4g26700 PCR product 2717bp	
pFIM1F +AscI	5'- CTTGGCGCGCCCGTTCAAACGCGCAAACCAGTC-3'
pFIM1 R +AscI	5'- CTCTCACTGTCTTACCATTCACGGGGGGCGCGCCTTC-3'
Actin7 PCR At5g09810 PCR product 4937bp	
pAct7F + AscI	5'- CTTGGCGCGCCCAAAAGCTTTTATTCTCTGTTTAAACCACT TGG-3'
pAct7R +AscI	5'-TTTTCACTAAAAAAAAAGTAAAATGAAACCGAAATGG GGCGCGCCTTC-3'
THI Promoter PCR product 1200bp	
At5g54770F+TAscI	5'-CACACCGGCGCGCCCAGGCTGGTTTGGTGAAGAAGG-3'
At5g54770R+AscI	5'-GTTGGCGCGCCTTTTGTTTTCTTTAGGGTTCGAGTTT-3'
EMB promoter PCR product 1500bp	
At1g80070F+TAscI	5'-CACACCGGCGCGCCGACACATCCAACCTACCATACAAGG-3'
At1g80070R+HindIII	5'-GTTAAGCTTCGTTGTTGTTCCACTTAGCTTAGGG-3'
UBC2 promoter PCR product 1500bp	
At2g02760F+TAscI	5'-CACACCGGCGCGCCCCATGAAGTTGCCTACCATACTGGAG-3'
At2g02760R+AscI	5'-GTTGGCGCGCCCCTTCTCTTACTCTATCAAATCCCAGTAACC-3'
FABD2 PCR product 2800bp	
FABD2 GF AttB1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACC AAGGGATCCTCTTGAAAGAGCTGAATTGGTTC-3'
FABD2 GR AttB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA TCAT GACTCGATGGATGCTTCCTC-3'

Table 3. Primer sequences used for PCR promoter amplification. GGCGCGCC = AscI restriction site. AAGCTT = HindIII restriction site. CACACC = topo site (backup strategy, not used). AttB# = GateWay Att recombination site (BP reaction). Grey = frame correction. Green = border extension.

Germination experiment setup

Germination experiments were done according to the methods of Joosen et al. (2010). Harvested seeds were stored for one week after ripening. Germination experiments were performed in plastic trays (15 x 21 cm, DBP Plastics, http://www.dbp.be) containing 49 ml water and two layers of blue filter paper (Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com). Two more trays were added on top and under the stack to assure equal evaporation and light conditions in all trays. 123 µl absolute ethanol was added to the trays with alcohol inducible AIP1 RNAi seeds, resulting in a final ethanol concentration of 0.25% (Ketelaar et al., 2004). For the experiment at 20 °C, the alcohol was pipetted on the wet blotting paper. For the experiment at 25 °C the alcohol was added to the water in the tray before adding the paper, to reduce evaporation. The stack of trays of each treatment were packed in a separate bag. Trays with stratification treatment were put in the dark at 4 °C for three days. Hereafter, trays without stratification were prepared and both treatments were put in an incubator at 20 °C, under continuous light. Trays were lit from the sides. The high temperature experiment was performed two weeks later at 25 °C. Newly harvested ABI3-5 seeds were also included in this experiment. *ga1-1* seeds were germinated in, and sprayed weekly with 10 µM GA. The germination trays were photographed at regular intervals. The photos were rotated and cropped with Adobe Photoshop. The number of seeds and germinated seeds were scored automatically by the germinator plug-in in ImageJ. The ImageJ plug-in was used with the following settings: Saturated = 0.02; Y= 50-255; U= 0-85 or 120; V= 0-255. The germination data was imported into Excel. In excel, curves were fitted to the data points. From these curves the following parameters were extracted: maximal germination (gMAX, the plateau at which the curve stabilizes); uniformity, the time between 16% and 84% germination (u8416); time to 50% germination (t50); the area under the curve (AUC, a cumulative parameter that combines the previous mentioned parameters). The settings used for data processing in Excel were: area= 60 pixels; Xy = 0.8; r2 limit: 0.2; minimal germination: 6 seeds.

Plant material and growth conditions

Plants were grown on 4x4x4 cm rockwool plugs (MM40/40, Grodan B.V., http://www.grodan.com) and watered with 1 g/l Hyponex fertilizer (NPK = 7:6:19

http://www.hyponex.co.jp) in a climate chamber (20 °C day, 18 °C night) with 16 h of light (35 Wm^{-2}) at a relative humidity of 70%.

Imaging

Gels were photographed with Bio Rad Molecular imager ChemiDoc™ XRS+. Leafs of two week old transformed seedlings were analysed with a Zeiss LSM510 META confocal laser scanning microscope, using standard GFP detection settings.

Results

Production of the construct

Arabidopsis seeds were transformed with a construct containing an embryonic promoter, a F-actin binding protein, a fluorescent protein and a stop/terminator sequence. The individual sequences first were cloned in Gateway entry plasmids and finally assembled in a destination plasmid by a multisite Gateway recombination reaction. Finally, plants were transformed and seedling leafs were observed under a confocal microscope.

PCR amplification of promoters sequences

The provided plasmids with the GFP and mCherry sequence (421 pENTR p4p1r MCS GFP) and (388 pENTR p4p1r MCS RFP Cherry) contained a Cauliflower Mozaik Virus 35S ribosomal subunit promoter sequence (35S promoter). The 35S promoter is not active in *A. thaliana* seed and could therefore not be used. The *FIM1* and *ACT2* promoters were chosen instead. However, the PCR amplification of both promoters did not give the desired result (too large *pFIM1* product, no *pACT2* product). Therefore, three strong embryonic promoters were selected from expression array data, and used instead. These were the promoters of At5g54770 (thiamine), At1g80070 (embryo defective) and At2g02760 (ubiquitin) (Table 3). The amplification of the three new promoters and FABD2 succeeded (Fig. 4).

Replacement of the 35S promoter in GW421 and GW388 with the embryo active promoters

The obtained embryo active promoters were introduced into the plasmids. A single restriction site (AscI) was used to ligate the promoter fragments (pTHI and pUBC) into the entry plasmids (pENTR p4p1r MSC GW421 (GFP) or GW388 (mCherry)). Half of the ligated fragments was expected to be in the reverse orientation. To select the plasmids with the correct insert orientation, the plasmid DNA was digested with two restriction enzymes. These were XhoI and SpeI for the pTHI plasmids, resulting in a small fragment of 500 bp when the inert is in the sense orientation, and 700 bp when in the antisense orientation. To digest the pUBC plasmids, KpnI and BamHI were used, this would result in a fragment of 980 bp when the fragments was in the sense orientation and 2000 bp otherwise. However, out of the 24 colonies tested, no restriction fragment size corresponding with the correct

orientation of the insert was found. The success rate of the ligation was low, as most colonies contained a self-ligated or undigested plasmid. To quickly screen the colonies on containing the insert, a PCR directly on bacterial cells was performed (Fig. 5). Twelve colonies per promoter and fluorescent protein combination (48 in total) were picked and the plasmid DNA was amplified by a PCR reaction (see tables 1 and 2). From these 48 samples, five samples with insert were chosen to determine the insert orientation (Fig. 6). The same restriction enzymes were used as before. Four colonies with the inserts in the correct orientation were found. Two of the pTHI:GFP construct and one for both the pUBC:GFP and pTHI:mCherry construct.

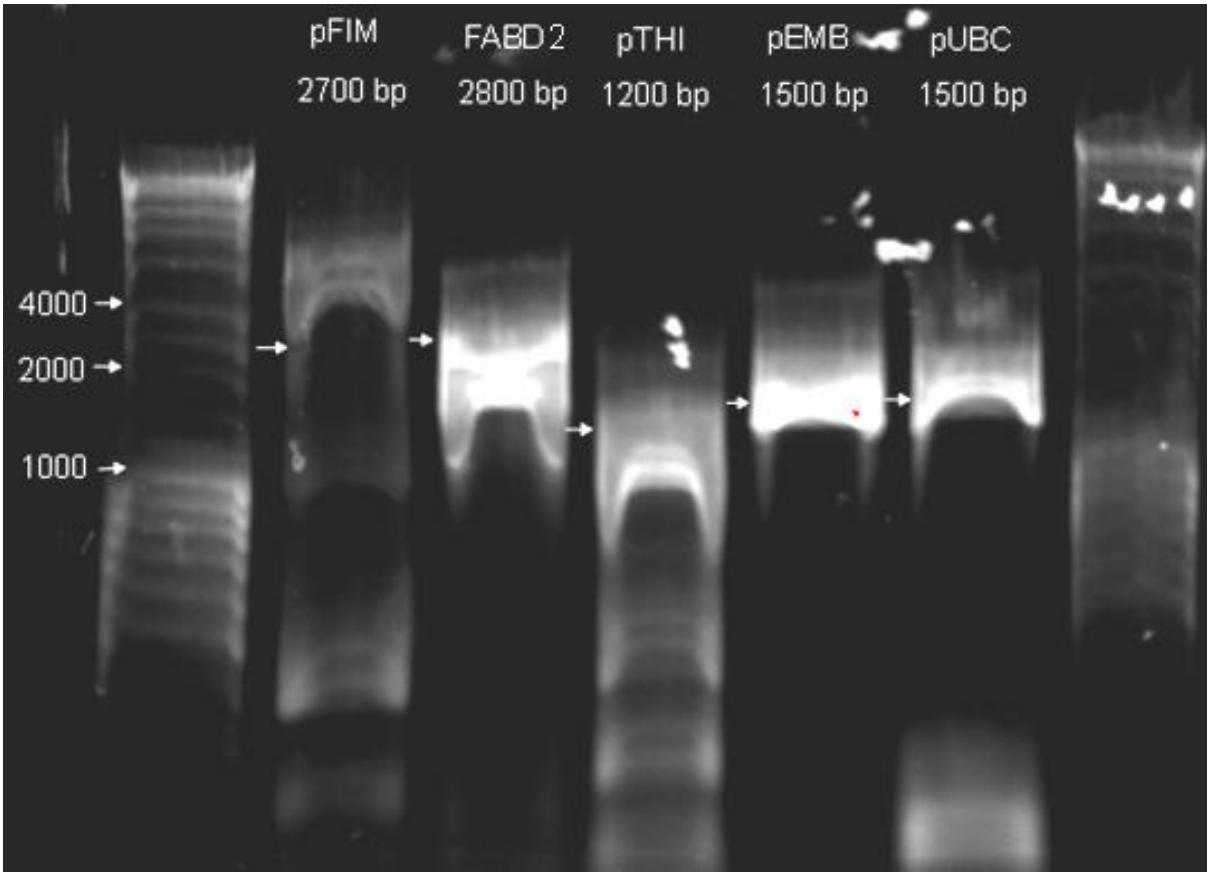


Fig. 4. Products of PCR amplification. Gene or promoter names (see also table 3) and the expected fragment length are indicated above the lanes. pFIM: *FIM1* promoter (At4g26700); *FABD2*: Fimbrin1 Actin Binding Domain 2; *pTHI*: Thiamine promoter (At5g54770); *pEMB*: Embryo defective At1g80070. *pUBC*: Ubiquitin promoter (At2g02760). Outer lanes contain massruler #sm0403. The three arrows and numbers at the far left indicate the fragment size at that ladder position. The remaining arrows indicate the expected position of the fragment per lane. Increasing the annealing temperature by 2 °C resulted in one single band for *pTHI*.

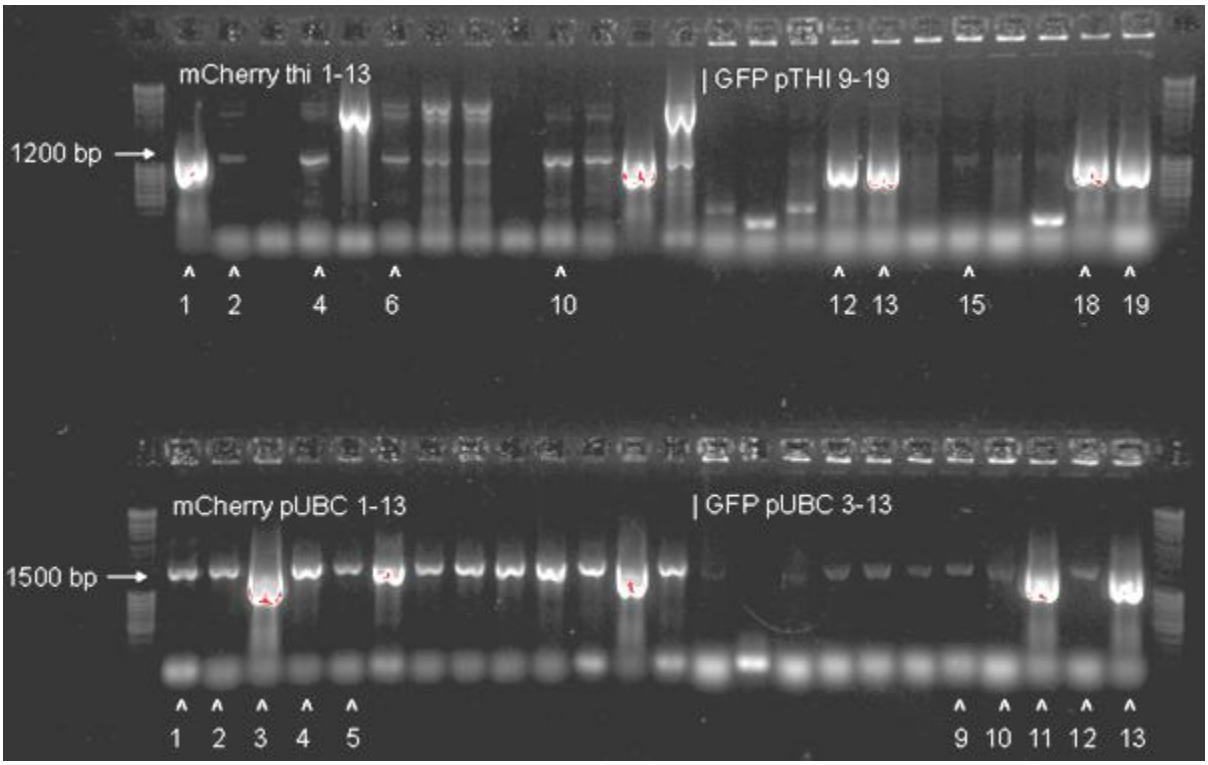


Fig. 5. Results of PCR done directly on bacterial colonies, which were transformed with promoter sequences pTHI or pUBC, in order to screen the plasmids pENTR p4p1r MSC GW421 (GFP) and GW388 (mCherry) on containing the promoter sequence. Horizontal arrows indicate the expected fragment size. ^ and the number indicate the selected plasmids of which the promoter orientation was determined afterwards.

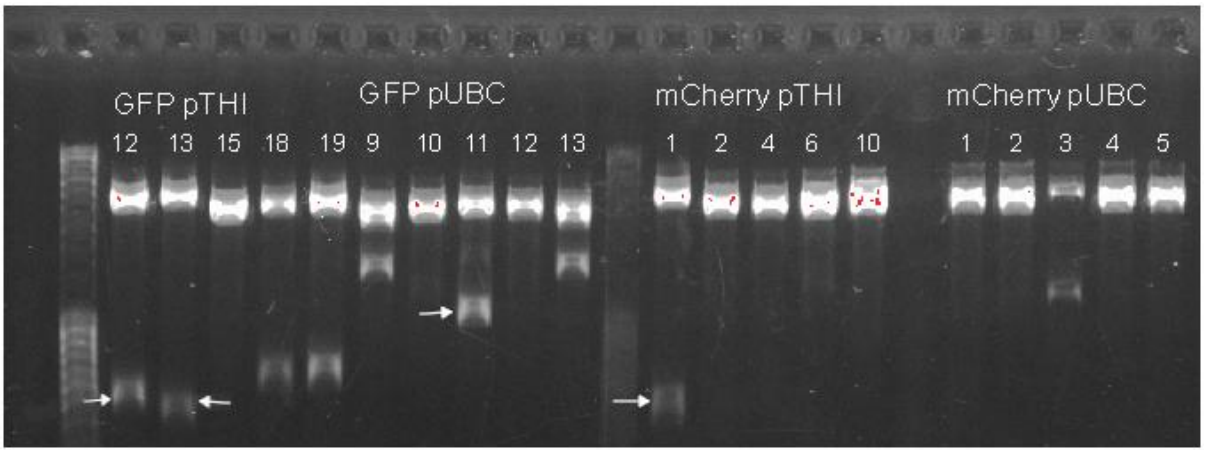


Fig. 6. Gel of digested plasmids pENTR p4p1r MSC GW421 (GFP) and GW388 (mCherry) to determine the orientation of the ligated promoter sequences pTHI and pUBC. The plasmids were digested with XhoI and SpeI (pTHI plasmids), and KpnI and BamHI (pUBC plasmids). Arrows indicate the fragments with the correct size (pTHi 500 bp, pUBC 980 bp).

Introducing FABD2 into pDONR221 by a BP reaction

To generate a binary plasmid expressing FABD2 under the control of the selected promoters, FABD2 was cloned and inserted into pDONR221. The PCR primers were designed with gateway AttB1 and AttB2 sites. The PCR product of FABD2 (Fig. 4, table 1-3) was recombined into pDONR221 by a BP reaction. The principle of the Gateway cloning technology ensures a single insert orientation. After the recombination, a diagnostic digestion was performed to verify the correct insertion of the FABD2 sequence into pDONR221 (Fig. 7). This was done by digesting the plasmids with EcoRI and PvuI, which should result in one fragment, and with BgiII and PvuI as negative control, which should result in two fragments. When the same restriction enzymes are added to pDONR221 without FABD2, the opposite number of fragments are expected. The FABD sequence was introduced successfully into pDONR221.

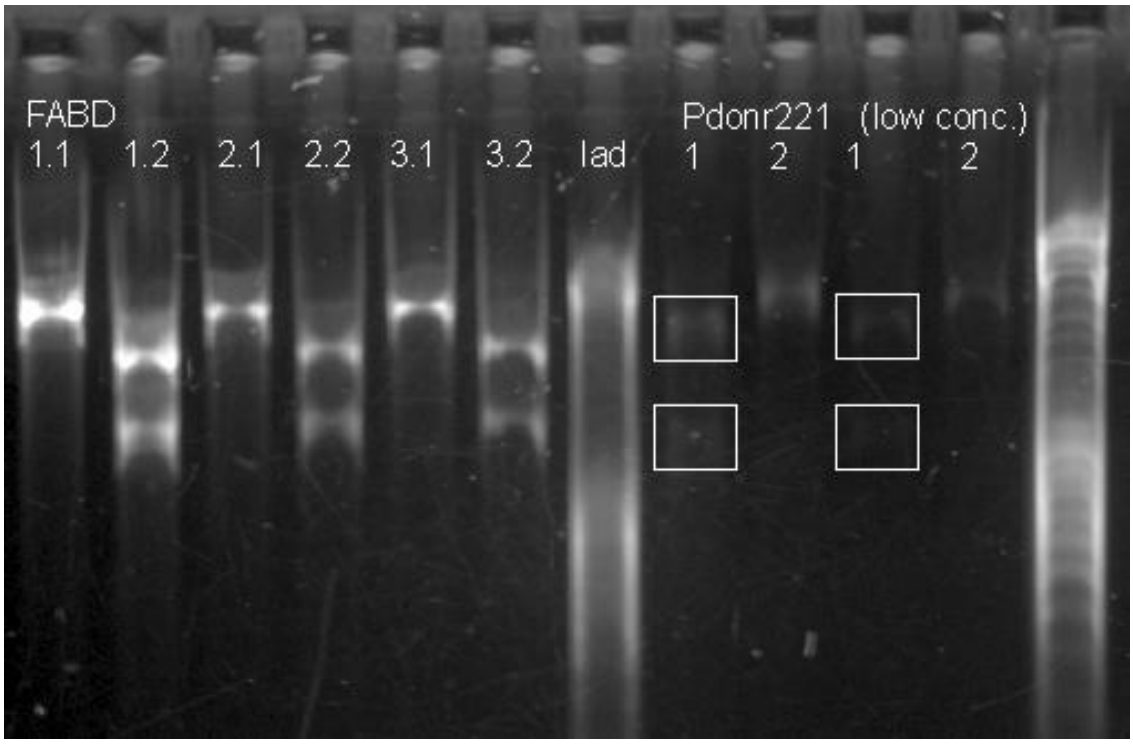


Fig. 7. Digestion of pDONR221 to confirm the correct insertion of FABD2 in pDONR211. FABD: the pDONR211 plasmid after FABD2 insertion by a BP reaction. pDONR221: the original plasmid without the FABD2 sequence. Restriction enzymes used in lanes 1.1, 2.1, 3.1, 1 as positive control resulting in one fragment: EcoRI and PvuI. Restriction enzymes used in lanes 1.2, 2.2, 3.2, 2 as negative control resulting in 2 two fragments: BgiII and PvuI. The numbers FABD 1.-3. refer to the picked colonies.

Check of Multisite Gateway product

Before the final construct was introduced into *Agrobacterium,* in order to transform the plants, a last check was done to verify that the Multisite Gateway reaction successfully combined the cloned promoters, FABD2, GPF and terminator DNA fragments, with the binary destination plasmid (GW238 pBGW - MGW). This was verified by performing a PCR with the FABD2 and promoter primers that were used before to obtain the fragments (Table 1 dreamtaq, 2 and 3). Figure 8 shows that the plasmids did contain the FABD2 and promoter sequences, as those were amplified. This was considered sufficient evidence that the we had obtained our desired plasmid.

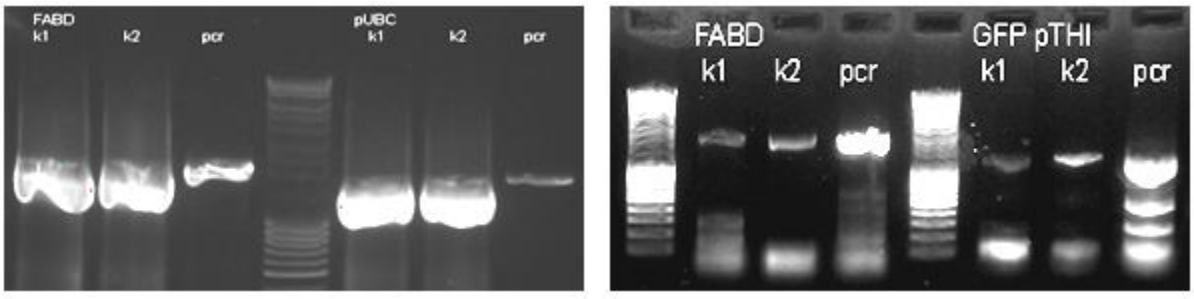


Fig. 8. Fragments obtained by a PCR on the MSGW product, to verify the success of the MSGW reaction. The presence of the promoters and FABD2 are an indication that the reaction went sucesfull. Left: pUBC:GFP. Right: pTHI:GFP. K1 and k2 refer to the bacterial colonies the plamids were isolated from. The PCR columns contiains the reference PCR product (FABD2, pUBC or pTHI) that were obtained before (Fig. 4). See tables 1-3 for primer sequence and PCR settings.

Microscopy

To test if the plant transformation was successful, small parts of the leaves of two week old seedlings were screened under the confocal laser scanning microscope for GPF fluorescence (Fig. 9). The two selected promoters are also active in the leaves. In all the selected seedlings GFP was expressed, as was confirmed by the susceptibility of the fluorescence to photobleaching. The actin filament are represented by thin, mostly straight lines (Fig. 9). Only one seedling was obtained that contained the thiamine (pTHI) promoter. In the images takes from this plant, the lines representing labelled actin filaments were better visible compared to the images from plants with the pTHI promoter.

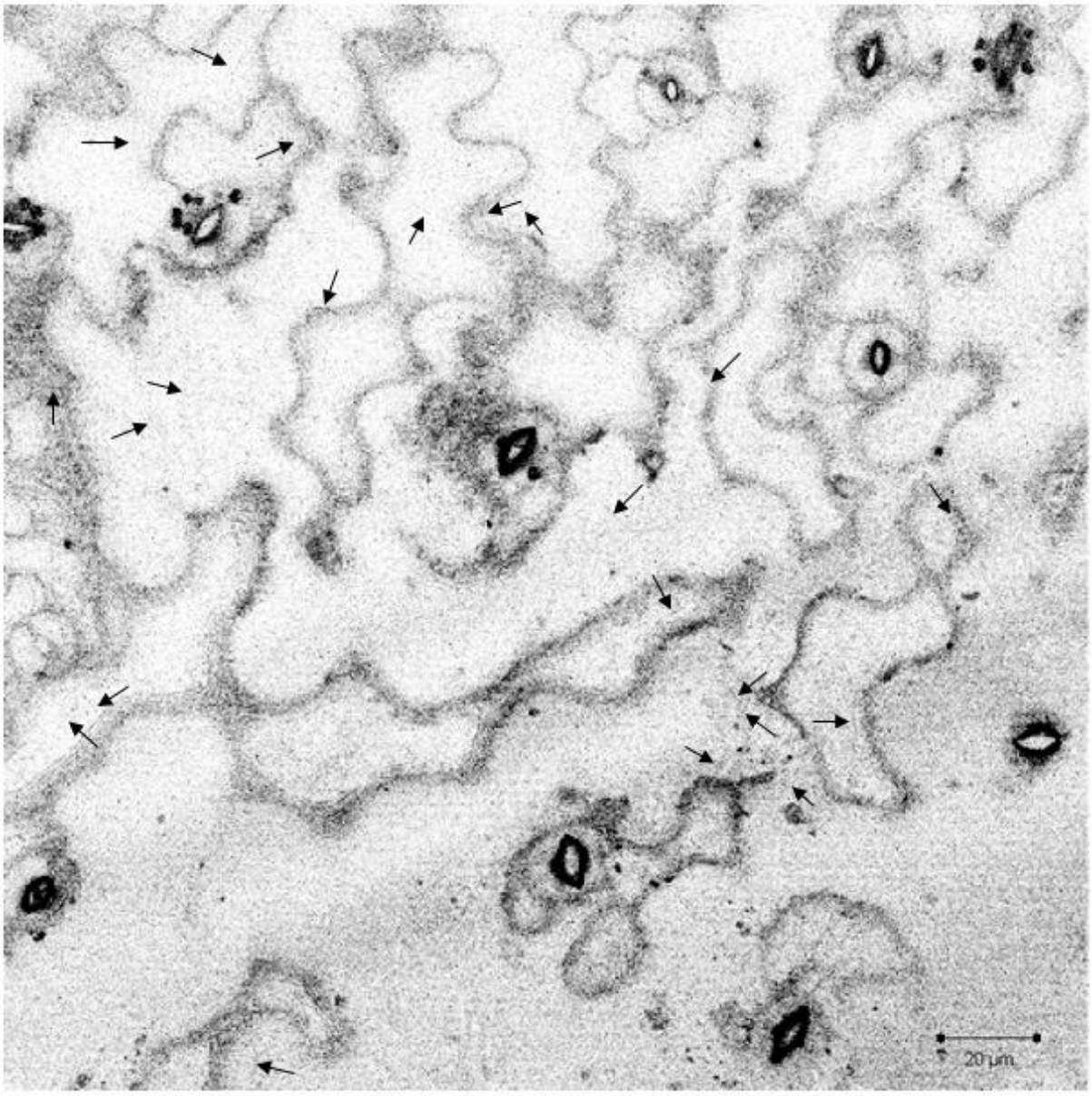


Fig. 9. Microscopic image taken of the bottom of two week old Arabidopsis leaves, transformed with pUBC:GFP:FABD2. Arrows are pointing perpendicular to actin filaments. (Colors are inverted to improve filament visibility)

Germination experiments

fim1 mutation decreases the dormancy index

Besides the labelling of the actin filaments for microscopic observations, the effects of *fim*1 mutation on seed germination characteristics were tested by performing germination experiments. Three seed germination treatments were conducted: with stratification at 4 °C and germination at 20 °C, without stratification and germination at 20 °C and finally, without stratification and germination at 25 °C. All stratified seeds germinated and germinated the

fastest at 20 °C (time until 50% germination (t50): 26 hours col-0, 29 hours *fim1*. See Fig. 10). Without stratification, the maximum germination (gMAX) was 84% for col-0 and 97% for *fim1*, which is a significant difference (p=0.002). The seeds of both lines germinated slower without stratification (t50: 60 hours col-0, 54 hours *fim1*) and less uniform then with stratification (average time between 16% and 84% germination (u8416) with stratification 15 hours, without stratification 35 hours). The time needed for half of the seeds to germinate (t50) was comparable between col-0 and *fim1* within each experiment. The differences between col-0 and *fim1* within each treatment, for the so far described parameters except for gMAX, were not significantly different (p>0.05).

The Area Under the Curve (AUC) is a parameter that combines the maximum germination, t50 and u8416 (Joosen et al., 2010). Without stratification, the AUC of col-0 and *fim1* differ significantly (82 and 99 hours respectively, p=0.03). The Dormancy Index (DI) is calculated as the AUC with stratification minus the AUC, of the same line, without stratification. This resulted in a DI of 51.2 for col-0 and 30.4 for *fim1*, which are significantly different (p = 0.022, appendix II, Fig. 10).

Col-0 seeds have a very low dormancy breakage in the first couple of weeks after harvest. The freshly harvested seeds were given just one week for after ripening before imbibition. Without stratification, the maximum germination percentage plateau of 84% is reached around 120 hours after imbibition. At the last measured time point (210h), delayed dormancy breakage of col-0 seeds was observed (see appendix I). For an equal comparison concerning dormancy breakage, the data presented here was analysed without the last data point (until 160h). The small change of the last data point caused a change in the gMAX value, which is the equal to highest germination percentage found, but it hardly influenced the fitted curve.

Germination at 25 °C did not enhance the dormancy phenotype

Dormancy effects are usually stronger when *A. thaliana* seeds are germinated at 25 °C (Kazmi et al., 2011). Therefore, the germination assay was also done at 25 °C. The stronger dormancy is reflected here in a decreased maximum germination and longer t50. *Fim1* seeds had a maximum germination of 69% and col-0 84%. Although the *fim1* seeds tend to germinate less well then col-0 at 25 °C compared to 20 °C, the difference is due to a large standard deviation not significant. The t50 at 25 °C was seven times longer compared to 20

°C, while the uniformity was reduced by a half. The pronounced differences caused by the higher temperatures were comparable for both *fim1* and col-0, and can therefore not be assigned to the *fim1* gene mutation. The germination experiment at 25 °C was continued for 434 hours, to allow also the more dormant seeds to germinate. No significant differences were found between col-0 and *fim1*, even though the gMAX appears less for *fim1* (Fig. 10). The results indicate that FIM1 is involved during dormancy and germination, because differences are found in the maximum germination and the dormancy index, although germination at 25 °C did not enhance the effect as was expected.

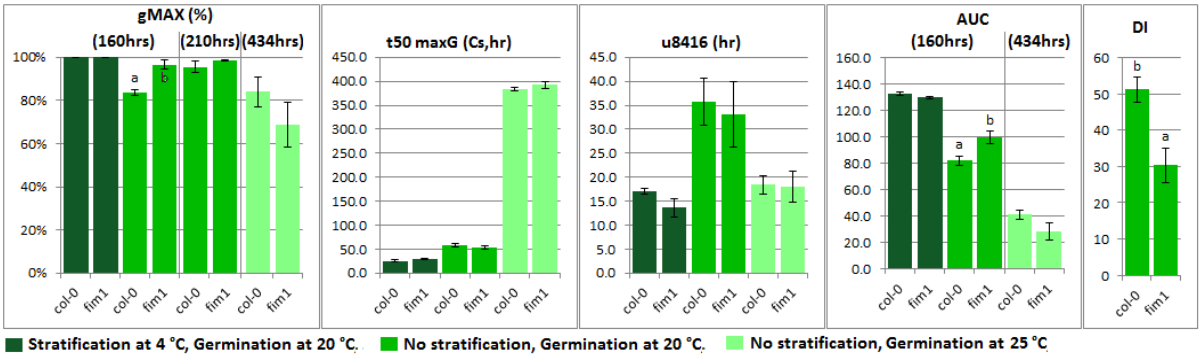


Fig. 10. Germination parameters. gMAX: Maximum germination, t50: time to 50% of maximum germination. u8416: time between 16% and 84% germination. AUC: area under the germination curve. DI: dormancy index. *fim1:* fimbrin1 mutant seeds. Col-0: control seeds. Statistically significant differences between col-0 and *fim1* are indicated with 'a' and 'b'. The hours between brackets indicate the last data point that was included during the curve fitting.

Germination experiment with AIP1 RNAi seeds

Because not only the *FIM1* gene expression decreases upon imbibitions, but also *ADF* expression increases, we repeated the germination assays with seeds with a decreased ADF activity via AIP1 RNAi. Two germination tests were conducted, without stratification, at 20 °C and 25 °C with two AIP1 (Actin Interacting Protein 1) alcohol inducible RNA interference lines compared with Col-0 (Ketelaar et al., 2004). At 20 °C, the AIP1 RNAi 1 line had a significantly shorter germination time (t50, p=0.006), and therefore also a larger AUC than the control (Fig. 11, p=0.03). There were no significant differences in maximum germination (75% average) and uniformity (39h average, p>0.05). The combination of 25 °C and alcohol treatment did have a severe impact on the germination capability, less than 6% germinated of all three lines (including control). Stratification (four days at 4 °C) ten days after

imbibition and changing to 20 °C, did not induce germination. The experiment was stopped after 18 days. Because of the low germination percentage, no analysis of germination parameters could be done at 25 °C.

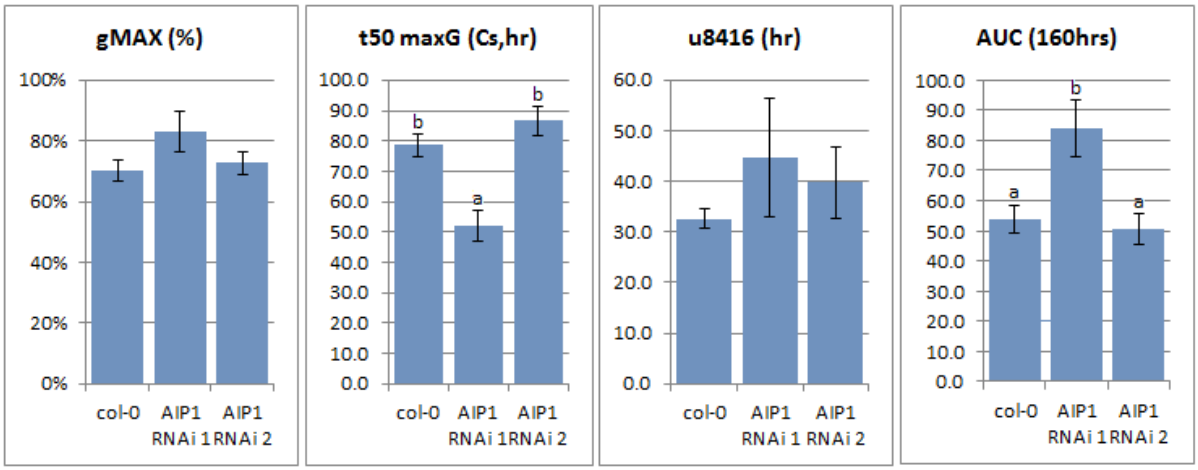


Fig. 11. Germination parameters for AIP1 alcohol induced RNAi 1, 2 and control seeds at 20 °C. gMAX: Maximum germination, t50: time to 50% of maximum germination. u8416: time between 16% and 84% germination. AUC: area under the germination curve. Statistically significant differences between the three lines are indicated with 'a' and 'b' (t50 p =0.006, AUC p=0.03).

Discussion

Here, we have generated Arabidopsis lines with GFP-FABD2 expression in embryonic tissue. We show that the actin bundling protein FIM1 is involved in seed dormancy. According to our germination assays, the dormancy index is decreased in the *fim1* knockout seeds.

The onset of germination is the elongation of a small group of cells in the hypocotyl-radicle transition zone (Sliwinska et al. 2009). Actin and actin binding proteins are of major importance of cell growth, which is the main process during germination. Upon imbibition, the expression of *FIM1* decreases and the expression of actin depolymerization factor 6 (ADF6) increases (Baxter et al., 2007; Bassel et al., 2008). Where FIM1 is expected to create a static actin filament network by bundling, ADF6 does the opposite by severing actin filaments. Severing of actin filaments adds actin monomers to the G-actin pool, from which new filaments can be formed, and thereby creating a dynamic environment. A dynamic network is needed for delivery of vesicles to the elongation zone. Chemical treatments blocking the cytoskeleton dynamics are known to result in cessation of growth. For cell growth it is therefore essential that the amount and location of actin filaments corresponds with the amount and location of required transport.

In vitro biochemical analyses showed that FIM5 bundles and stabilizes actin filaments (Wu et al., 2010). Loss of function of FIM5 in pollen rendered the actin filaments hypersensitive to the actin-depolymerization drug latrunculin B (Wu et al., 2010). In living stamen hair cells, FIM1 protects actin filaments from *Zea mays* profilin-induced depolymerization (Kovar et al., 2000). In addition, villins, which are another major family of bundling proteins, have shown to protect actin filaments against depolymerization by ADF (Huang et al., 2005). In embryos with a low fimbrin content, it is likely that the actin filaments are less protected and more dynamic. In *fim1* seeds it is expected that upon imbibition, actin is quicker available for dynamic processes. Considering the expression profiles and the effect of the actin binding proteins, we hypothesized that *fim1* mutant seeds are less dormant.

The effect of *fim1* mutation on the germination parameters

We have done germination assays with Arabidopsis fimbrin mutant *fim1* seeds. By comparing germination characteristics with and without stratification, the dormancy index

was calculated. We found a statistically significant lower dormancy index for the *fim1* mutant seeds, compared to the col-0 control seeds. Therefore, we conclude that the FIM1 protein has an effect on dormancy.

The dormancy index is calculated from the area under the curves (AUC) with and without stratification per line. The AUC represents a combination of the other germination parameters and has therefore a high discriminative power (Joosen et al., 2010). A high AUC is composed of a quick, uniform and high total germination. Here, the AUC of *fim1* seeds without stratification at 20 °C is larger than the AUC of col-0 seeds mainly because of a higher maximum germination percentage of *fim1* seeds. The *fim1* mutation increased seed maximum germination by 13%. In addition, the germination of *fim1* seeds is slightly more uniform and the t_{50} is a bit shorter.

If seeds are germinated at high temperatures, the dormancy effects are usually more extreme. Therefore, we germinated the seeds at 25 °C. The gMAX of *fim1* seeds was 15% lower than for col-0 seeds, which is in contrast with the previous experiment. Due to a high standard deviation this difference is not statistically proven. There is a possibility that fimbrin is not directly involved with dormancy, but has an effect on dormancy via related processes such as germination or maintenance processes. Nonetheless, it is in line with the decrease in gMAX of *fim5* pollen *in vitro* at 25 °C described by Wu et al., (2010).

The used seeds were after ripened for one week after harvest. A number of seeds lost their dormancy during the experiment. This prolongs the time between the start and end of germination (defined here as 16% and 84% of total germination). Strong dormancy leads to a decreased maximum germination. We see this with the Col-0 seeds in the experiment without stratification and germination at 20 °C. There, gMAX stabilizes around 80% around 120 hours after imbibition. At the final measurement at 220 hours, there is dormancy breakage of the remaining seeds.

High temperatures delay germination

Environmental stress conditions such as high germination temperature, are known to delay germination and decrease the germination uniformity (Kazmi et al., 2011; Joosen et al., 2012). The germination is indeed delayed, as the t50 is seven times longer compared to germination at 20 °C. The germination is on the other hand very uniform, at a comparable level with seeds that were stratified. A possible explanation is that high temperatures

generally speed up biochemical processes. As 25 °C is just three degrees above the optimum, the negative effects could be minor. Interestingly, the uniformity is similar for both *fim1* and col-0 seeds for all treatments.

It has to be noted that the fitted curve for the seeds at 25 °C was not S-shaped, but rather a near-linear line between the last two measure points. For a more conclusive result, the experiment should be repeated with more measure points between 300 and 500 hours. The experiment should be done as sterile as possible, as growth of fungi enforced the end of the experiment.

Magnifying the observable effects of *fim1* mutation

The effect of *FIM1* mutation can possibly be enhanced by creating fimbrin double or even triple knockouts. Even though the expression of the other fimbrin(-like) proteins are not as strong as that of *FIM1,* or, are expressed outside the embryonic tissue (Dekkers et al., unpublished data). Alternatively, double knockouts of *FIM1* and other actin bundling proteins can be made. Functional substitution of FIM1 functions by other bundling proteins is likely to occur, as also suggested by Wu et al., (2010). It has to be kept in mind that the actin dynamics and gene expression profiles in relation to dormancy breakage should be studied in the small group of cells in the elongation zone. If larger tissues are used for microarray experiments, the gene expression in the few cells of the elongation zone can be obscured by the surrounding cells. Also the timing of observation or measuring is important, as seed vary in their rate of germination. Ideally a marker is used that marks the elongation zone and the onset of elongation.

In the embryo during seed maturation, *FIM1* has the highest expression of the fimbrin gene family (Winter et al., 2007; Bassel et al., 2008). Several studies have shown similarities between FIM1 and FIM5. Both fimbrin proteins create higher order structures when added to actin strands *in vitro* (see Fig. 1 and 2). Nevertheless, the structures linked by FIM1 were larger, less compact and had the appearance of an aggregate instead of bundles (Kovar et al., 2000; Wu et al., 2010). The *in vivo* actin filament structure and FIM1 action in seeds needs to be observed to gain more knowledge on the actual function of the actin cytoskeleton in seed dormancy. The amount of (cross-)linking could be measured in maturing, dormant, germinating and germinated seeds. The right equilibrium of actin binding proteins is important for 'optimal' functioning of the actin cytoskeleton. During

germination the equilibrium is changed towards a more dynamic state. Shortage or abundance of fimbrin might shift the equilibrium below or beyond the temporary optimum for organelle transport. It is known that the actin binding protein regulation is dependent on crosstalk and self-regulation (Hussey et al., 2006). This can be the reason for the seen differences between the fimbrin knockouts in pollen and seeds.

The effect of actin cross-linking on desiccation tolerance

It is known that the degree of actin cross-linking and bundling strongly alters the physical properties of the actin network (Ketelaar et al., 2010). The high *FIM1* expression during seed maturation and high abundance of FIM1 in dry seeds suggests that it may be involved in desiccation tolerance. Analysis of gene expression data from the Plant Physiology group has linked the FIM1 gene with drought tolerance (unpublished data). Late embryogenic abundant proteins have previously been associated with protection of drying cell compartments (Franchi et al., 2011). Apparently, the stiff network is not required anymore in wet conditions, because after imbibition, the expression of *FIM1* rapidly decreases. Programmed cell drying allows longer seed and pollen survival. It has been estimated that Arabidopsis seeds can be viable for up to 2000 years, when kept with 5% moisture content at -20 °C (Clerkx, 2003). Also pollen of most plant species show a decrease in water content before dispersal (Franchi et al., 2011). The *fim5* mutant pollen might therefore be less tolerant to desiccation. A special actin configuration may be involved in the survival of dry seeds. In the case of the *fim1* mutant, the actin configuration may be suboptimal for storage conditions, and hence result in decreased dormancy. At the same time, it probably shortens the storage life of the *fim1* seeds.

Two Arabidopsis mutant lines that have short seed longevity are *abi3* and *aba1. Abi3* is an abscisic acid insensitive mutant of which the seeds are green at the maturation stage. *Aba1* is deficient in abscisic acid production. Both lines are known to lack dormancy, as abscisic acid is the main regulator of dormancy (Nakamura and Toyama, 2001; Ogawa, 2003; Bentsink et al., 2006; Huang et al., 2007). *FIM1* is one of the many genes that is up regulated by ABA. We tried to transform a number of *abi3* and *aba1* plants with our GFP:FABD2 construct to study the effect of ABA on the actin cytoskeleton organization. However, the *aba1* plants did not produce viable seeds, and no transformed *abi3* seedlings were found in the offspring. Therefore, we were not able to observe the actual actin organization in these

mutants. The observation of a distorted actin organization in these seeds would support the hypothesis that the actin organization plays a role in dormancy. In the *aba1* mutant, a reduced cross-linking phenotype would be expected, or at least an actin organization similar to the actin organization of *fim1* seeds. As there is an interaction between ABA and GA, the opposite phenotype, a strong cross-linked or bundled actin filament network, would be expected in *ga-1* mutant seeds. These seeds do not germinate without addition of exogenous GA. The fact that GA increases the growth potential of embryos (Ogawa, 2003) could suggest that less bundling of actin filaments in seeds is beneficial for growth. GA has an effect on the expression of many genes. Still, excessively bundled actin filaments could be the reason of delayed germination. Besides the effect of phytohormones on the expression of *FIM1*, also the expression of *ACT7* is known to respond differently to many of the phytohormones (McDowell et al., 1996). As the balance of phytohormones regulate the expression of many genes, it is difficult to allocate their effect on individual genes. Instead, microscopic observation of the cells' actin cytoskeleton organization in these phytohormone mutants will give insight in the overall dynamics.

Germination experiments with AIP1 RNAi seeds

To visualize the actin organization, we used the second actin binding domain of FIM1 coupled to GFP. This method has been used for live cell imaging of actin filaments in all seedling cells (Voigt et al., 2005). Seed-expressed fluorescent proteins have been used, but not to label the actin cytoskeleton in seeds (Stuitje et al., 2003; Fuji et al., 2007). Mostly, easily accessible tissues such as root hairs, leaf cells and pollen have been studied. To visually correlate the changes in actin organization to the presence of fimbrin, another fluorescent protein could be fused to the FIM1 protein. The co-localization of FIM1 and actin (cross-)linking sites could be observed and measured. Complementation of the *fim1* line with this construct (with FIM1 promoter) would be expected to result in the wild-type phenotype. Otherwise it is an indication of interference by the fluorescent proteins. Previous studies did not indicate such interference (Ketelaar et al., 2004; Sheahan, 2004; Wang et al., 2004; Voigt et al., 2005). Labelling can provide information about the presence, abundance, specific location, interaction and network structure of the labelled proteins. These aspects can be compared between dormant and non-dormant seeds. For as far as

time-lap series are meaningful on dissected seeds, these might show interesting changes in several of these aspects.

Severing of an actin filament by ADF happens in a very short timeframe, after which ADF disperses from the actin filament. Although ADF has a high affinity for actin filaments, in its active form it does not accumulate along actin filaments, but is rather diffusely distributed throughout the cytoplasm (Allwood, 2002). In Lily pollen tubes, this diffuse distribution was indeed observed (Allwood, 2002). However, in dormant pollen, LiADF1 decorated actin filaments and aggregates, and had a very low severing activity (Allwood, 2002). Upon pollen germination, the activity of LiADF1 was increased by the presence of Actin Interacting Protein 1 (AIP1). The combination of LiAFD1 and AIP1 increased the severing activity by 60 percent. It has been found that the *in vitro* severing activity of ADFs is strongly dependent on the presence of AIP1 (Allwood, 2002; Ketelaar et al., 2004; Clement et al., 2009). Consequently, shortage of the AIP1 protein decreases the activity of ADFs. LiADF1 is Lilly pollen specific and has important structural differences with AtADF5 and AtADF6 (Allwood, 2002). AtADF1 is not expressed in Arabidopsis pollen and only moderately in seeds (Winter et al., 2007; Bassel et al., 2008). Research by Ketelaar et al., (2004) has shown that AIP1 is also of importance in cells with (non-mutated) active ADF. So instead, we examined the germination characteristics of AIP1 RNA interference mutants to study the effect of reduced ADF activity.

Variations in AIP1 RNAi concentration may account for differences in seedling cell growth.

We used Arabidopsis seeds that were transformed by Ketelaar et al., (2004) with an alcohol inducible RNAi construct against AIP1. They obtained four lines, with differences in the interference severity. According to the leaf size and AIP1 protein scarcity, AIP1 RNAi 1 was considered the least severe mutant. In contrast, the root hair length of AIP1 RNAi 1 was more reduced than of AIP1 RNAi 2. Diffuse cell stretching and (root)tip growth have been seen as distinct growth processes, but in both processes a variety of common actin binding proteins are involved, including ADF and AIP1 (Smith and Oppenheimer, 2005). According to our hypothesis, the increase of *ADF6* expression upon imbibition creates a dynamic actin network that facilitates growth. Reduced actin turnover would then result in slower germination.

The results we obtained with AIP1 RNAi mutants do not support this. The seeds of AIP1 RNAi 2, which had the lowest amount of AIP1 protein of the two lines used here, did not show deviant germination characteristics from the control seeds. In contrast, AIP1 RNAi 1 seeds did have a statistically significant shorter t50 compared to Col-0. Whether the (assumed) lower amount of AIP1 protein is indeed the cause for the differences between the two RNAi lines is uncertain, this was not measured. The differences in effects between roots and leaves of the AIP1 RNAi lines reported by Ketelaar et al., (2004) indicate that the response is tissue dependent. It is likely that the lacking response of RNAi 2 seeds is caused by transcription differences. Especially in our case, because the AIP1 RNAi construct relies on a 35S:AlcA transcription factor, which does not work efficient in young tissue. So if in the AIP1 RNAi 1 line the F-actin turnover is indeed reduced, we would expect slower germination, but we see the opposite happen.

Underexpression of ADF has been reported to increase seedling cell growth, by increasing the number of actin cables (Dong et al., 2001). Because we expect an initial high degree of bundled F-actin in seeds, it seems unlikely that more F-actin would stimulate growth. There remains the possibility that the 'storage form' bundles are somehow different from the bundles formed during cell growth. Then, these bundles would need to be degraded before they could be reassembled, then possibly involving other proteins. Besides the severing effect of ADF, also a bundling effect was described by *in vivo* experiments with onion and tobacco cells (Dong et al., 2001) and by d-cofilin (different name for ADF) in *Dictyostelium* (Aizawa et al., 1996). Thick actin bundles found in dark grown hypocotyls were associated with the rapid elongation of that tissue (Dong et al., 2001). If this bundling activity occurs as well in the AIP1 RNAi seeds, the reduction of ADF severing could explain the faster germination. Still, severing is needed to maintain a pool of actin monomers for continuous polymerization. An alternative explanation for the fast germination of AIP1 RNAi 1 seeds could be that the ADF concentration upon imbibition increases beyond the optimal level for cell growth in the wild-type. This is not the most likely explanation, because one can reason that most processes are optimized by natural selection. Still, overexpression of ADF is known to reduce growth (Dong et al., 2001) and can cause the disappearance of transvacuolar strands (Ketelaar et al., 2010). Moderate decrease of the cellular ADF levels would in this case be beneficial for growth. Possibly, the decrease of ADF activity in RNAi 2 seeds is too strong to be beneficial. The increased dormancy phenotype we expected would

then be more obvious in the AIP1 RNAi 3 and 4 lines. To better understand the development in the actin cytoskeleton configuration during imbibition, repetition of this experiment, including microscopic observation is needed.

We repeated the experiment at 25 °C, at which almost none of the seeds germinated. The fact that even the control seeds did not germinate, indicate that this was caused by the treatment (25 °C and 0.25% alcohol) instead by AIP1 RNAi or ADF inactivity only. It may not only be caused by the temperature change, but also by a higher alcohol concentration then at the same experiment but at 20 °C. The same amount of alcohol was added, but the amount of vaporization during preparation and sowing was reduced (see m&m section). It is nonetheless an indication that AIP1 and ADF are of importance for germination. The mismatch of phenotype severity and concentration of AIP1 protein in the mutant lines indicate that multiple processes occur at the same time. In addition, there are controversial results on the *in vitro* and *in vivo* effects of both ADF and AIP1 (Dong et al., 2001; Ketelaar et al., 2004). To study the effect of ADF in seed germination, the direct approach used by Dong et al. (2001) appears more suitable. The 35S promoter they used should be replaced by an embryonic active and inducible promoter, to be able to obtain healthy seeds. Finally, it is very likely that the balance in concentration, activity and interaction of multiple proteins are important for the effects we have seen.

Plant transformation

At the end of this project, plants expressing our GFP:FABD2 construct were obtained, but these were still in an early development stage. Both used promoters are also active in leafs. So instead of the seeds, small leaf parts of the transformed seedlings were observed under the confocal microscope. GFP expression was found, as confirmed by rapid photo bleaching. Labeled actin filaments were observed, although the labeling was not very strong. Only one seedling was transformed with the thiamine promoter construct. The gene expression by the thiamine promoter is six times stronger than that of the ubiquitin promoter. The high expression of the construct could have caused some interference, although an abnormally high GFP concentration was not seen in the leaves. When the seeds are developed, further research on these seeds will provide answers to the points addressed in this report, and most likely provide information for new questions.

To conclude, in this report we have shown that actin filament bundling by fimbrin 1 is of importance during seed dormancy and germination. We have made a link between the actin cytoskeleton, seed dormancy and germination, phytohormones and seed longevity. This project has resulted in a tool which can be used in further studies to increase our understanding of seed physiology. The results also indicate that there is probably a lot of interplay between different actin binding proteins. An optimal balance of actin binding proteins is required for optimal growth. In any case, cellular observations, especially in the radical elongation zone, and germination experiments are needed to make further conclusions about the functioning of fimbrin and ADF in seeds.

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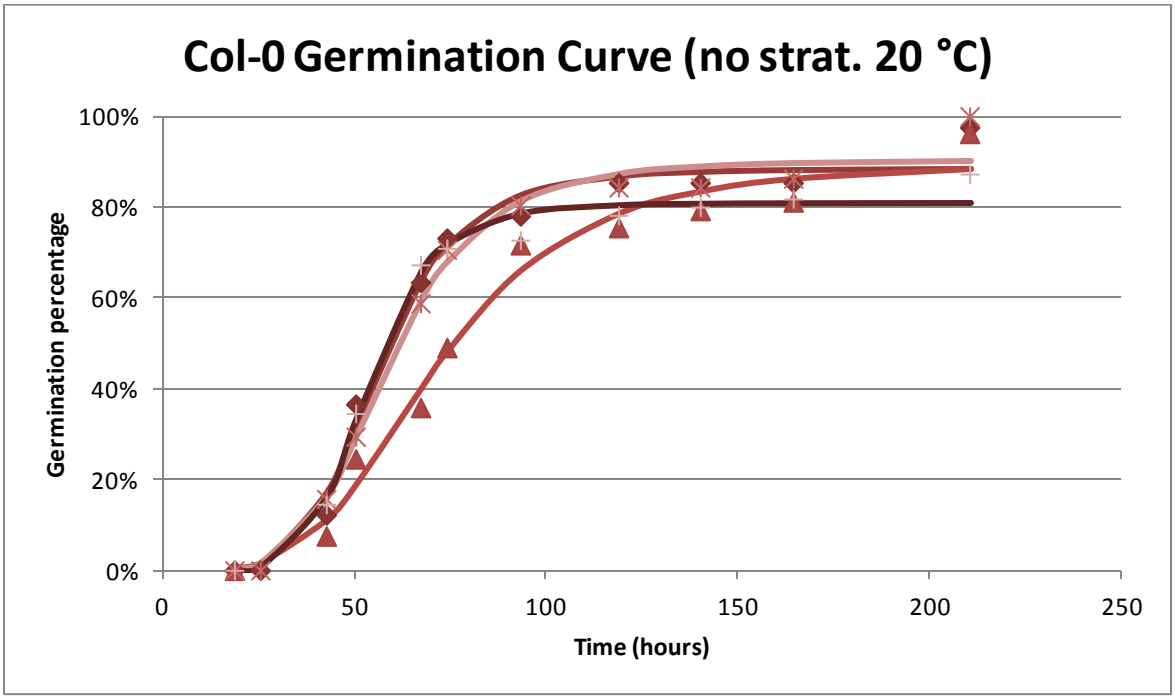
APPENDIX I 20 °C Germination Curves



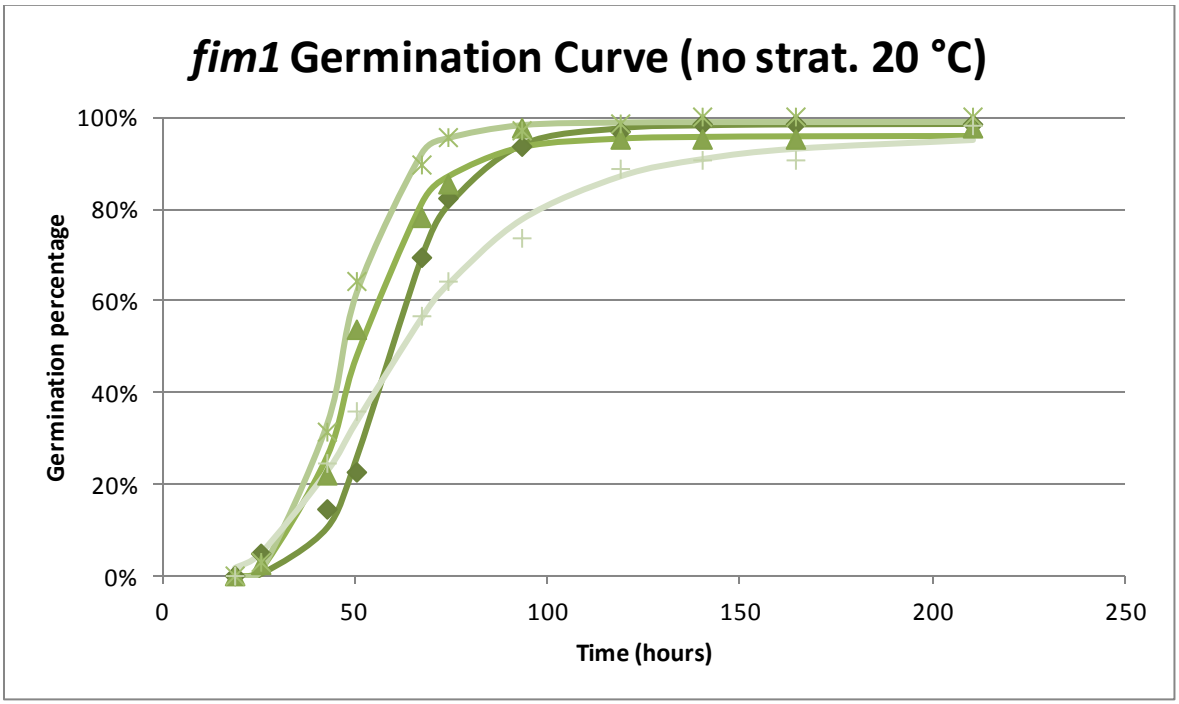


Fig. I. Germination curves of Arabidopsis col-0 and *fim1* seeds, without stratification, germination at 20 °C. The different lines are repetitions, each representing the average of approximately 50 seeds. Late dormancy breakage is seen for the col-0 seeds after 160 hours after imbibition.

APPENDIX II Dormancy Index Statistics

T-Test 160h

Group Statistics

	line	N	Mean	Std. Deviation	Std. Error Mean
DI	col-0	3	51.167	6.1076	3.5263
	fim1	4	30.350	9.5389	4.7694

Independent Samples Test

Equal variances assumed

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
DI	1.196	.324	3.269	5	.022	20.8167	6.3679	4.4474	37.1860

T-Test 210h

Group Statistics

	line	N	Mean	Std. Deviation	Std. Error Mean
DI	col-0	3	53.800	6.4954	3.7501
	fim1	4	36.675	8.0901	4.0450

Independent Samples Test

Equal variances assumed

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
DI	.429	.542	2.992	5	.030	17.1250	5.7229	2.4138	31.8362