Regulation of storage protein gene expression in *Arabidopsis*

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Accepted 4 August 2003

Development 130, 6065-6073
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doi:10.1242/dev.00814

**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 capacity to bind DNA in a sequence-specific manner has been determined. Obvious candidates for binding the RY 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**

The 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 $7 \times 10^6$ 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'-ATCAC-TCATGCATGCATGCATTCTTACACGTGATTGCCATGCAAATC- TCCC-3') and the B-box (−169 to −132) (5'-ATCTGGTCGACT-TGTCAGCTTCTCTTCCAACACTAATCCC-3') were cloned into the Xhol 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-amino-triazol (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 B 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'-ATGATATCCATTTTGATGAAAAAATGTTGAAAAACCC-3' and 5'-ATGATATCTCTTAGAAGTCATCGGAG-3' for Fus3 and 5'-TCTAGAAAAAGGATCTTCCTCTACCTCT-3' and 5'-GTCGACCATATACCACTUAAAGT-3' for Lec2, cloned into pCR7/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):

1. 5'-acacactcatgcatgcattcttacacgtgattgcgtacg-3' and 5'-gga-gatttcatttcacacgtgattgcgtacg-3' and 5'-gga-gatttcatttcacacgtgattgcgtacg-3' (At2S3 wild-type sequence –98 to –48 relative to the transcription start), 5'-acacactcatgcatgcattcttacacgtgattgcgtacg-3' and 5'-gga-gatttcatttcacacgtgattgcgtacg-3' (At2S3 wild-type sequence with the indicated mutations),

2. 5'-acacactcatgcatgcattcttacacgtgattgcgtacg-3' and 5'-gga-gatttcatttcacacgtgattgcgtacg-3' and 5'-gga-gatttcatttcacacgtgattgcgtacg-3' (At2S3 wild-type sequence with the indicated mutations),

**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 (pFP91.54.3) 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’-CCTGCACTTCCCATTCTCCT-3’ and 5’-TCTTAATTGTAAGTGTCTT-3’ (At2S1), 5’-TCCAGGACACATCCCTTTCTTCTT-3’ and 5’-GACAGACCTTAGGACACGAGTGAT-3’ (At2S2), 5’-TCCAGAATCTCCTTACTG-3’ and 5’-AAACTAAACAACTCTCCTTTA-3’ (At2S3), 5’-GCCGCCTTTGGAGGGGCCAGA-3’ and 5’-CCTTGTTGTTACCGGCTTATGAG-3’ (CRB), 5’-CACCTTGAGACGGCCGAA-3’ and 5’-CCTTGTTGCAGCATGTTAA-3’ (CRB) and 5’-AGACCTTCTAATCCGGAGCAATCAG-3’ and 5’-GCATTGACAGGAACTCTGTGTTG-3’ (CRC). At2S3-specific probe was obtained by subcloning a fragment corresponding to nucleotides 536 to 690 from gene At4G27160.1 into pCR2-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’-GGACCTTCAAGTAATGGTACTGG-3’ and 5’-CCATCGAA-TGTCCGAGCTTGGAGT-3’ for FUS3,

5’-ATGCCCGAGACATCGTATCCATCT-3’ and 5’-TGCTGCCGACCTCCTCATTG-3’ for EFL1

5’-GTATGTCTCCGAGACACACAGAGG-3’ and 5’-TCAAGTGGGTCGTTAACACATGATT-3’ for LEC2. We verified that amplification was still in its linear range by monitoring amplification products at multiple cycles. Western blot analysis of AB13 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.

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, 7x10⁶ 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...
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,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 F2 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 designed specific probes for four *At2S* genes and three cruciferin genes 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.
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).

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.
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.

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 (Guérche 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; Vicent 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; Vicent 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; Vicent 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.

We thank D. Thomas for help with the one-hybrid screen, L. Lepinec and P. McCourt for the generous gift of seeds, Plant Genetic System for the pDE1000 plasmid, J. Vicente-Carbojosa, P. Carbonero, L. Rask and I. Ezcurra for sharing data before publication, S. Rippa...
for sequencing the ABI3-6 locus, R. Blanvillain and P. Gallois for the GFP construct, D. Jublot and V. Pautot for in situ hybridisation, the ISV cell biology platform for help with microscopy, M. Blazquez, S. Thonine, M. Delseny, R. Khush and D. Weigel for critical reading of
the manuscript and members of the Giraudat lab for discussions. This work was supported by the Centre National de la Recherche Scientifique, the Genoplante program AF1999-08, the European Community Forschungsgemeinschaft (grant KR 2004/1-1).

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