Regulation of storage protein gene expression in *Arabidopsis*

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Summary

The expression of seed storage proteins is under tight developmental regulation and represents a powerful model system to study the regulation of gene expression during plant development. In this study, we show that three homologous B3 type transcription factors regulate the model storage protein gene, *At2S3*, via two distinct mechanisms: FUSCA3 (FUS3) and LEAFY COTYLEDON2 (LEC2) activate the *At2S3* promoter in yeast suggesting that they regulate *At2S3* by directly binding its promoter; ABSCISIC ACID INSENSITIVE3 (ABI3), however, appears to act more indirectly on *At2S3*, possibly as a cofactor in an activation complex. In accordance with this, *FUS3* and *LEC2* were found to act in a partially redundant manner and differently from *ABI3* in planta: *At2S3* expression is reduced to variable and sometimes only moderate extent in *fus3* and *lec2* single mutants but is completely abolished in the *lec2 fus3* double mutant. In addition, we found that *FUS3* and *LEC2* expression patterns, together with an unsuspected regulation of *FUS3* by *LEC2*, enable us to explain the intriguing expression pattern of *At2S3* in *lec2* or *fus3* single mutants. Based on these results, we present a model of *At2S3* regulation and discuss its implications for other aspects of seed maturation.

Key words: Seed maturation, Transcriptional regulation, Storage protein, ABI3, FUS3, LEC2

Introduction

The emergence of seeds during evolution allowed the plant kingdom to colonise the emerging continents as water started to become limiting (Steeves, 1983). As a dry seed, the plant is able to pause its life cycle and withstand unfavourable conditions. This selective advantage is mainly conferred by a process called seed maturation that starts after embryo morphogenesis. During this developmental phase, the embryo acquires desiccation tolerance, enters a quiescent stage and accumulates important quantities of storage compounds in order to resume growth when conditions become more favourable (Goldberg et al., 1994; McCarty, 1995). Storage compounds such as lipids, carbohydrates and proteins accumulate in the endosperm, the aleurone layer or the embryo depending on the species (Gatehouse and Shirsat, 1993; Higgins, 1984). As in other brassicaceae, seeds of the model plant *Arabidopsis thaliana* mainly accumulate lipids and storage proteins (Mansfield and Briarty, 1992). 2S and 12S proteins are the major seed storage proteins (SSP) and represent up to one third of the *Arabidopsis* seed's dry weight (Baud et al., 2002; Guerche et al., 1990; Pang et al., 1988). SSP genes are highly expressed and tightly regulated both spatially and temporally. How this regulation is achieved is still poorly understood. Although important cis-elements have been mapped in different SSP promoters (Bustos et al., 1991; Chandrasekharan et al., 2003; Gatehouse and Shirsat, 1993), only a few trans-acting factors have been identified. Regulation of the maize zein SSP gene by OPAQUE2 and prolamin-box binding factor (PBF) is one of the best understood paradigms of SSP gene regulation (Vicente-Carbajosa et al., 1997). In dicotyledonous plants, an extensive analysis of the *napA* promoter from *Brassica napus* has been performed (Ezcurra et al., 1999; Stalberg et al., 1996). This promoter contains a B-box made of DistB (GCCACTTGTC) and ProxB (CAAACACC) elements, two RY (CATGCA) elements and a G-box (CACGTG). Mutations in each of these elements lead to a strong reduction of *napA* promoter activity in seeds but the transcription factors binding these elements remain to be determined. Obvious candidates for binding the RY elements are transcription factors from the B3 family. The B3 domain was first defined as a conserved domain between VIVIPAROUS-1 (VP1) from maize and ABI3 from *Arabidopsis* (Giraudat et al., 1992; McCarty et al., 1991). Its capacity to bind DNA in a sequence-specific manner has been shown in vitro using the isolated B3 domain of VP1 and the Sph motif from maize, which contains an RY element (Suzuki et al., 1997). The *Arabidopsis* ABI3 protein has never been shown to bind DNA but its role in regulating SSP genes has been established using *abi3* mutants and ectopic *ABI3* expression in vegetative tissues (Nambara et al., 1995; Parcy et al., 1997; Parcy et al., 1994). In addition, *ABI3* expression in tobacco seedlings activates *napA* expression via the B-box and the RY elements suggesting that ABI3 does bind directly to SSP gene promoters (Ezcurra et al., 2000). In *Arabidopsis*, the two B3 factors FUS3 and LEC2 are the closest ABI3 homologues and, like ABI3, they control various facets of seed maturation such as desiccation tolerance, developmental arrest of the embryo and storage compound accumulation (Bäumlein

et al., 1994; Keith et al., 1994; Luerssen et al., 1998; Meinke et al., 1994; Nambara et al., 2000; Nambara et al., 1995; Parcy et al., 1997; Parcy et al., 1994; Raz et al., 2001; Stone et al., 2001). Since SSP gene expression is reduced in *fus3* mutants and since FUS3 binds in vitro to the RY element present in the *Vicia faba* legumin promoter, it is very likely that FUS3 directly activates SSP gene expression (Reidt et al., 2000). In contrast, a direct role of LEC2 on SSP gene regulation has never been established. Storage protein inclusion bodies are missing from the tip of *lec2* cotyledons but this phenotype might be a consequence of *LEC2* early function in promoting cotyledon identity (Meinke et al., 1994; Stone et al., 2001). ABI3, FUS3 and LEC2 are thus potential direct regulators of SSP gene expression but their respective roles, modes of action and the nature of their interactions are still to be determined (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1997; Raz et al., 2001). Finally, the transcription factors acting through the distB and proxB regulatory elements of the *napA* promoter are unknown.

We combined the information obtained from the exhaustive characterisation of the *napA* promoter and the power of *Arabidopsis* genetics to study the regulation of the *At2S3* promoter in *Arabidopsis*, which is very similar to *napA* promoter. We initiated a one-hybrid screen in yeast to identify transcription factors able to interact with the different cis-regulatory elements. This screen yielded the two B3 factors FUS3 and LEC2 but not ABI3. This result led us to investigate the respective roles of all 3 homologous transcription factors in planta. We propose that ABI3 regulates *At2S3* expression indirectly and that *FUS3* and *LEC2* regulate *At2S3* directly in a partially redundant manner. The extent of this redundancy is determined by the overlapping but not identical expression patterns of *FUS3* and *LEC2* and by a so far unsuspected regulation of *FUS3* by *LEC2*.

Materials and methods

Plant material and culture conditions

fus3-3 glabrous1 and *abi3-6 glabrous1* mutants are derived from the *Arabidopsis thaliana* Columbia-0 (Col-0) accession, *lec2-1, -2, -3, -4* from the Wassilewskija (Ws) accession (Stone et al., 2001). Plants were grown in controlled growth chambers or a greenhouse as previously described (Bensmihen et al., 2002).

Yeast one-hybrid experiments

cDNA library

RNA was extracted from 8 g of 5-12 DAP (days after pollination) Col-0 siliques using a phenol-chloroform extraction (Parcy et al., 1994) followed by a clean-up with RNeasy midi kit (Qiagen, Hilden, Germany). After mRNA purification using an mRNA purification kit (Amersham-Pharmacia, Little Chalfont, UK), the cDNA library was built in the pAD-GAL4-2.1 vector with HybriZAP-2.1 XR cDNA synthesis kit and HybriZAP-2.1 XR library construction kit (Stratagene, La Jolla, USA). The cDNA library represented 7×10^6 independent cDNA clones.

Yeast reporter strains

The reporter constructs were made in the plasmid pYi2267OHIS (Blaiseau et al., 1997), which carries the *URA3* selection marker and the minimal *CYC1* promoter upstream of the *HIS3* reporter gene. The region –170 to –45 of the Col-0 *At2S3* promoter (relative to the transcription start), the RY-G-box complex (–95 to –48) (5′-ATCACTCATGCATGCATGCATTCTTACACGTGATTGCCATGCAAATCTCCC-3′) and the B-box (–169 to –132) (5′-ATCTGTTCGTCACTTGTCACTCTTTTCCAACACATAATCCC-3′) were cloned into the *Xho*I site of pYi2267OHIS, giving rise to the plasmids pYi22-1, pYi22-G and pYi22-B, respectively. These plasmids and the empty plasmid pYi2267OHIS were linearised and transformed into the yeast strain YM954 (Blaiseau et al., 1997) generating the strains At2S3::HIS3, RY-G::HIS3, B::HIS3 and Yi22-C, respectively. Stable prototroph transformants were selected on SD medium (Ausubel et al., 1989) lacking uracil, and analysed for correct integration by PCR analysis.

One-hybrid screen

The At2S3::HIS strain was transformed with the hybrid expression library, and colonies growing on SD medium –Leu, –His, +10 mM 3-aminotriazol (3-AT) were isolated. Plasmid DNA was recovered from these colonies and retransformed into At2S3::HIS3 and Yi22-C. Transformants were selected on SD medium –Leu and then tested on SD medium –Leu, –His, +10 mM 3-AT.

One-hybrid analysis

LEC2 and *FUS3* coding sequences were cloned into pCV70, a derivative of pRS315 (Sikorski and Hieter, 1989), which allows expression of HA-tagged proteins under the control of the *ADH* promoter. For GAL4-AD fusions, *LEC2*, *FUS3* and *ABI3* coding sequences were cloned in pDON201 and recombined in pDEST22-PC86 using the Gateway technology (Invitrogen, La Jolla, USA). The fusion between the VP16 activation domain (Parcy et al., 1998), a short ABI3 N-terminal piece (amino acids 3-13) and ABI3 B3 domain (aa 556-720) did not lead to a detectable activation of an *At2S3::LACZ* reporter construct built in the pKF1 vector (Parcy et al., 1998).

Electrophoretic mobility shift assays (EMSA)

EMSA were performed essentially as previously described (Bensmihen et al., 2002). *FUS3* and *LEC2* coding sequences were PCR amplified using oligonucleotides 5′-ATGATATCCATGGTTGATGAAAATGTGGAAACC-3′ and 5′-ATGATATCTAGTAGAAGTCATCGAGAG-3′ for *FUS3* and 5′-TCTAGAAAAATGGATAACTTCTTACCCT-3′ and 5′-GTCGACCCATATCACCACCACTCAAAGT-3′ for *LEC2*, cloned into PCRT7/CT-TOPO (Invitrogen) and sequenced. The resulting plasmids were used for in vitro transcription and translation in TNT®-rabbit reticulocyte system (Promega, Madison, USA). Probes and competitor DNA was obtained by annealing the following oligonucleotides (mutant bases are underlined):

5′-acacactcatgcatgcatgcattcttacacgtgattgccatgcaaat-3′ and 5′-ggagatttgcatggcaatcacgtgtaagaatgcatgcatgcatgagtgtgt-3′ (*At2S3* wild-type sequence –98 to –48 relative to the transcription start), 5′-acacactgtagacctgtagcattcttacacgtgattgccatgcaaat-3′ and 5′-ggagatttgcatggcaatcacgtgtaagaatgctacaggtctacagtgtgt-3′ (mutant RY1),

5′-acacactcatgcatgcatgcattcttacacgtgattgcgtacgaaat-3′ and 5′-ggagatttcgtacgcaatcacgtgtaagaatgcatgcatgcatgagtgtgt-3′ (mutant RY2),

5′-acacactgtagacctgtagcattcttacacgtgattgcgtacgaaat-3′ and 5′-ggagatttcgtacgcaatcacgtgtaagaatgctacaggtctacagtgtgt-3′ (mutant RYs),

5′-acacactcatgcatgcatgcattcttactgtccattgccatgcaaat-3′ and 5′-ggagatttgcatggcaatggacagtaagaatgcatgcatgcatgagtgtgt-3′ (mutant G-box).

Generation of transgenic plants

All transgenic plants were obtained by floral dip of *Arabidopsis* Col-0 (Clough and Bent, 1998). Plasmids were built according to standard molecular biology procedures (Ausubel et al., 1989). *At2S3::GFP* plasmid (pFP91) was built by assembling *At2S3* promoter fragment (–310 to +35 relative to the transcription start) to an optimised green fluorescent protein (GFP) coding sequence with a translational enhancer and an endoplasmic reticulum targeting signal (obtained from R. Blanvillain and P. Gallois) and the 35S terminator. Over 60 independent lines were generated and one representative line (FP91.54.3) was chosen for subsequent analyses. LEC2::GUS

plasmid (pTK-DE111) was built by inserting the *LEC2* promoter (–2020 to +5 relative to ATG of *LEC2*) into pDE-GUS vector (Parcy et al., 1994). Three independent lines were generated that showed the same expression profile. The FUS3::GUS reporter plasmid was generated by inserting the *FUS3* promoter (–2100 to +44 relative to ATG of *FUS3*) into pDE-GUS. 12 plants were generated of which 11 showed a similar expression profile.

Expression analyses

Northern blot analysis was performed as described previously (Parcy et al., 1997). Gene-specific probes were PCR amplified from genomic DNA with the following oligonucleotides: 5′-CTTCAACATC-CCTTCATTCCCTT-3′ and 5′-TCTTATTTATTAAGTAGTGCTT-3′ (*At2S1*), 5′-TCCAGACCACCATCCCTTTCTT-3′ and 5′-GACAA-CCTAGAGAGAGCATA-3′ (*At2S2*), 5′-TTCCAGATCCCTTCAAT-CCCTT-3′ and 5′-AACATAAACAAACCTCTCTTA-3′ (*At2S4*), 5′-GCCGCCTTTGAGGGGCCAGA-3′ and 5′-CCTTGTGGTACGGC-TATGAG-3′ (*CRA*), 5′-CACCCTTGAGACGCGGCGAA-3′ and 5′-CCTTGTGGCACGACTAGTAA-3′ (*CRB*) and 5′-AGACCTTCAT-GGACTCGCAG-3′ and 5′-GCATGTCACGGAACCCTTGTTG-3′ (*CRC*). *At2S3*-specific probe was obtained by subcloning a fragment corresponding to nucleotides 536 to 690 from gene At4G27160.1 into PCRII-TOPO (Invitrogen, La Jolla) generating pTOPAT2S3. RT-PCR analysis was performed on silique total RNAs extracted using the RNeasy mini kit (Qiagen). The following oligonucleotides were used for PCR amplification:

5′-GGACCTTCAAGTATAGGTACTGG-3′ and 5′-CCATCGAA-TGTTCCGAACTTGGAGT-3′ for *FUS3*,

5′-ATGCCCCAGGACATCGTGATTTCAT-3′) and 2 (5′-TTGGC-GGCACCCTTAGCTGGATCA-3′) for *EF1α* and

5′-GTATGTCCTCGAGAACACAGGAG-3′ and 5′-TCAAAGT-CGTTAAAGCTCACCTGAT-3′ for *LEC2*. We verified that amplification was still in its linear range by monitoring amplification products at multiple cycles. Western blot analysis of ABI3 protein was performed as previously described (Parcy et al., 1994). A total protein extract corresponding to 30 seeds at 15 DAP was loaded from each genotype. In situ hybridisation was performed as described previously (Bensmihen et al., 2002) using the *At2S3*-specific probe. GUS assays were performed as described (Parcy et al., 1998). Stained embryos were cleared in Hoyers solution (2.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerol and 30 ml water) and observed under differential interference contrast microscopy. In order to observe both *At2S3::GFP* and FUS3::GUS signals, embryos were extracted from the seed coat and maintained between a slide and a coverslip spaced by pieces of coverslips in order to create a thin cavity. Buffers were changed by capillarity.

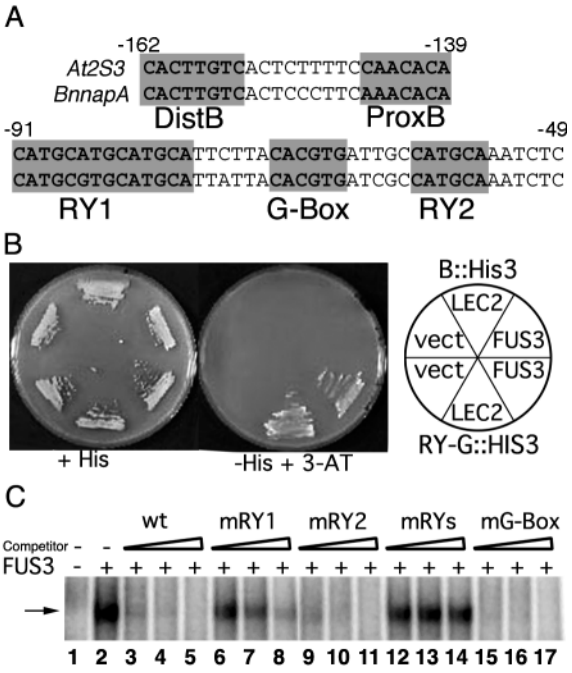

Fig. 1. FUS3 and LEC2 activate the *At2S3* promoter. (A) Comparison of nucleotide sequences of cis-elements present on *At2S3* (upper sequence) and *Brassica napus napA* (bottom sequence) promoters. The B-box element contains DistB and ProxB, the RY-G-box complex contains a G-box surrounded by 2 RY motifs. (B) FUS3 and LEC2 bind to the RY-G-box complex in the *At2S3* promoter. Yeast reporter strains B::HIS3 and RY-G::HIS3 carrying either the control plasmid pCV70 or expressing FUS3 or LEC2 were streaked (as depicted on the right) on medium containing histidine (+His) or restrictive medium (–His, + 1 mM 3-AT). (C) FUS3 protein forms a gel retardation complex (arrow) with both RY elements. FUS3 protein was incubated with a radiolabelled fragment of the *At2S3* promoter (–98 to –48 relative to transcription start) containing both RY elements and the G-box. The reactions contain in vitro transcribed and translated control plasmids (lane 1) or *FUS3* expression plasmid (lanes 2-17). Non-labelled competitor DNA (see Materials and methods) was added in 5-fold molar excess (lanes 3, 6, 9, 12, 15), 10-fold molar excess (lanes 4, 7, 10, 13, 16) and 20-fold (lanes 5, 8, 11, 14, 17). Competitor DNA used was wild type (lanes 3-5), mutant RY1 (lanes 6-8), mutant RY2 (lanes 9-11), mutant RYs (lanes 12-14) and mutant G-box (lanes 15-17).

Results

FUS3 and LEC2 bind and activate the *At2S3* promoter in yeast

To identify factors binding the *At2S3* promoter, we generated a reporter construct containing *At2S3* regulatory sequences (Fig. 1A), upstream of the *HIS3* gene. This construct was integrated into the yeast genome and one yeast strain (At2S3::HIS3) was chosen that exhibited a leaky His+ phenotype that was suppressed by the addition of 1 mM 3-AT to the medium. We also constructed a yeast expression library using cDNAs synthesised from maturing silique mRNAs in a vector allowing fusions to the yeast GAL4 activation domain (GAL4-AD). After transformation of the library into the At2S3::HIS3 strain, 7×10^6 transformants were screened for their ability to grow in the absence of histidine and in the presence of 10 mM 3-AT. From the 37 clones able to grow under these restrictive conditions, 17 were confirmed by recovery of plasmid DNA and retransformation of the At2S3::HIS3 strain. Sequencing of the 3′ and 5′ ends of the 17 plasmid inserts showed that 13 clones contained *FUS3* and four *LEC2* cDNAs (Luerssen et al., 1998; Stone et al., 2001). All of the *LEC2* cDNAs were out of frame with the fragment encoding the GAL4-AD, suggesting that LEC2 might possess its own activation domain. We tested this hypothesis by expressing both LEC2 and FUS3 without the heterologous activation domain in the At2S3::HIS3 strain. Both of them conferred growth on restrictive medium, showing that LEC2 and FUS3 were able to both bind to and activate the *At2S3* promoter in yeast (data not shown). In order to determine which cis-element in the *At2S3* promoter was essential for FUS3 and LEC2 transactivation, we generated 2 yeast reporter strains carrying either the RY-G-box complex (RY-G::HIS3) or the B-box element (B::HIS3) upstream of the *HIS3* reporter gene. While the RY-G::HIS3 strain grew on restrictive medium

when transformed with *FUS3* or *LEC2*, the B::HIS3 strain did not (Fig. 1B). This indicated that both factors could bind the RY-G-box complex but not the B-box nor any other sequence motifs upstream of the *HIS3* gene. In order to map more precisely the binding sites of these factors, we produced FUS3 and LEC2 proteins in vitro and performed electromobility shift assay using the RY-G-box complex as probe (Fig. 1C). While we did not succeed in obtaining any retardation with the LEC2 protein, FUS3 was found to bind both RY motifs in the RY-G-box complex with a preference for RY1. The G-box was not required for FUS3 binding.

ABI3 does not activate the *At2S3* promoter in yeast

FUS3 and LEC2 both belong to a family of plant-specific transcription factors that contain a B3 DNA-binding domain (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). Comparisons of their B3 amino-acid sequence with other family members revealed that LEC2, FUS3 and ABI3 define a subgroup in the family of B3 domain proteins, with each of them being equally homologous to one another (data not shown). Although ABI3 is known to be a positive regulator of *At2S3* expression in seeds, it was not identified in our one-hybrid screen, suggesting that ABI3 does not interact directly with the *At2S3* promoter. In order to test this possibility, we expressed GAL4-AD fusions of all three B3 factors in the At2S3::HIS3 yeast strain and tested the transformants for growth on restrictive medium. While GAL4-AD:LEC2 and GAL4-AD:FUS3 were found to transactivate the *At2S3* promoter, Gal4-AD:ABI3 was inactive (data not shown). We repeated this experiment using different reporter constructs and different ABI3 expression vectors, and we verified ABI3 expression by western blot analysis in some cases (data not shown). We never obtained *At2S3* activation by ABI3 (nor by the isolated B3 domain fused to an activation domain), which supports our hypothesis that ABI3 does not bind to the *At2S3* promoter in yeast.

ABI3, *FUS3* and *LEC2* regulate *At2S3* expression differently in planta

The results of our yeast experiments, which suggested a direct and similar role for *FUS3* and *LEC2* and an indirect role for *ABI3* in regulating *At2S3*, prompted us to analyse their respective roles in planta. We analysed *At2S3* regulation in severe *abi3*, *fus3* and *lec2* mutant alleles. We used the *abi3-6* mutant allele that was previously shown to contain a large deletion at the *ABI3* locus (Nambara et al., 1994). We sequenced this locus and found that the C terminus of the *ABI3-6* coding sequence is not in frame with the short N-terminus. *Abi3-6* is thus the only *abi3* allele that lacks the three B1, B2 and B3 domains. We used the null *lec2-1* allele in the Ws background (Stone et al., 2001). We observed, based on the anthocyanin accumulation patterns in mutant seeds, that the phenotype of *lec2-1* seeds is extremely variable from seed to seed. The same was true for *lec2-2*, *-3* and *-4* (data not shown). We used *fus3-3* in the Col-0 background (Luerssen et al., 1998) which also shows a phenotype of variable intensity. Unless otherwise indicated, *abi3*, *lec2* and *fus3* will, from now on, refer to *abi3-6*, *lec2-1* and *fus3-3*, respectively.

In order to easily follow the temporal and spatial patterns of *At2S3* activation, we built a transgenic *Arabidopsis* line carrying a fusion between the *At2S3* promoter and the coding sequence of the GFP, and crossed this *At2S3::GFP* line to the *abi3*, *lec2* and *fus3* mutants. We first used northern blot analysis to measure the quantitative effects of all 3 mutations on *At2S3* and *At2S3::GFP* mRNA levels (Fig. 2A). We found that *At2S3* and *GFP* expressions were most reduced in *abi3* (over 60 fold) and to a lesser extent in *fus3* (8-9 fold) and *lec2* (1.5-2.5 fold) (Fig. 2 and data not shown). Because each of the mutations reduced the *At2S3* and *GFP* mRNA levels by a similar factor, we concluded that all three B3 factors reduced *At2S3* mRNA steady-state level by affecting the *At2S3* promoter activity. We used the *At2S3::GFP* line to determine how the three different mutations differentially affect the spatial expression of *At2S3*. In the wild-type background, *At2S3::GFP* fluorescence started at torpedo stage in the embryo axis, spread in the whole embryo at the end cotyledon stage and ended up stronger in cotyledons than in the axis of the dry seed (16-18 DAP) (data not shown and Fig. 3I). In addition, fluorescence was absent from the root meristem and detectable in the endosperm layer of the dry seed. This dynamic expression pattern is similar to what has been described for *Brassica napus* SSP genes (Fernandez et al., 1991). In *abi3* mutant embryos, *At2S3::GFP* fluorescence was consistently and strongly reduced as compared to wild-type embryos (Fig. 3B,F). It was detected at low levels in the embryo axis and in the centre of cotyledons and undetectable

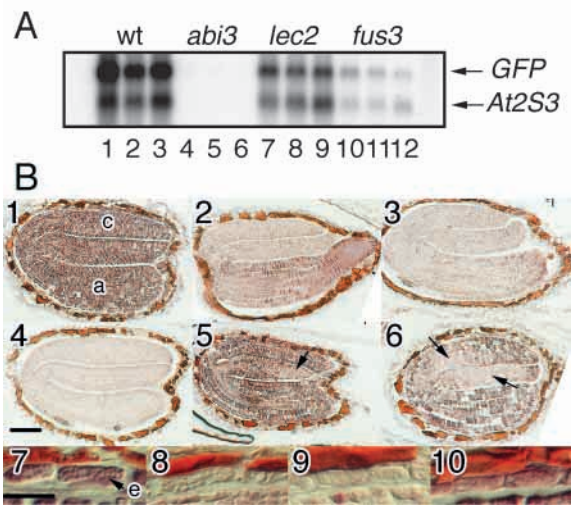

Fig. 2. *abi3*, *fus3* and *lec2* mutations affect *At2S3* expression in seeds. (A) Northern blot analysis of *At2S3* and *At2S3::GFP* expression. All plants analysed are homozygous for the *At2S3::GFP* transgene introduced in wild-type Col-0 (lanes 1-3), *abi3* (lanes 4-6), *lec2* (lanes 7-9) and *fus3* (lanes 10-12). Total RNA was extracted from 11 DAP siliques (lanes 1, 4, 7, 10), 12 DAP siliques (lanes 2, 5, 8, 11) and 13 DAP siliques (lanes 3, 6, 9, 12). (B) The *At2S3* expression pattern is altered in *abi3*, *fus3* and *lec2* mutants. Non-radioactive in situ hybridisation with an *At2S3* probe was performed on 11-13 DAP seeds of the same genotypes as in A: Col-0 (panels 1 and 7), *fus3* (panels 2, 3, 8), *abi3* (panels 4 and 9), and *lec2* (panels 5, 6, 10) homozygous for the *At2S3::GFP* transgene. In wild type, expression was detected in both cotyledons (c), the embryo axis (a) and endosperm cell layer (e). Arrows in panels 5 and 6 indicate sectors of *lec2* cotyledons with reduced *At2S3* expression. Scale bars: 50 μm (1 to 6) and 25 μm (7-10).

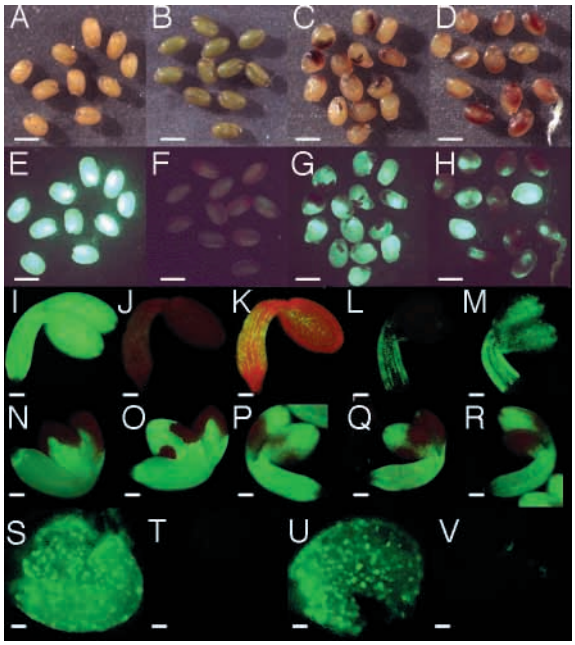

Fig. 3. *At23S::GFP* expression pattern in *abi3*, *fus3* and *lec2* mutants. Seeds homozygous for the *At2S3::GFP* transgene were used to visualise the *At2S3* promoter activity in wild-type Col-0 (A,E,I,S), *abi3* (B,F,J,K,T), *fus3* (D,H,L,M,V), *lec2* (C,G,N-R,U). (A-D) Seeds harvested just before complete desiccation (yellow siliques, 15-16 DAP) visualised under white light. (E-H) Green fluorescence of the seeds shown in A-D. (I-R) Green fluorescence of isolated embryos extracted from seed coats at 11-13 DAP. All embryo images were taken with the same exposure except K ,which is identical to picture J but exposed 10 times longer to visualise the low *At2S3::GFP* fluorescence present in *abi3*. (S-V) Green fluorescence signal present in the seed envelope at 16 DAP and arising from the endosperm layer. Scale bars: 500 μm (A-H) and 50 μm (I-W).

in the endosperm (Fig. 3J,K,T). *Fus3* and *lec2* mutations lead to very variable phenotypes (Fig. 3C,D,G,H). In the *lec2* mutant, the fluorescence was often slightly reduced throughout the embryo but the most striking phenotype was the presence of sectors accumulating anthocyanins where the fluorescence was totally absent (Fig. 3N-R). These sectors varied in shape and size between embryos from the same silique and between cotyledons of the same embryo (Fig. 3O,Q,R). In mature *lec2* seeds, endosperm fluorescence was not reduced (Fig. 3U). *At2S3::GFP* fluorescence was also reduced in *fus3* mutant embryos. This reduction was sometimes very mild, making *fus3* seeds hardly distinguishable from wild types (data not shown) while in the most severe *fus3* embryos, the fluorescence was completely absent at the cotyledon periphery, very weak in the cotyledon centre and reduced in the embryo axis (Fig. 3L,M). Endosperm fluorescence was almost abolished in *fus3* mutants (Fig. 3V). Our analyses of the *At2S3::GFP* transgenic line were essentially confirmed by in situ hybridisation showing that the *At2S3::GFP* reporter faithfully reproduced *At2S3* expression. *At2S3* mRNA was undetectable in *abi3* embryos (Fig. 2B-4) and *fus3* cotyledons (Fig. 2B-2). It was either undetectable or reduced in *fus3* axis and in discrete regions of *lec2* cotyledons (Fig. 2B-3, -5, -6). Expression in the endosperm layer, which was more difficult to detect with confidence, was often observed in Col-0 and *lec2* but always absent from *abi3* and *fus3* endosperm (Fig. 2B-7 to 2B-10). In summary, our analysis of *At2S3* expression in seeds showed that all three B3 regulators are important but to different extents: *ABI3* has a major role in regulating *At2S3*, whereas, *FUS3* and *LEC2* appear to be dispensable in some parts of the embryo.

At2S3 expression is abolished in *lec2 fus3* double mutants

The variable and sometimes mild alterations of *At2S3* expression in *fus3* and *lec2* mutants, together with the similar activity of the FUS3 and LEC2 proteins in yeast, suggested that both factors might act in a redundant manner in plants. We tested this hypothesis by generating a *lec2 fus3* double mutant in the *At2S3::GFP* background. *lec2 fus3 At2S3::GFP* embryos were small, not folded and never showed any *At2S3::GFP* fluorescence (Fig. 4A-E). Among F_2 seeds from the *lec2* × *fus3* cross, we also observed some small seeds with a very low, but detectable level of *At2S3::GFP* fluorescence. Seedlings derived from these seeds were genotyped as *fus3/fus3 lec2/LEC2* or *fus3/FUS3 lec2/lec2* showing a gene dosage effect in *fus3* or *lec2* mutant background. Northern blot analysis performed on *lec2 fus3* siliques confirmed that *At2S3* mRNA was drastically reduced in this double mutant (data not shown and Fig. 4). The complete absence of *At2S3::GFP* fluorescence in *lec2 fus3* seeds suggested that ABI3 was not sufficient to induce *At2S3*. To test whether ABI3 was still expressed in this double mutant, we performed western blot analysis with antibodies directed against ABI3 (Fig. 4G). Because *lec2 fus3* embryos are smaller and contain reduced level of protein compared to wild-type embryos, we normalised the loading with respect to seed number and not to protein content. We found a detectable, albeit reduced, ABI3 level in *lec2 fus3* indicating that ABI3 protein is present but unable to induce *At2S3* expression in the absence of functional FUS3 and LEC2 proteins. We wondered whether other SSP genes are regulated in the same way as *At2S3*. In order to test this, we designed specific probes for four *At2S* genes and three cruciferin genes (Guerche et al., 1990; Pang et al., 1988) and analysed SSP gene expression in *abi3*, *fus3*, *lec2* and *lec2 fus3* mutants. We found that the expression of all genes was reduced to a variable extend in *fus3* and *lec2* single mutants but drastically reduced in *lec2 fus3* (Fig. 4F).

FUS3 and *LEC2* expression patterns

At2S3 expression was abolished in the *lec2 fus3* double mutant but still detectable in *fus3* or *lec2* single mutants suggesting that *FUS3* and *LEC2* acted in a partially redundant manner. If this were the case, and one regulator could compensate for the loss of the other, the presence of sectors devoid of *At2S3* expression in *lec2* and *fus3* single mutants would indicate that *FUS3* and *LEC2* expression were not uniform. In order to investigate the spatial expression patterns of *FUS3* and *LEC2*, we generated transgenic Col-0 plants carrying the *GUS* reporter gene under the control of 5′ regulatory sequences of *FUS3* or *LEC2*. FUS3::GUS and LEC2::GUS activities were very similar during early embryo development: they were first detected in the suspensor of early globular embryos and then present throughout the embryo from the late globular until the heart seed stage

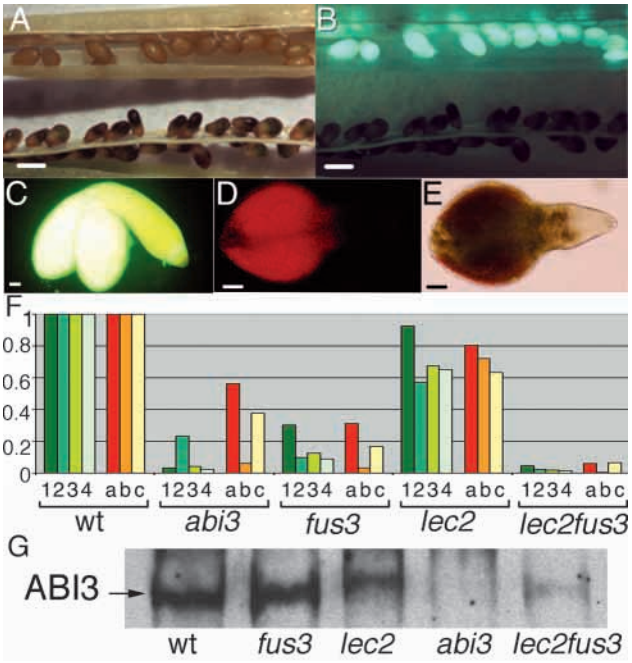

Fig. 4. Storage protein gene expression is dramatically reduced in *lec2 fus3* double mutant. (A,B) *At2S3::GFP* seeds in Col-0 background (top) and *lec2 fus3 At2S3::GFP* seeds (bottom) at 16 DAP visualised under white light (A) or blue light (B). (C-E) Green fluorescence in *lec2 fus3 At2S3::GFP* (D) embryos as compared to *At2S3::GFP* Col-0 (C) at 14-16 DAP. (E) Same embryo as in D but under white light to show the presence of green and purple pigments in cotyledons. (F) Quantification of storage protein gene expression by northern blot analysis of seed RNA from wild-type, *abi3*, *fus3*, *lec2* and *lec2 fus3* seeds in the *At2S3::GFP* background. Specific probes used were *At2S1* (lanes 1), *At2S2* (lanes 2), *At2S3* (lanes 3), *At2S4* (lanes 4), *CRUCIFERIN A* (lanes a), *CRUCIFERIN* B (lanes b), *CRUCIFERIN* C (lanes c). (G) Analysis of ABI3 expression by western blot of total protein extracts from wild-type, *abi3*, *fus3*, *lec2* and *lec2 fus3* seeds.

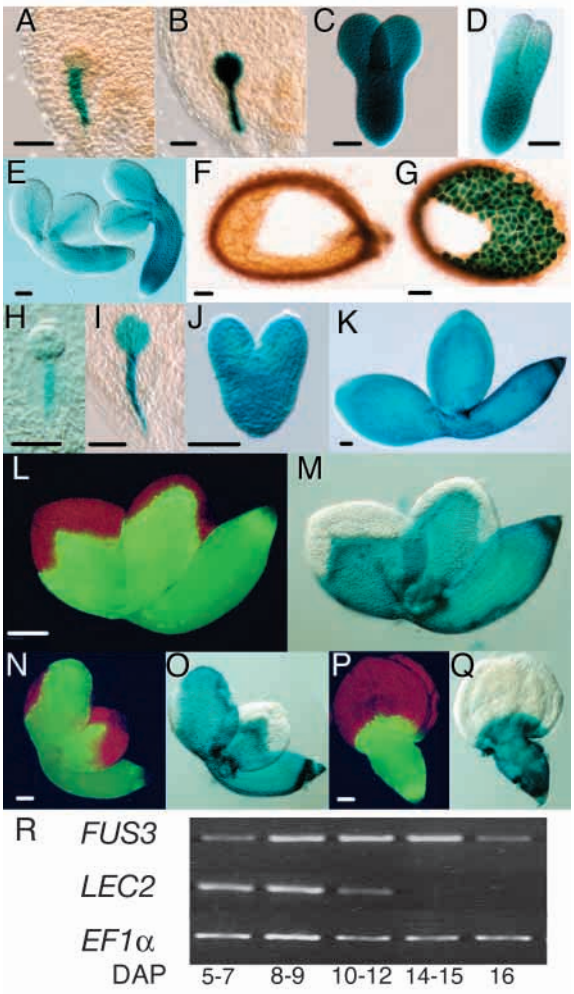

Fig. 5. *FUS3* and *LEC2* promoter activities in embryos and endosperm. LEC2::GUS (A-F) and FUS3::GUS (G-K) activities were assayed in whole seeds (A,B,H,I: globular stages), isolated embryos (C,D, torpedo stages; E, early mature embryos around 12 DAP; J, heart stage; K, mature embryo before desiccation) or sections of seed envelopes (F,G). FUS3::GUS (M,O,Q) expression pattern and *At2S3::GFP* green fluorescence (L,N,P) were compared in individual *lec2* mutant embryos just before desiccation (at 15-16 DAP). Scale bars: 50 μm. (R) Time course of *FUS3* and *LEC2* mRNA expression during silique development analysed by RT-PCR. The developmental stage of samples is indicated in DAP. *EF1α* was used as a control.

(Fig. 5A,B,H,I,J). Afterwards FUS3::GUS activity remained uniform throughout the embryo until the dry seed stage (Fig. 5K). In contrast, *LEC2::GUS* expression began diminishing in the tip of nascent cotyledons at the early torpedo stage (Fig. 5C,D) to become absent from the outer part of the cotyledons at the bent-cotyledon stage (Fig. 5E). In nearly dry seeds, LEC2::GUS activity was either undetectable or confined to the embryo axis and the vascular tissues of cotyledons (data not shown). FUS3::GUS activity was also detectable in the endosperm until the dry seed stage (Fig. 5G) whereas LEC2::GUS activity was weak in the endosperm around 11-12 DAP and undetectable in the endosperm of dry seeds (Fig. 5F). Analysis of *FUS3* and *LEC2* mRNA levels by RT-PCR were consistent with our results obtained from GUS reporter plants: *FUS3* mRNA was detectable from 5 DAP until the dry seed stage, whereas *LEC2* mRNA levels decreased at 12 DAP to become undetectable in dry seeds (Fig. 5R). The *LEC2::GUS* spatial expression pattern after the bent cotyledon stage is extremely similar to the *At2S3::GFP* expression domain in *fus3* embryos (compare Fig. 3M and Fig. 5E), suggesting that the *LEC2* expression pattern determines *At2S3* expression in the *fus3* mutant. In contrast, the uniform expression of *FUS3* in wild-type embryos was in sharp contrast with *At2S3::GFP* expression in *lec2* mutants and, therefore, did not provide an explanation for why *At2S3* was missing from sectors of *lec2* mutant embryos. We then examined whether *FUS3* expression might be modified by the *lec2* mutation by introducing *FUS3::GUS* into a *lec2 At2S3::GFP* background and found that FUS3::GUS activity was absent from sectors of varying size in *lec2* cotyledons. We designed an experimental protocol (see Materials and methods) to observe both *At2S3::GFP* fluorescence and FUS3::GUS activity in individual embryos. This experiment revealed a striking coincidence between sectors expressing *FUS3::GUS* and sectors expressing *At2S3::GFP* (Fig. 5L-Q).

Discussion

FUS3 and LEC2 function similarly and differently from ABI3 in yeast

We have studied how the three B3 proteins ABI3, FUS3 and LEC2 regulate the expression of SSP genes such as *At2S3*. Despite the fact that they are the most closely related B3 factors, only FUS3 and LEC2, and not ABI3, activated the *At2S3* promoter through the RY-G-box complex in yeast. LEC2 and FUS3 were able to activate the *At2S3* promoter without any heterologous activation domains, showing that they are capable of binding this cis-element and activating transcription (possibly via their C-terminal acidic domain). We have shown, in vitro, that FUS3 binds to both RY motives present in the *At2S3* promoter. FUS3 protein was previously shown to bind the RY element from a *Vicia faba* legumin promoter (Reidt et al., 2000) but had never been tested on the *At2S3* promoter that contains two RY elements interspaced by a G-box. Since FUS3 interactions with the *At2S3* promoter do not require the G-box, this regulatory sequence probably mediates the binding of another transcription factor. We did not succeed in obtaining any gel retardation of *At2S3* promoter sequences with LEC2 protein despite the fact that the protein was efficiently produced in vitro. Nevertheless we suspect that LEC2 is able to bind to the RY element directly and that the in vitro synthesised LEC2 protein was inactive or that our in vitro conditions were not suitable for detecting LEC2 binding. Also, we cannot exclude the possibility that proteins present in yeast enhance this binding in one-hybrid assays. Comparison of the amino acid sequence of the B3 domains of ABI3, FUS3 and LEC2 proteins shows that they are equally related to each other and, consequently, do not provide any explanation for why ABI3 does not work in yeast. Direct DNA binding has been shown in vitro for a truncated version of VP1, the maize orthologue of ABI3 (Suzuki et al., 1997). We have tested several truncated versions of the ABI3 protein in yeast and none of them activated the *At2S3* promoter (C.V., T.K. and F.P., data not shown). It is therefore possible that ABI3, on its own, is unable to bind RY motives directly. Emerging models of late embryogenesis abundant (LEA) gene regulation by ABI3 suggest a basic leucine zipper (bZIP) protein (such as ABI5) that would interact with a G-box and recruit ABI3 at the promoter level (Nakamura et al., 2001). Along the same line, recent work shows that two *Arabidopsis* bZIP proteins related to OPAQUE2 induce SSP gene expression synergistically with ABI3 and are capable of binding to ABI3 and to a G-box present in the *At2S1* promoter (Lara et al., 2003). These results complement our data and are in favour of ABI3 acting as a co-activator, tethered to the SSP promoter by BZIP proteins (Fig. 6B). Based on the presence of a B3 domain in ABI3 and of RY elements in the *At2S* promoters, it had been previously proposed that ABI3 would act by binding the RY motives (Ezcurra et al., 2000). We now speculate, based on the additional data, that the RY motives might be bound by LEC2 and FUS3 but not by ABI3.

In plants, *FUS3* and *LEC2* act in a partially redundant manner and differently from *ABI3*

Our in planta results also suggest that *LEC2* and *FUS3* function similarly, whereas *ABI3* acts in a different manner. Using various techniques, we have shown that *ABI3*, *FUS3* and *LEC2* positively regulate the expression of *At2S3*. However, whereas the *abi3* mutation has a strong and consistent effect on *At2S3* expression, *fus3* and *lec2* have milder and very variable effects. This variability is also observed for mutant phenotypes such as the accumulation of anthocyanins and was previously noted for *lec2* but not for *fus3* (Meinke et al., 1994; Stone et al., 2001). Because we observed strong variability in anthocyanin accumulation for all four of the *lec2* alleles, including two bearing large deletions, we are convinced that variability is a true feature of loss of *LEC2* function and is not due to leakiness of the mutations. The variability disappears in a *lec2 fus3* double mutant where *At2S3* expression is consistently abolished. This result, together with the similar FUS3 and LEC2 activities in yeast, strongly suggests that *FUS3* and *LEC2* act in a partially redundant manner in planta. The *lec2 fus3* phenotype also shows that *LEC2* is able to partially compensate the loss of *FUS3* in *fus3* mutants and, similarly, that *FUS3* compensates for the loss of *LEC2* in *lec2* mutants. The phenotypic variability of single mutants thus appears to be due to a variable redundancy between these two genes.

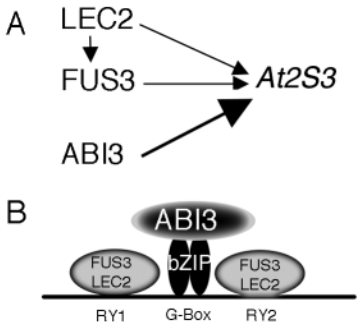

Fig. 6. Models for SSP gene regulation. (A) *ABI3*, *FUS3* and *LEC2* positively regulate *At2S3* expression. We propose that FUS3 and LEC2 act directly in a similar and partially redundant manner. ABI3 is also crucial for *At2S3* expression but functions differently from FUS3 and LEC2. In addition, *LEC2* is needed to ensure *FUS3* uniform expression in wild-type embryos. (B) Speculative model of how FUS3, LEC2 and ABI3 proteins might be acting according to our study and data presented by Lara et al. (Lara et al., 2003).

Several lines of evidence indicate that *ABI3* regulates *At2S3* expression by a different mechanism than *LEC2* and *FUS3*. First, the ABI3 protein present in the *lec2 fus3* double mutant is unable to compensate for the loss of *FUS3* and *LEC2* functions, indicating that, despite its B3 and activation domains, ABI3 is not sufficient to activate *At2S3*. In agreement with this conclusion, we observed that transformation of *lec2 fus3 At2S3::GFP* plants with a *35S::ABI3* construct did not yield any fluorescent seed, whereas transformations with *35S::LEC2* or *35S::FUS3* did (G.S. and F.P., unpublished). Similarly, we observed using RT-PCR analysis that *FUS3* and *LEC2* are normally expressed in *abi3* mutant seeds (data not shown) and yet unable to compensate for the lack of ABI3 and fully induce *At2S3*. However, FUS3 and LEC2 are not completely inactive in the absence of ABI3 since they are responsible for the low *At2S3* expression level present in *abi3* mutant (F.P., unpublished). Based on these results and on previous studies on the regulation of the *At2S* and *napA* promoters (Ezcurra et al., 2000; Lara et al., 2003) we propose a speculative model (Fig. 6B), which predicts that the activation of *At2S3* would require three types of proteins: ABI3, FUS3/LEC2 and bZIP10/bZIP25. While, according to the model, only FUS3/LEC2 and the bZIPs interact directly with the RY-G-box complex, ABI3 is tethered to the promoter

through interactions with the bZIPs. FUS3/LEC2 are necessary for *At2S3* activation. When the FUS3/LEC2 binding sites are mutated (Ezcurra et al., 2000), or in the *lec2 fus3* double mutant, *At2S3* expression is abolished. When the G-box motif is mutated, so that the bZIP proteins cannot recruit ABI3 to the RY-G-box complex or when ABI3 itself is inactivated, transactivation of the promoter is drastically reduced. Nevertheless, FUS3 and LEC2 alone are sufficient to slightly activate *At2S3* even in the absence of ABI3 as observed in yeast or in the *abi3* mutant. Other SSP genes seem to be regulated via the same mechanisms as *At2S3* since their expressions levels in the *lec2 fus3* double mutant are also drastically reduced when compared to wild type. Their regulation might, however, differ slightly from one SSP gene to another because the effects of single mutations on individual genes are not identical. *At2S1*, for example, is less affected by *fus3* or *lec2* single mutations than other *At2S* genes, probably because *At2S1* is specifically expressed in the embryonic axis (Guerche et al., 1990) where the effects of the *fus3* and *lec2* single mutations are the weakest (as judged by *At2S3* expression).

FUS3 and *LEC2* expression patterns explain *lec2* and *fus3* mutant phenotypes

If the FUS3 and LEC2 proteins are indeed functionally equivalent and if they are both expressed throughout the embryo, we would predict that they regulate *At2S3* in a completely redundant manner. The local lack of *At2S3* expression in *lec2* or in *fus3* single mutants, however, indicated that this is not the case. Indeed, we have shown that phenotypes of *fus3* and *lec2* single mutants can be explained by non-uniform *FUS3* and *LEC2* expression patterns. From 12 DAP on, *LEC2::GUS* expression was absent from the periphery of the cotyledons. This pattern was strikingly similar to the *At2S3::GFP* expression pattern in *fus3* embryos of the same age, as one would expect if *At2S3* expression totally depends on LEC2. We thus propose that *LEC2* transcription is a limiting factor for *At2S3* expression in the *fus3* background. However, we do not yet understand why the *fus3* phenotype is variable and why some dry *fus3* mutant seeds still bear a high level of fluorescence. As opposed to *LEC2*, *FUS3* is uniformly expressed throughout the wild-type embryo. This expression pattern was difficult to reconcile with the lack of *At2S3* expression in sectors of *lec2* mutant embryos. We have solved this apparent paradox by showing that FUS3::GUS activity becomes very heterogeneous in a *lec2* mutant and is absent wherever *At2S3* expression is missing. The absence of both essential factors (*LEC2* is inactive and *FUS3* not expressed) explains why *At2S3* expression is abolished in these sectors. The precise coincidence between FUS3::GUS activity and *At2S3* expression strongly suggests that *FUS3* expression is the limiting factor for *At2S3* expression in *lec2*. In support of this conclusion, transformation of *lec2* mutants with a *35S::FUS3* construct almost completely suppresses the presence of sectors devoid of *At2S3::GFP* fluorescence (G.S. and F.P., unpublished). Our analysis of *FUS3* and *LEC2* promoter activities are consistent with RT-PCR experiments (Fig. 5R) and the expression of the *At2S3* target gene in *fus3* or *lec2* mutants. For these reasons, we think FUS3::GUS and LEC2::GUS activities faithfully reflect the expression of *FUS3* and *LEC2* genes. However, the confirmation of the precise expression patterns of *FUS3* and *LEC2* will require immunolocalization or in situ hybridisation.

In the endosperm, *FUS3* and *LEC2* expression patterns also provide an explanation for the *fus3* and *lec2* phenotypes. In the mature endosperm, we have detected FUS3::GUS and ABI3::GUS activities (Parcy et al., 1994) but no LEC2::GUS activity. In agreement with these expression profiles, *At2S3* expression is almost abolished in *fus3* or *abi3* but unaffected in *lec2*. Our model (Fig. 6) therefore also applies for *At2S3* expression in the endosperm with FUS3 and ABI3 as major actors. In conclusion, we think that *FUS3* and *LEC2* expression patterns and their functional similarity explain *At2S3* expression in *lec2* and *fus3* mutants. However, it is likely that FUS3 and LEC2 are not completely interchangeable. We have, for example, observed that a *35S::LEC2* construct induces ectopic *At2S3* expression in leaf or floral tissue while a *35S::FUS3* construct does not (T.K., G.S. and F.P., unpublished).

Implications of *FUS3* regulation by *LEC2* for seed maturation

Our analysis of FUS3::GUS activity showed that *LEC2* controls *FUS3* expression. However, since *FUS3* expression is not abolished in *lec2* mutant, other factors must be involved in *FUS3* activation. The variability of the *lec2* phenotype is probably due to variable activation of *FUS3* by this unknown factor. The observation that *LEC2* regulates *FUS3* has implications for seed maturation in general: the existence and the nature of interactions between the four major regulators of maturation (ABI3, FUS3, LEAFY COTYLEDON1 and LEC2) has often been raised but never explained (Bäumlein et al., 1994; Keith et al., 1994; Meinke, 1992; Meinke et al., 1994; Parcy et al., 1997; Raz et al., 2001; Vicient et al., 2000). *FUS3* regulation by *LEC2* is the first demonstrated interaction between two of these regulators. It is likely that the lack of *FUS3* expression in sectors of *lec2* cotyledons is responsible for other *lec2* phenotypes such as anthocyanin accumulation. In agreement with this assumption, constitutive expression of *FUS3* in *lec2* almost abolishes anthocyanin accumulation (G.S. and F.P., unpublished). The *lec1* mutation also results in a reduced expression of the SSP gene (Parcy et al., 1997; Vicient et al., 2000). The nature of the interactions between *LEC1* and *ABI3*, *LEC2* and *FUS3* has never been clearly elucidated. According to available data, *lec1* is unlikely to completely abolish the expression of one of the three B3 genes (Meinke et al., 1994; Parcy et al., 1997; Raz et al., 2001; Stone et al., 2001; Vicient et al., 2000). However, as suggested by the effect of *lec2* on *FUS3* expression, it is possible that *LEC1* regulates the expression of some B3 genes only locally.

In conclusion, by focusing on *At2S3* regulation, we have shown that FUS3 and LEC2 have a similar mode of action that differs from that of ABI3. This finding is consistent with the emerging model of an *At2S3* regulatory complex containing several DNA binding proteins (FUS3, LEC2 and bZIPs) and ABI3 as coactivator (Fig. 6B). Many experiments are now possible to test this model biochemically, in yeast, or genetically in planta. Finally, we think that discovering a local regulation between two major players in seed maturation (FUS3 and LEC2) was not only useful for understanding the *lec2* phenotype but also suggests the existence of other regulations of this type in the embryo.

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