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RESEARCH PAPER

The *Arabidopsis* mutant, *fy-1*, has an ABA-insensitive germination phenotype

Shiling Jiang, Santosh Kumar, Young-Jae Eu*, Sravan Kumar Jami, Claudio Stasolla and Robert D. Hill†

Department of Plant Science, University of Manitoba, Winnipeg, Canada R3T2N2

* Present address: Department of Applied Chemistry, Chungnam National University, Daejeon, 305-764, Korea.
† To whom correspondence should be addressed. E-mail: rob_hill@umanitoba.ca

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Abstract

***Arabidopsis* FY, a homologue of the yeast RNA 3' processing factor Pfs2p, regulates the autonomous floral transition pathway through its interaction with FCA, an RNA binding protein. It is demonstrated here that FY also influences seed dormancy. Freshly-harvested seed of the *Arabidopsis fy-1* mutant germinated readily in the absence of stratification or after-ripening. Furthermore, the *fy-1* mutant showed less ABA sensitivity compared with the wild type, L*er*, under identical conditions. Freshly-harvested seed of *fy-1* had significantly higher ABA levels than L*er*, even though L*er* was dormant and *fy-1* germinated readily. The PPLPP domains of FY, which are required for flowering control, were not essential for the ABA-influenced repression of germination. *FLC* expression analysis in seeds of different genotypes suggested that the effect of *FY* on dormancy may not be elicited through *FLC*. No significant differences in *CYP707A1*, *CYP707A2*, *NCED9, ABI3*, and *ABI4* were observed between freshly-harvested L*er* and *fy-1* imbibed for 48 h. *GA3ox1* and *GA3ox2* rapidly increased over the 48 h imbibition period for *fy-1*, with no significant increases in these transcripts for L*er*. *ABI5* levels were significantly lower in *fy-1* over the 48 h imbibition period. The results suggest that FY is involved in the development of dormancy and ABA sensitivity in *Arabidopsis* seed.**

Key words: ABA, ABI5, dormancy, flowering, *FY*, germination.

Introduction

A highly conserved eukaryotic gene, *FY*, was first identified in one of the late-flowering mutants of *Arabidopsis thaliana* and shown to be involved in the autonomous flowering pathway (Koornneef *et al.*, 1991). FY shares homology to a yeast 3′ end-polyadenylation factor Pfs2p, required for maintaining transcript stability by polyadenylation of the 3′ ends of the nascent transcripts (Ohnacker *et al.*, 2000). The *FY* gene is comprised of 18 exons and 17 introns and is localized on chromosome V in *Arabidopsis*. The encoded protein of 84 kDa contains seven WD repeats and two Pro-Pro-Leu-Pro-Pro (PPLPP) domains in the C-terminal region (Simpson *et al.*, 2003). The WD region is highly conserved among different species, whereas the PPLPP region is relatively less conserved.

The FY protein was first purified from cauliflower extracts through its interaction with FCA, an RNA binding protein involved in regulating flowering in *Arabidopsis*. This interaction occurred through the WW domain present on FCA and the PPLPP domains present on FY (Simpson *et al.*, 2003). FCA negatively autoregulates its expression by modulating the site of 3′ polyadenylation in its own pre-mRNA, leading to the formation of four different transcripts (Quesada *et al.*, 2003). A lack of negative autoregulation of *FCA* in *fy-1* mutants, that lack the PPLPP domains, suggested that *FY* functions as a 3′-end processing factor in *Arabidopsis* (Simpson *et al.*, 2003). FCA-FY interaction leads to the repression of Flowering Locus C (FLC), a MADS-box transcription factor that is a potent repressor of flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 2000). FCA and FY can operate independently of each other to affect the regulation of *FLC* through RNA-mediated chromatin silencing (Manzano *et al.*, 2009).

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FY may have pleiotropic effects within the autonomous pathway, as suggested by genetic analysis (Koornneef *et al.*, 1991). FY may undertake various functions depending on its interacting partners (Henderson *et al.*, 2005; Manzano *et al.*, 2009), although no specific functions have been identified. The *FY* mutants, *fy-1*and *fy-2*, both lack PPLPP motifs but retain the WD repeats intact. These mutants are late flowering due to the lack of FCA interaction, resulting in continued *FLC* expression. The interaction of FCA with FY which was thought to be strong and stable, is now considered transient, leading to altered organization of splicing machinery on FY (Manzano *et al.*, 2009). Studies with various *fy* mutants have shown that *FY* functions in flowering and embryo development and may have phenotypic effects dependent or independent of *FCA* (Koornneef *et al.*, 1991; Henderson *et al.*, 2005; Manzano *et al.*, 2009).

In addition to flowering, *FLC* also appears to regulate temperature-dependent seed germination in *Arabidopsis* (Helliwell *et al.*, 2006; Chiang *et al.*, 2009). This is the only documented evidence of any of the components of the autonomous flowering pathway functioning in germination. Increased germination at cool, but not warm, temperatures may be caused by highly expressed natural *FLC* alleles in *Arabidopsis* that result in increased expression of *CYP707A2* (Chiang *et al.*, 2009), a gene encoding an ABA 8'-hydroxylase (Kushiro *et al.*, 2004). ABA 8'-hydroxylase genes have been purported to play a pivotal role in regulating seed dormancy (Millar *et al.*, 2006), metabolizing ABA and relieving the block on germination. ABA is known to be a factor inhibiting both endosperm rupture and radicle growth (Finch-Savage and Leubner-Metzger, 2006), with the effect being regulated through several transcription factors identified by examining ABA-insensitive mutants in *Arabidopsis* (Koornneef *et al.*, 2002). One of these, ABI5, is a basic leucine zipper transcription factor that regulates a number of ABA-inducible responses, such as certain late embryogenesis-abundant genes (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000), embryo lipid mobilization and repression of seed germination in the endosperm (Penfield *et al.*, 2006). *ABI3* and *ABI5* are expressed in both *Arabidopsis* endosperm and embryo, with *ABI5* expression confined to the micropylar region of the endosperm (Penfield *et al.*, 2006). *ABI4* is expressed only in the embryo. Piskurewicz *et al.* (2008) suggest that ABI5 acts as the final common repressor of germination in response to changes in ABA and GA levels. They note, however, that expression of ABI5 alone was not sufficient to repress germination as ABI5 phosphorylation is required for activity.

In this work, evidence is provided to demonstrate that another autonomous flowering pathway gene, *FY*, is also involved in regulating germination. Our experiments show that the *Arabidopsis fy-1* mutant lacks seed dormancy, characterized by insensitivity to ABA. The results suggest that reduced levels of *ABI5* in *Arabidopsis fy-1* may be linked to this ABA-insensitivity.

Materials and methods

Plant materials and growth conditions

Arabidopsis *fy-1* mutant (CS57) seeds and its wild-type background parent (L*er*) were obtained from the *Arabidopsis* Biological Resource Centre (ABRC, http://www.arabidopsis.org). Identification of the *fy-1* mutant by genomic PCR and sequencing was according to Simpson *et al.* (2003). *Arabidopsis* seeds were surfaced-sterilized in 50% (v/v) bleach before sowing on Murashige and Skoog (MS) medium with 1% (w/v) sucrose, stratified for 4 d at 4 °C, and kept in a tissue culture cabinet (22 °C, 16 h photoperiod) for germination. Seven-day-old seedlings were transferred to soil and grown in growth chamber under constant conditions (20 °C, 70% relative humidity, 16 h photoperiod, light intensity of 100 $\mu E\ m^{-2}\ s^{-1-}$. All genotypes were grown side by side in a growth chamber. The seeds were harvested and used for further studies.

Seed germination assays

Mature seeds of different genotypes were harvested at the same time from dry siliques (moisture content of the harvested seeds was approximately 9%). Fresh seed denotes seed that was sown directly after harvesting. To afterripen, harvested seed was stored at 22 °C for 4 weeks in darkness. Cold stratification was performed by incubating the imbibed seeds at 4 °C for 3 d in darkness. Batches of 40–70 seeds from each genotype were sown in triplicate in Petri dishes with two layers of water-saturated filter paper (Whatman) or were supplemented with different concentration of ABA (Sigma-Aldrich). Germination was scored daily by radicle emergence for up to 5 d, and the germination frequency (%) was calculated. Each germination experiment was repeated at least three times using biological replicates.

RNA extraction, Northern blots, and real-time RT-PCR

The extraction of total RNA from seeds and Northern blots were performed as previously described by Baumbusch *et al.* (2004). The primers used for amplifying the probe for Northern blots analysis were as follow: *FY,* forward 5′-ATGTACGCCGGCGGCGA-TATG-3′ and reverse 5′-TCCGAATAGGCAACTGTGGG-3′; *FLC,* forward 5′-GTAGCCGACAAGTCACCTTCTC-3′ and reverse 5′-CTAATTAAGTAGTGGGAGAGTCAC-3′; *ACTIN* , forward 5′-GAYTCTGGWGATGGTGTG-3′ and reverse 5′-ACCTGYTGKAAHGTRCTGAG-3′. For the real-time RT-PCR, the extracted total RNA was treated with DNaseI (Qiagen, http://www1.qiagen.com) for digestion of genomic DNA, and was further purified using the RNeasy plant mini kit (Qiagen, http://www1.qiagen.com) according to the manufacturer's instructions. First-strand cDNA was synthesized with 5 μg of total RNA in 50 μl reaction by M-MLV Reverse Transcriptase (Promega, http://www.progema.com). Quantitative RT-PCR was performed, in triplicate, with 1 μl of cDNA template in 25 μl reaction using QuantiTect® SYBR Green PCR kit (Qiagen, http://www1.qiagen.-com) and run on the Applied Biosystem 7500 Fast real-time PCR system (http://www.appliedbiosystems.com) according to the manufacturer's instructions. A melting curve was used to verify the specificity of the amplified fragment. *ACTIN7* was used as an internal standard to normalize the amplification products. The following primers were used: *FLC*, forward 5′-CTGTTCTCTGTGACG-CATCC-3′ and reverse 5′-AGTGTGAACCATAGTTCAGAGC-3′; *AtCYP707A1*, forward 5′-CCATCGCTCAAGACTCTCTC-3′ and reverse 5′-CCTCGTCTTTTCCGAAGATCG-3′; *AtCYP707A2*, forward 5′-CAATTCCTTCTTCGCCACTCG-3′ and reverse 5′-GCCTCTGGTCCAATCATACG-3′; *AtNCED9*, forward 5′-CG-ACCGGAGAGATTCGAAAG-3′ and reverse 5′-CACCTTCT-CCTCGTCGTGAAC-3′; *AtABI3*, forward 5′-ACGTCAGCAG-GTGGTACCAG-3′ and reverse 5′-GGCAAGTGTGTCT-CAGCTTC-3′; *AtABI4*, forward 5′-ATGGACCCTTTAGCTTC-

CCAAC-3′ and reverse 5′-CTTTGCGTTTGCGTTGAGCG-3′; *AtABI5*, forward 5′-AGTCTGCTGCTAGATCTAGAG-3′ and reverse 5′-TGTTGCTTCCTCTTCCTCTCC-3′; *AtGA3ox1*, forward 5'-TTCACCATCACTGGCTCGCC-3' and reverse 5′-CCACATCAATTTCGATGCCAAC-3′; *AtGA3ox2*, forward 5′-TAAGAAGATGTGGTCCGAAGG-3′ and reverse 5′-CACATCAACTTGGCTGCCAAC-3′; *AtACTIN7*, forward 5′-AGGGAGAAGATGACTCAGATC-3′ and reverse 5′-GTGTGAGACACA CCATCACC-3′.

Construction of pFY::GUS *transgene and GUS histochemical staining*

The promoter of *AtFY* was amplified by PCR with genomic DNA from *Arabidopsis* (Columbia ecotype), using the primers *PAtFY*-F, 5′-AAGCTTCTCGGTGTGTTTTTGGGG-3′ and *PAtFY*-R, 5′-GGATCCCATTGTTGCCCACGGAG-3′. The 1.33 kb PCR product was amplified and cloned into pGEM-Teasy vector (Promega,http://www.promega.com) and confirmed by sequencing. The *FY* promoter fragment was then introduced upstream of β-glucuronidase (GUS) at the *Hin*dIII/*Bam*H1 site by replacing the CaMV 35S promoter in the pBI121 binary vector (Clontech, http://www.clontech.com). The *pFY::GUS* was mobilized into *Agrobacterium* GV3101 and then introduced into *Arabidopsis* by the floral dip transformation (Clough and Bent, 1998). Transformed lines were selected using kanamycin (50 μg ml^{-1}) in MS medium with 1% sucrose. Plants were stained for GUS expression as described (Stomp, 1992). Briefly, the tissues were incubated overnight at 37 °C in staining buffer (100 mM phosphate buffer, pH 7.5, 10 mM EDTA, 0.1% TritonX-100, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.5 mg ml^{-1} X-gluc). Seeds were dissected into the embryo and endosperm under a dissecting microscope before staining. After staining, tissues were transferred to a solution of ethanol: acetic acid (3:1 v/v) and photographed using a Leica DC500 dissecting microscope (http://www.leica-microsystems.com).

Construction of 35S::FY/AAFY *complementation transgene*

The opening reading frame of *FY* was amplified using the cDNA templates prepared from L*er* leaves with the primers Nflag-F (5′-TCTAGAATGG ACTACAAAGACGATGACG-3′) and FY3Xb-R (5′-TCTAGACTACTGATGTTGCTGATTGTTG-3′). The two PPLPP domains were mutagenized to AAAPP by using two steps of overlapping PCR with the following primers: Nflag-F (5′-TCTAGAATGGACTACAAAGACGATGACG-3′); 1PP2AA-F (5′-GGGCTGCAGCTGCACCACCTGGTCCCCACCCATCG-3′); 1PP2AA-R (5′-GTGGTGCAGCTGCAGCCCCCAGTGCCATGGAACCTGGAAG-3′); 2PP2AA-F (5′-AGCAGGCAGCTGCACCTCCTGGCCCTCCACCAAAC-3′); 2PP2AA-R (5′-GAGGTGCAGCTGCCTGCTGCTGCTGTTGGAAAG-3′); and FY3Xb-R (5′-TCTAGACTACTGATGTTGCTGATTGTTG-3′). The mutant *FY* with two AALPP instead of two PPLPP was designated as *AAFY*. The PCR products were cloned into pGEM-Teasy (Promega, http://www.promega.com) and sequenced. The target genes (*FY*/*AAFY*) were subcloned into pFGL 779, a derivative of pPZP201 at the *Xba*I site. The *35S::FY*/*AAFY* were transformed into the *Arabidopsis fy-1* mutant by *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998). Glufosinate-ammonium (10 mg l^{-1}) was used for selecting transformed lines.

Analysis of ABA

ABA levels were measured using the Phytodetek competitive ELISA kit (Agdia, Elkhardt, IN, USA) following the manufacturer's instructions. Tissue was powdered and extracted overnight in 1 ml of ABA extraction buffer (methanol, containing 100 mg l^{-1} butylated hydroxyl toluene, 0.5 g l^{-1} citric acid monohydrate). Samples were then centrifuged at 2500 *g* and the supernatant was dried with N_2. Dry extracts were re-suspended in 100 μl pure methanol followed by the addition of 900 μl of 1× TRIS-TBS buffer. For ABA ELISA 10–20 μl of the final extracts were further diluted in 1× TBS in order to obtain a final volume of 100 μl. Analysis was then conducted following the Phytodetek protocol. ABA levels were expressed as pmol mg^{-1} dry tissue.

Results

Arabidopsis fy-1 *displayed a lack of seed dormancy and decreased sensitivity to ABA during germination*

Freshly-harvested seed of *fy-1* and L*er* were imbibed on water-soaked filter paper, devoid of nutrients, under fluorescent light in the growth chamber for a period of 5 d. At the end of 5 d, seeds of the *fy-1* mutant germinated while most of the L*er* seed failed to germinate under the same conditions (Fig. 1A). The *fy*-1 seed completed germination by the third day of imbibition, while L*er* showed approximately 10% germination after 5 d. L*er* seeds required 4–5 weeks of dry after-ripening to reach complete germination after 5 d imbibition, while *fy-1* seeds germinated readily without after-ripening (Fig. 1B).

Stratified seed of *fy-1* was less sensitive to ABA than stratified L*er* (Fig. 1C). Ninety per cent inhibition of L*er* germination was attained with 3 μM ABA, while *fy-1* germination was inhibited by 25%. Embryo radicle elongation also showed a differential response to ABA between L*er* and *fy-1* (Fig. 1D). Post-germination growth of the *fy-1* mutant was, however, affected by 3 μM ABA (Fig. 1Ea), as the untreated seedlings continued to develop producing green cotyledons (Fig. 1Eb).

PPLP repeats within the carboxyl terminus of FY are not required for acquisition of seed dormancy and ABA sensitivity

Complementation studies were performed, introducing the *FY* open reading frame of ~2.2 kb, driven by the constitutive CaMV 35S promoter, into an *fy-1* loss-of-function background. Two independently transformed lines (*C2* and *C6*) were selected on glufosinate–ammonium in the T_2 generation showing a 3:1 segregation ratio indicating a single insertion of *35S::FY* transgene in the genome. The homozygous lines (*C2, C6*), obtained in the T_3 generation, were used for further functional analysis. Northern blot analysis from the total RNA obtained from freshly harvested dry seeds revealed a higher level of expression of *FY* in the two transgenic complemented lines (*C2* and *C6*) when compared to L*er* and *fy-1* mutant (Fig. 2A). The *fy-1* mutant is known as a late-flowering phenotype (Koornneef *et al.*, 1991; Simpson *et al.*, 2003) and total leaf number at flowering is correlated with flowering time (Koornneef *et al.*, 1991). Both complemented lines possessed similar leaf numbers as L*er* at flowering (Fig. 2B). Thus, the introduced *FY* gene restored the flowering phenotype and was fully functional.

Using freshly harvested seeds, the complemented lines showed a similar level of germination (~10%) to L*er*

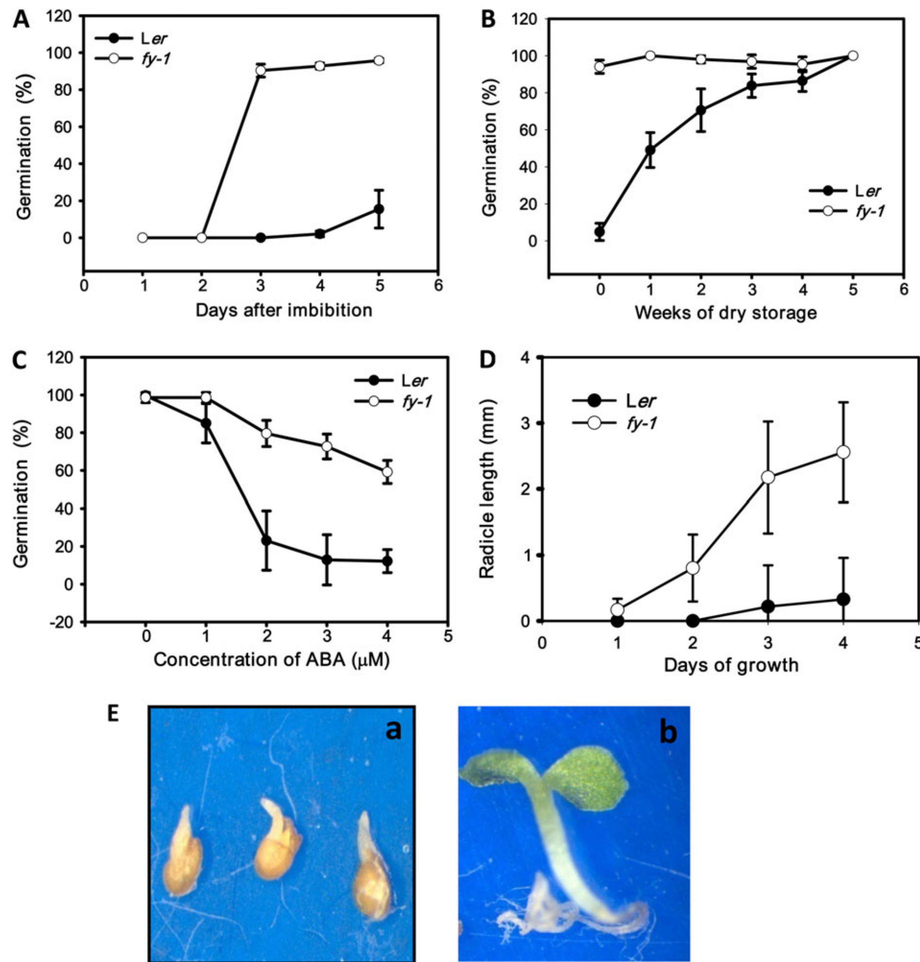
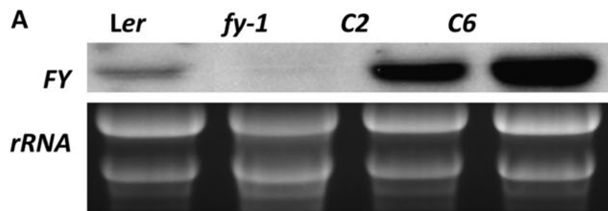


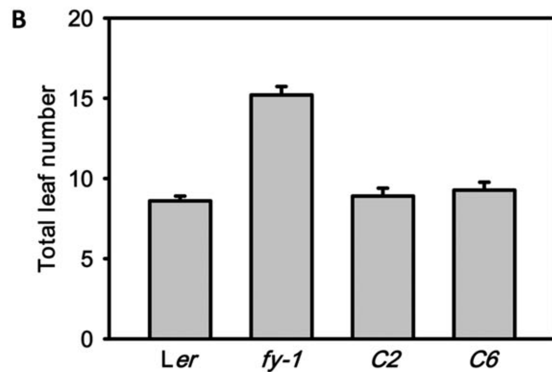
Fig. 1. Germination characteristics of L*er* (wild type) and *fy-1*. (A) Germination (%) of freshly-harvested L*er* and *fy-1* mutant seeds. (B) Germination (%) of L*er* and *fy-1* mutant after different periods of dry storage. Seed germination was scored after 5 d imbibition. (C) Germination (%) of stratified L*er* and *fy-1* seeds at 5 d in the presence of ABA. (D) Changes in radicle length of isolated embryos of L*er* and *fy-1* in the presence of 4 μM ABA. The imbibed seeds were kept at 4 °C for 3 d before embryo isolation. (E) Stratified *fy-1* seed at 7 d imbibition with 3 μM ABA (a) or H_2O (b). Germination frequencies are means of triplicate assays. Radicle lengths are means of 15 embryos for each genotype. Error bars represent ±1.96 SE.

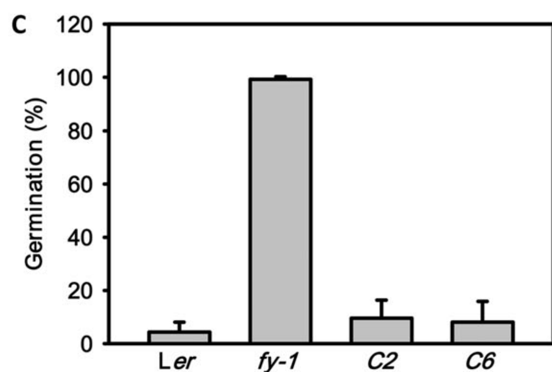
(Fig. 2C) after 5 d of imbibition, while the *fy-1* mutant germinated completely. Also, both complemented lines (*C2*, *C6*) restored ABA sensitivity of *fy-1* with a similar germination percentage to L*er* (∼10%) when imbibed with 3 μM ABA after 5 d of imbibitions (Fig. 2D). These results demonstrate *FY* involvement in the acquisition of dormancy and ABA sensitivity in *Arabidopsis* seeds.

Arabidopsis FY contains seven conserved WD repeats at the N-terminus and two non-conserved domains (PPLPP) at the C-terminal region. It has been reported that these two PPLPP domains are required for *in vitro* FY–FCA interaction and floral transition (Henderson *et al.*, 2005). The *fy-1* mutant lacks these PPLPP domains due to the shift in the splice acceptor site within exon 16, resulting in a premature stop codon (Simpson *et al.*, 2003). In order to determine whether these two PPLPP domains of FY are important to acquire ABA sensitivity and dormancy, the first two proline (P) residues of both PPLPP domains were each mutated to alanine (A) and a construct, in which both PPLPP domains were mutated, was generated. The mutant, *AAFY*, driven constitutively by the CaMV 35S promoter, was introduced into *fy-1*. Two homozygous transgenic lines (*AAC1* and *AAC3*) in the T_3 generation were obtained, based on glufosinate–ammonium selection. Northern blot analysis detected the presence of *AAFY* transcripts (Fig. 3A). Seed germination was assayed using freshly-harvested seeds from *AAC1* and *AAC3* transgenic plants along with L*er* and the *fy-1* mutant. Both seed dormancy and ABA-insensitivity of the *fy-1* mutant were complemented by both transgenic lines expressing *AAFY* (Fig. 3B, C). However, neither of the transgenic lines expressing *AAFY* could rescue the late-flowering phenotype of the *fy-1* mutant (Fig. 3D). This suggests that intact PPLPP repeats are not required for FY function in seed dormancy and ABA sensitivity, but are essential in floral transition.

The interaction of FCA–FY affects flowering by down-regulating *FLC* levels in leaves (Simpson *et al.*, 2003) and it has recently been shown that *FLC* affects







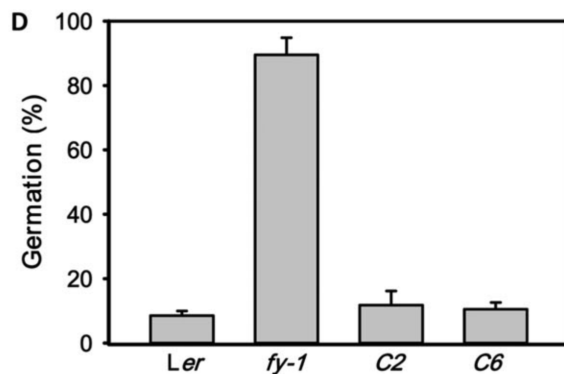
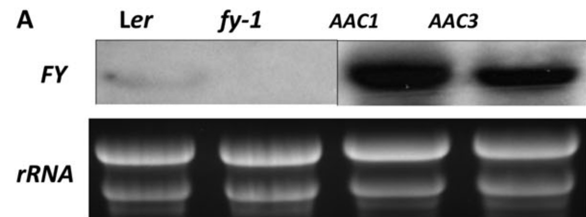
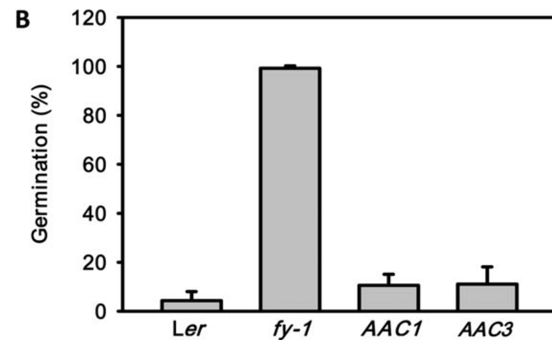
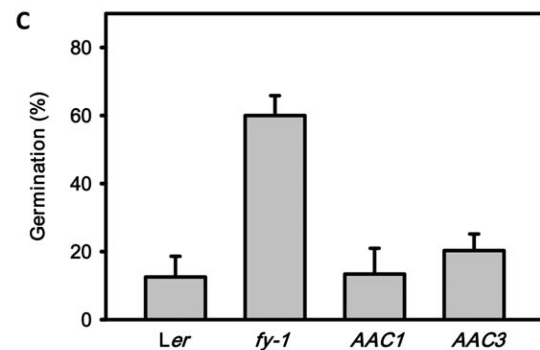


Fig. 2. Effect of introduction of *FY* into *fy-1* on seed dormancy. (A) Transcript level of *FY* in L*er*, *fy-1*, and complementation lines (*C2, C6*). RNA was extracted from freshly-harvested seed. Each lane contained 10 μg total RNA. (B) Total leaf number of L*er, fy-1*, and complementation lines (*C2*, *C6*) at the time of flowering. Values are means of 10 plants and error bars represent ±1.96 SE. (C) Germination (%) of freshly-harvested seeds from *Ler, fy-1*, and complementation lines (*C2*, *C6*). (D) Germination (%) of *Ler, fy-1*, and complementation lines (*C2, C6*) with 3 μM ABA. The imbibed seeds were kept at 4 °C for 3 d before moving to a growth chamber. Germination was scored at 5 d. Germination percentages are means of triplicate and error bars represent ±1.96 SE.







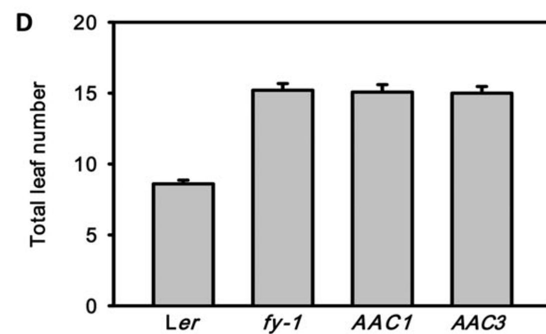


Fig. 3. PPLPP repeats of FY are not important for seed dormancy. (A) Northern blot showing the expression of *FY* in L*er*, *fy-1*, and homozygote transgenic lines with *35S::AAFY* (*AAC1, AAC3*). RNA was extracted from freshly-harvested dry seeds. Each lane contained 10 μg total RNA. (*AAFY*: *FY* with 2× PPLPP→AALPP). (B) Germination (%) of fresh seeds from L*er, fy-1*, *AAC1,* and *AAC3*. The seeds were scored after 5 d imbibition on filter paper. Germination percentages are means of triplicate and error bars represent ±1.96 SE. (C) Germination (%) of L*er, fy-1*, *AAC1*, and *AAC3* in the presence of 3 μM ABA. The imbibed seeds were kept at 4 °C for 3 d before moving to a growth chamber. Germination was scored at 5 d. Germination percentages are means of triplicate and error bars represent ±1.96 SE. (D) Total leaf number at flowering of L*er*, *fy-1*, *AAC1*, and *AAC3*. Values are means of 10 plants and error bars represent ±1.96 SE.

temperature-dependent germination in *Arabidopsis* (Chiang *et al.*, 2009). Real-time RT-PCR of total RNA extracted from freshly-harvested dry seeds of *Arabidopsis* genotypes L*er, fy-1, C2, C6, AAC1*, and *AAC3* showed that the *AAFY* transgenic lines (*AAC1*and *AAC3*) had similar levels of *FLC* to the *fy-1* mutant while *FLC* levels were reduced in *FY* complementation lines (*C2* and *C6*) and L*er* (Fig. 4). A similar trend was observed in Northern analysis (see Supplementary Fig. S1 at *JXB* online).

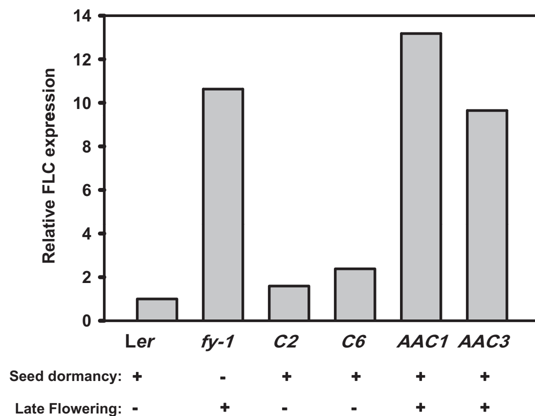


Fig. 4. *FLC* expression in *Arabidopsis* genotypes. Real-time RT-PCR of *FLC* in freshly-harvested seed of *Arabidopsis* genotypes L*er, fy-1*, *C2*, *C6*, *AAC1*, and *AAC3*. The phenotypes associated with the lines are indicated.

Effect of FY *on genes involved in ABA signalling and GA metabolism*

The regulation of seed germination is determined by a balance between the phytohormones, ABA and GA (Debeaujon and Koornneef, 2000; Finkelstein *et al.*, 2002). Genes involved in ABA and GA signalling and metabolism were analysed to determine whether there was altered expression of these genes in the *fy-1* mutant and how ABA influenced this expression. Three time periods of up to 48 h imbibition time, just prior to the first appearance of germinated seed, were chosen. The genes *CYP707A1, CYP707A2, NCED9, GA3OX1, GA3OX2, ABI3, ABI4, ABI5*, and *RGL2* were selected for testing. *CYP707A1* and *CYP707A2* encode an ABA 8'-hydroxylase, responsible for ABA catabolism (Okamoto *et al.*, 2006) and *NCED9* is required for ABA biosynthesis in seeds (Lefebvre *et al.*, 2006). *GA3ox1* and *GA3ox2* are two central GA biosynthesis genes that form bioactive GA in *Arabidopsis* (Yamaguchi *et al.*, 1998; Yamauchi *et al.*, 2004). *RGL2* codes for a DELLA protein that is a GA signal repressor inhibiting germination by stimulating ABA and ABI5 synthesis (Piskurewicz *et al.*, 2008). *ABI3, ABI4*, and *ABI5* are ABA signalling genes that are involved in the regulation of seed germination by ABA (Finkelstein and Lynch, 2000; Söderman *et al.*, 2000). ABA biosynthesis and catabolism genes were not significantly different between L*er* and the *fy-1* mutant over the 48 h of imbibition (Fig. 5). *ABI3* was not significantly different over the same period, but *ABI4* was significantly higher in *fy-1* at

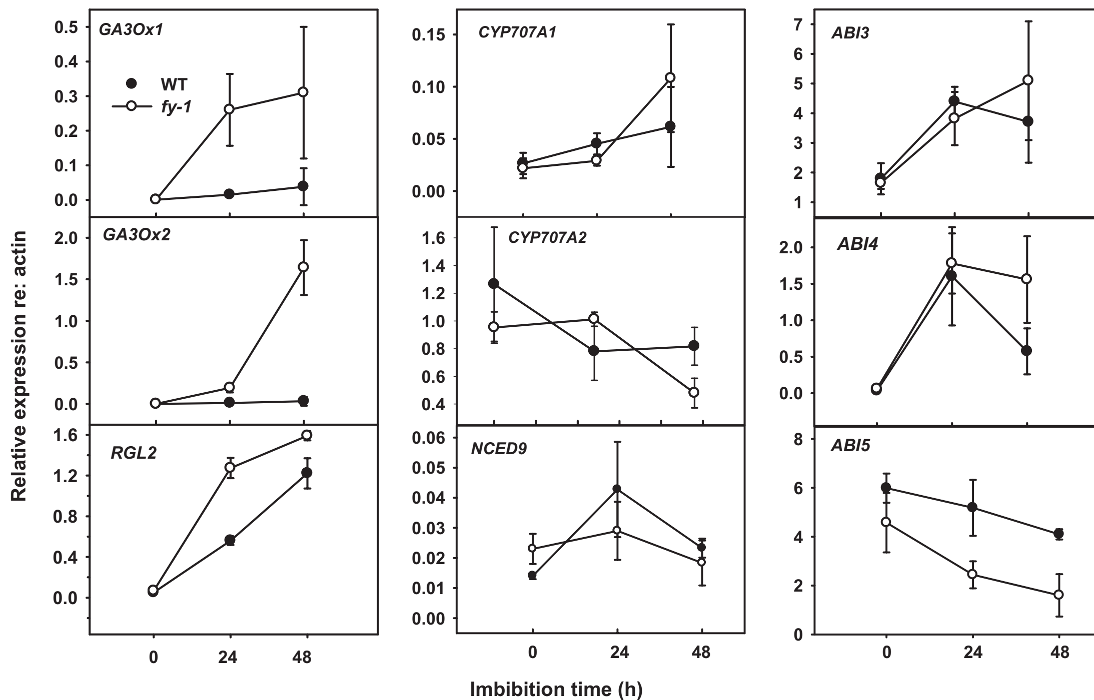


Fig. 5. Expression of ABA- and GA-related genes during imbibition in L*er and fy-1*. Transcript levels of *CYP707A1*, *CYP707A2*, *NCED9*, *ABI3*, *ABI4*, *ABI5*, *GA3ox1*, *GA3ox2*, and *RGL2* analysed by quantitative RT-PCR. CDNA was prepared from freshly-harvested seeds of L*er* and *fy-1* during the first 48 h imbibition. The expression value of the individual gene was normalized to *ACTIN7* as an internal control. Values, with the exception of *ABI5* are means from three replicates from different seed lots. The data for *ABI5* are means from four different seed lots. Error bars represent ±1.96 SE.

48 h imbibition. *ABI5* was consistently lower in *fy-1* over the course of the imbibition and significantly lower after 1 d of imbibition (Fig. 5). *GA3ox1* increased throughout imbibition in *fy-1* and was significantly higher in the *fy-1* mutant from 24 h imbibition (Fig. 5). *GA3ox2* began to increase rapidly in *fy-1* after 24 h imbibition and was significantly higher than L*er* by 48 h imbibition (Fig. 5). There were no changes in expression of either gene in L*er* seeds throughout imbibition. The differences between L*er* and *fy-1* in the *GA3ox* genes are characteristic of the differences between dormant and non-dormant seeds (Finch-Savage and Leubner-Metzger, 2006), consistent with an effect of FY on dormancy. *RGL2* levels increased during imbibition of freshly-harvested seeds of both L*er* and *fy-1*, with *fy-1* having significantly higher transcript levels than L*er* at 24 h and 48 h imbibition.

ABA levels were measured in freshly-harvested seed to determine whether changes in ABA could explain the differences in dormancy between L*er* and *fy-1* and the changes observed in the examined transcripts during imbibition. ABA levels were significantly higher in *fy-1* than L*er* at the start of imbibition and remained higher, although with considerable variability between replicates, in *fy-1* at all sample times (Fig. 6). L*er* ABA moderately declined throughout imbibition while a sharp decline in *fy-1* ABA levels occurred in the interval between 24 h and 48 h imbibition.

Expression of ABI5 *in relation to* FY *during seed germination*

In view of the differences in *ABI5* transcripts in the two lines, *ABI5* transcripts were examined in freshly-harvested seed of L*er*, *fy-1*, and the previously described complemented lines, *C2*, *C6*, *AAC1*, and *AAC3*, that had rescued seed dormancy (Fig. 4). If the restoration of seed dormancy in the complemented lines was the result of restored *ABI5*, this should be apparent from the levels of *ABI5* in the tissue. Complementation of *fy-1* with either complete *FY* or PPLPP-mutated *FY* restored *ABI5* levels to that of the wild type, L*er* (Fig. 7).

Stratified L*er* and *fy-1* seeds were examined to determine how the two non-dormant lines responded, relative to *FY* and *ABI5* expression, to an ABA concentration sufficient to repress their germination. Stratified seed imbibed in water germinated rapidly, with complete germination occurring within 48 h. Ten μM ABA completely inhibited this germination. After stratification, *Fy* levels in L*er* were about 6-fold higher than in *fy-1* (Fig. 8A). Over the 72 h germination at 22 °C, there was no change in the *Fy* levels of the *fy-1* mutant, irrespective of the presence of ABA. In the case of L*er* stratified seeds imbibed in water, *Fy* levels declined to the levels in *fy-1* seed over the first 48 h of imbibition, remaining constant over the remaining 24 h. For L*er* seeds imbibed in ABA, *FY* levels were not significantly different from water-imbibed seed over the first 24 h, but were higher than water-imbibed seed over the remaining 48 h of measurement. *ABI5* levels in stratified seeds (Fig. 8A) were 5–20% of those in non-stratified seeds (Fig. 5). The *ABI5* levels in ABA-treated seeds were 2.5-fold higher than those in water-treated seeds at the end of stratification (Fig. 8A). The *ABI5* levels were relatively constant and similar for L*er* and *fy-1* over the course of the imbibition for water-treated seed. For ABA-treated seed, the two lines had the same *ABI5* levels at the start of the incubation. During the first 24 h in the presence of ABA, L*er* *ABI5* levels remained constant, while *fy-1* *ABI5* levels declined. After 24 h in ABA, the two lines showed parallel increases in *ABI5* levels over the remainder of the incubation, with the *fy-1* levels being consistently lower.

Histochemical analysis, using an *FY* native promoter fused to *GUS* (*pFY::GUS*) showed intense GUS staining in both the embryo and endosperm after 24 h imbibition of transgenic seeds in water (Fig. 8B).

Discussion

A component of the autonomous flowering pathway, FY, along with its interacting partners FCA, is known to regulate flowering in *Arabidopsis* (Simpson *et al.*, 2003). FY was identified through its interaction with FCA, and its molecular function was thought to be dependent on its

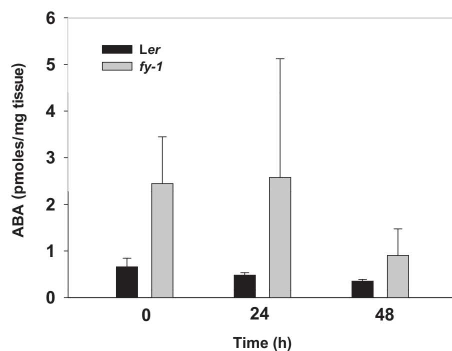


Fig. 6. ABA levels in freshly-harvested, imbibed L*er* and *fy-1*. Freshly-harvested seed of L*er* and *fy-1* were imbibed in water for various time periods and their ABA content measured. Three biological replicates of each line and time point were analysed. The results are expressed as means ±1.96 SE.

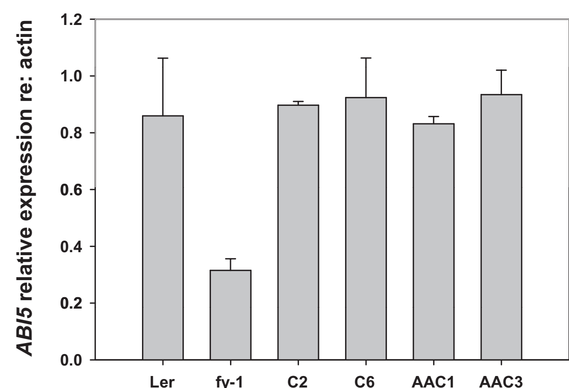


Fig. 7. *ABI5* levels in *FY*-complemented *fy-1*. Freshly-harvested seed of L*er*, *fy-1*, and the *FY*-complemented lines *C2*, *C6*, *AAC1*, and *AAC3* were imbibed in water for 24 h prior to extraction of RNA and real-time RT-PCR. Three replicates of each line were analysed and the results are presented as means ±1.96 SE.

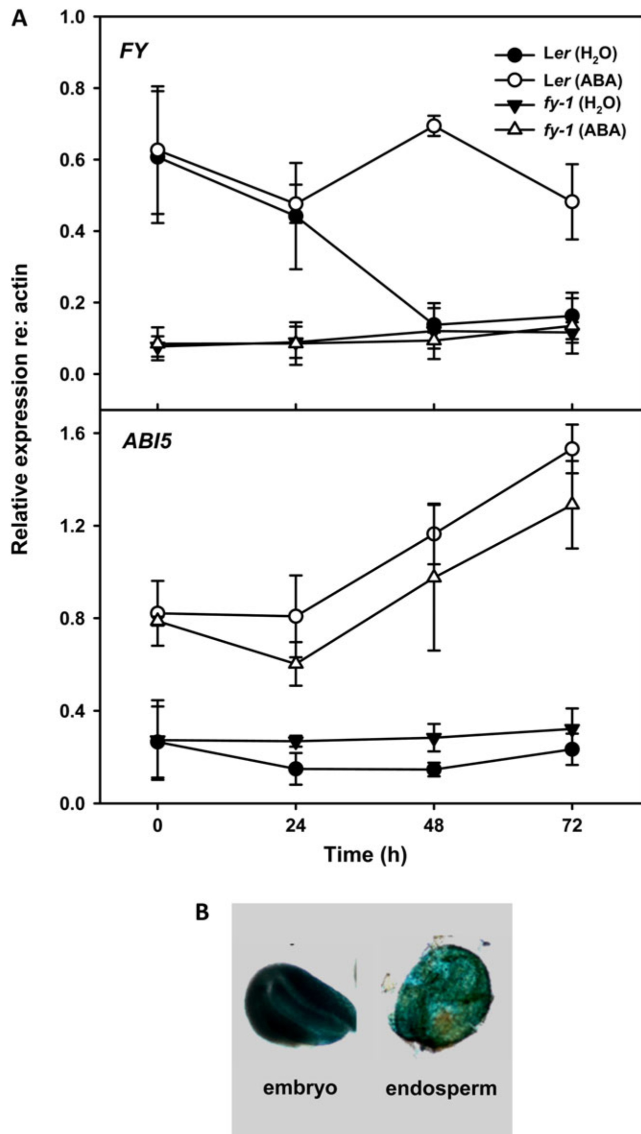


Fig. 8. *FY* and *ABI5* expression during seed germination. *FY* and *ABI5* transcript levels in stratified L*er* and *fy-1* seeds during imbibition in H_2O or 10 μM ABA. The values are means ±standard deviation from two biological replicates. (B) Tissue expression of *FY::GUS* in 1 d imbibed seeds.

interaction with FCA (Quesada *et al.*, 2003). Studies with *fy* mutants suggested that FY can affect various physiological functions (Henderson *et al.*, 2005) through the dynamic interaction complexes it can form with or without FCA (Quesada *et al.*, 2003; Manzano *et al.*, 2009).

The work reported here demonstrates that *FY* expression also affects seed dormancy and ABA sensitivity to germination in *Arabidopsis* seeds and that the mechanism by which FY accomplishes this affect differs from the mechanism by which FY regulates flowering in the autonomous pathway.

ABA insensitivity in Arabidopsis fy-1seeds

Freshly-harvested *fy-1* seeds germinated rapidly (Fig. 1A), whereas L*er* required 5 weeks of dry storage to achieve the same germination capacity as freshly-harvested *fy-1* (Fig. 1B). The two hormones, GA and ABA, are critical regulators of germination (Bentsink and Koornneef, 2008), with ABA considered to impose a block on germination. The differences in germination do not appear to be the result of ABA repression of germination as the ABA levels of freshly-harvested *fy-1* were significantly higher, not lower, than L*er* ABA at the start of imbibition (Fig. 6) and tended to be higher throughout the imbibition period. ABA levels in *fy-1* were considerably more variable than L*er*, probably reflecting variability in the effect of environment on seed status of the biological replicates relative to germination and dormancy in the non-dormant *fy-1*. ABA insensitivity in the *fy-1* mutant is the more likely cause of the phenotype as stratified *fy-1* seed had significantly less sensitivity to ABA than stratified L*er* (Fig. 1C) and *fy-1* embryo radicle growth was significantly less inhibited by ABA than L*er* (Fig. 1D). The germination phenotype of the wild-type parent can be rescued in both first generation progeny from reciprocal crosses between the mutant and L*er* (see Supplementary Fig. S2 at *JXB* online). This indicates nuclear control and suggests that ABA insensitivity in the mutant might be found in both the endosperm and embryo of the seed. *FY* is expressed both within the embryo and the endosperm of 1 d imbibed seeds (Fig. 7B). During seed development, *Arabidopsis FY* expression is confined to the embryo at the heart stage of development but is evident in both embryo and endosperm in the mature seed (Henderson *et al.*, 2005). The effect of *FY* expression on seed ABA sensitivity may, therefore, be the result of an event affected by *FY* during late embryogenesis and/or during stratification.

ABA catabolism and GA synthesis are considered key elements in controlling germination and dormancy (Debeaujon and Koornneef, 2000; Millar *et al.*, 2006). There were no significant differences in expression of two enzymes of ABA catabolism, *CYP707A1* and *CYP707A2*, between freshly-harvested, imbibed seed of L*er* and *fy-1* (Fig. 5). Similarly, there were no significant differences between L*er* and *fy-1* during imbibition for *NCED9*, an enzyme involved in ABA synthesis (Lefebvre *et al.*, 2006) in *Arabidopsis* seed.

Low GA levels result in an increase in ABA through stabilization of RGL2, a DELLA factor that represses germination. (Piskurewicz *et al.*, 2008). What is the relationship between FY, RGL2, and the regulation of germination? *RGL2* is low at the start of imbibition, with transcript levels increasing in both lines over the course of imbibition (Fig. 5), as increased metabolic activity commences. FY, as a 3'-polyadenylation factor, stabilizes transcript stability (Zhao *et al.*, 1999) and if *RGL2* stability were a factor in the ABA insensitivity of *fy-1*, then *RGL2* levels would be predicted to be lower than L*er* over the course of the imbibition and they are not. The higher relative transcript levels of the gene in *fy-1* during imbibition likely reflect the tendency towards higher levels of ABA in *fy-1* (Fig. 6), since ABA affects *RGL2* expression (Piskurewicz *et al.*, 2008). The significant increases in *GA3Ox1* and *GA30x2* in *fy-1* compared with L*er* in the first 24 h of imbibition (Fig. 5) suggest that significant increases

in *fy-1* GA levels might be expected. This should result in increased protein turnover of RGL2 (Piskurewicz *et al.*, 2008) in *fy-1*, but not in L*er*, during imbibition, which should relieve the *RGL2* repression which is observed (Fig. 1A). It has been suggested, however, that ABI5 acts as the final common repressor of germination in response to changes in ABA and GA levels (Piskurewicz *et al.*, 2008) and that *ABI5* levels are higher in L*er* at 24 h and 48 h of imbibition (Fig. 5). In this scenario, ABI5 would be the controlling factor regulating germination status. It is evident (Fig. 6) that a decline in ABA is not essential for the initiation of germination processes in *fy-1*.

Of the three ABA response transcription factors tested, *ABI3, ABI4*, and *ABI5*, only *ABI5* had consistently lower transcript levels during imbibition, with the differences being significant at the 24 h and 48 h imbibition periods (Fig. 5). There are two possible considerations as to why there is no significant difference in *ABI5* levels at the start of imbibition. The first possibility is that changes in *ABI5* that affect dormancy occur during imbibition when differences in the metabolism (Millar *et al.*, 2006) or amount (Ali-Rachedi *et al.*, 2004) of ABA between dormant and non-dormant seed influence ABA-induced synthesis of *ABI5* (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). Our examination of a few of the enzymes involved in ABA catabolism and biosynthesis (Fig. 5) are not definitive enough to support this possibility. The second possibility is that there is a relationship between *ABI5* levels in freshly harvested seed and seed dormancy, but the variable growth and harvest conditions of the various seed lots mask this effect in the biological replicates. Regardless of the lack of difference in the *ABI5* levels at time zero, the levels at 24 h and 48 h imbibition are significantly higher in L*er* (Fig. 5) and it is essentially dormant, while *fy-1* is in the process of germination (Fig. 1A). There are 5–15-fold differences in *ABI5* levels between dormant (Fig. 5) and non-dormant seed (Fig. 8), with the stratified seed having much lower levels. ABA promotes *ABI5* expression (Fig. 8), in agreement with previous work (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). This is also consistent with the tendency of *ABI5* to be lower in freshly-harvested *fy-1*, even though *fy-1* ABA levels tend to be higher than those in L*er* (Fig. 6), an effect that could be the result of lack of *ABI5* transcript stability in *fy-1*. ABI5 is expressed in the embryo and the micropylar region of the endosperm (Penfield *et al.*, 2006) and has been suggested to be the final common repressor of germination in response to changes in ABA and GA (Piskurewicz *et al.*, 2008), acting downstream of ABI3 (Lopez-Molina *et al.*, 2002). Furthermore, complementation of *fy-1*, with either fully-functional *FY* or PPLPP-mutated *FY* rescued both seed dormancy and wild-type *ABI5* levels.

ABA increased *ABI5* levels, as anticipated from previous studies (Lopez-Molina *et al.*, 2001; Finkelstein and Lynch, 2000), with no substantial changes in *FY* levels in stratified seeds imbibed in the absence and presence of ABA at sufficient concentrations to repress germination in both lines (Fig. 8). This is counterintuitive to what might be expected if *ABI5* levels were totally dependent on FY for transcript stability. *ABI5* may have been maintained as the result of ABA-induced increased *ABI5* transcription to offset the increased breakdown due to the inadequate 3'-polyadenylation. Furthermore, germination repression may be the result of the combined effects of ABA and ABI5 as it has been shown that the two can act synergistically in ABA-regulated events (Gampala *et al.*, 2002).

Based on the above considerations, *ABI5* transcript stability is a likely factor resulting in decreased ABA sensitivity of *fy-1*.

FY in relation to seed germination and flowering in Arabidopsis

Acknowledging that *fy-1*seeds do have an ABA insensitivity, possibly as a result of modified *ABI5* expression, does the mutation in FY that results in altered flowering in *Arabidopsis* operate via a similar mechanism to produce seed ABA insensitivity? Although there remain many unanswered questions, there is enough information to conclude that the pathways are different.

Complementation of *fy-1* with wild-type FY rescued both the germination and flowering phenotype (Fig. 2). PPLPP repeats in the C-terminal region of FY are important for regulating *FLC* levels in vegetative rosette tissue (Simpson *et al.*, 2003). The introduction of PPLPP-mutated *FY* (*AAFY)* into a *fy-1* loss-of-function background rescued inhibition of seed germination and ABA sensitivity, but not flowering in *fy-1* (Fig. 3), suggesting FY functions differently in seed germination than in the transition to flowering in the autonomous pathway. This is supported by the evidence that complementation of *fy-1* with wild type *FY* reduced *FLC*, late flowering, and inhibition of seed germination, whereas complementation with PPLPP-mutated *FY (AAFY)* in the transgenic *fy-1* mutant line had little effect on *FLC* or late flowering but still affected seed dormancy (Fig. 4). These findings suggest that the effect of *FY* on seed germination is not processed via *FLC* expression and that the PPLPP domains of FY are not required to regulate seed germination through ABA. FY interacts with FCA via the PPLPP motifs in the C-terminal region of FY and the WW domain of FCA, and this interaction is important for floral transition (Simpson *et al.*, 2003). FY, but not FCA, has a pleiotropic function in embryo development (Henderson *et al.*, 2005). The FCA–FY interaction is transient in *Arabidopsis* (Manzano *et al.*, 2009). PPLPP-mutated FY (*AAFY*), expressed in *fy-1*, is unable to interact with FCA, yet the expression of the mutated gene in *fy-1* rescued ABA-regulated seed germination, indicating that *FY* function relative to seed germination may not depend on *FCA.*

Acknowledgements

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