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A Network of Local and Redundant Gene Regulation Governs *Arabidopsis* Seed Maturation

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In *Arabidopsis thaliana*, four major regulators (ABSCISIC ACID INSENSITIVE3 [ABI3], FUSCA3 [FUS3], LEAFY COTYLEDON1 [LEC1], and LEC2) control most aspects of seed maturation, such as accumulation of storage compounds, cotyledon identity, acquisition of desiccation tolerance, and dormancy. The molecular basis for complex genetic interactions among these regulators is poorly understood. By analyzing *ABI3* and *FUS3* expression in various single, double, and triple maturation mutants, we have identified multiple regulatory links among all four genes. We found that one of the major roles of LEC2 was to upregulate *FUS3* and *ABI3*. The *lec2* mutation is responsible for a dramatic decrease in *ABI3* and *FUS3* expression, and most *lec2* phenotypes can be rescued by *ABI3* or *FUS3* constitutive expression. In addition, *ABI3* and *FUS3* positively regulate themselves and each other, thereby forming feedback loops essential for their sustained and uniform expression in the embryo. Finally, *LEC1* also positively regulates *ABI3* and *FUS3* in the cotyledons. Most of the genetic controls discovered were found to be local and redundant, explaining why they had previously been overlooked. This works establishes a genetic framework for seed maturation, organizing the key regulators of this process into a hierarchical network. In addition, it offers a molecular explanation for the puzzling variable features of *lec2* mutant embryos.

INTRODUCTION

The conquest of most terrestrial niches by land plants has been greatly facilitated by the appearance of seeds (Steeves, 1983). Seeds offer plants a unique opportunity to interrupt their life cycle by withstanding adverse environmental conditions in a desiccated state and then resuming growth using endogenous storage compounds. Seed-specific traits, such as desiccation tolerance, reserve accumulation, and entry into quiescence, are acquired during a developmental phase called seed maturation (Goldberg et al., 1994; Wobus and Weber, 1999; Vicente-Carbajosa and Carbonero, 2005). In *Arabidopsis thaliana*, this phase is genetically controlled by at least four genes, *FUSCA3* (*FUS3*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *LEAFY COTYLEDON1* (*LEC1*), and *LEC2*. *ABI3*, *FUS3*, and *LEC2* encode related plant-specific transcription factors containing the conserved B3 DNA binding domain (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001), whereas *LEC1* encodes a CBF transcription factor (Lotan et al., 1998). All four *abi3*, *lec1*, *lec2*, and *fus3* mutants are severely affected in seed maturation and share some common phenotypes, such as reduced expression of seed storage proteins (SSPs) (Table 1). However, they also show some specific phenotypes, such as the absence of chlorophyll degradation in the dry seed (*abi3*), a reduced sensitivity to abscisic acid (ABA) (*abi3* and, to a lesser extent, *lec1*), the accumulation of anthocyanins (*fus3*, *lec1*, and, to a lesser extent, *lec2*), an intolerance to desiccation (*abi3*, *fus3*, and *lec1*), or defects in cotyledon identity (*lec1*, *fus3*, and *lec2*) (Meinke, 1992; Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1994, 1997; West et al., 1994; Nambara et al., 1995; Lotan et al., 1998; Luerssen et al., 1998; Vicient et al., 2000a; Raz et al., 2001; Stone et al., 2001; Kroj et al., 2003). Despite numerous studies, the mechanisms through which these genes interact to control the various facets of seed maturation remain poorly understood (Bäumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994; Parcy et al., 1997; Nambara et al., 2000; Vicient et al., 2000a; Raz et al., 2001). Since *lec2*, *fus3*, and *abi3* single mutants have similar but distinct phenotypic traits, which are additive in double mutants, *ABI3*, *LEC2*, and *FUS3* may work in parallel pathways (Keith et al., 1994; Meinke et al., 1994; West et al., 1994). Other genetic

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Table 1. Main Phenotypes of Maturation Mutant Seeds

	Wild Type	*abi3*	*fus3*	*lec2*	*lec1*
Chlorophyll accumulation in dry seed	No	Yes (f and g)	No (c)	Yes, in sectors (c and j)	Yes (g)
Anthocyanin in cotyledons	No	No (g)	Yes (b and g)	Yes, in sectors (i, c, and j)	Yes (a, g, and c)
Storage protein expression	Normal	Reduced (d, f, g, and j)	Reduced (b, c, g, h, and j)	Reduced (c and j)	Reduced (a, c, g, e, and h)
Ectopic trichomes on cotyledons	No	No (f)	Yes (b and c)	Yes (i and c)	Yes (a, e, j, and c)
Seed ABA sensitivity	Normal	Reduced (g)	Normal (g)	Normal (c)	Reduced in cotyledons (g and c)
Desiccation-tolerant seeds	Yes	No (d)	No (b)	Yes (c)	No (e)

From this study and the following references: a, Meinke (1992); b, Keith et al. (1994); c, Meinke et al. (1994); d, Parcy et al. (1994); e, West et al. (1994); f, Nambara et al. (1995); g, Parcy et al. (1997); h, Vicient et al. (2000a); i, Stone et al. (2001); j, Kroj et al. (2003).

analyses have also suggested the existence of interactions between *ABI3* and *FUS3* but without elucidating their molecular nature (Parcy et al., 1997; Nambara et al., 2000; Vicient et al., 2000a). Recently, LEC1 was shown to regulate expression of *ABI3* and *FUS3* (Kagaya et al., 2005). Also, *FUS3* and *LEC2* were shown to act in a partially redundant manner to control SSP gene expression, and *LEC2* was shown to locally regulate *FUS3* expression in regions of the cotyledons (Kroj et al., 2003). These finding suggests that local and redundant regulation within this group of genes, which had been previously overlooked, might be a central requisite for the correct establishment of seed maturation and prompted us to systematically analyze their expression in various mutant combinations.

RESULTS

FUS3 Expression Is Regulated by *LEC2*, *ABI3*, and *FUS3*

We tested the effect of the *abi3*, *fus3*, and *lec2* mutations, alone and in combination, on the expression of *ABI3*, *FUS3*, and *LEC2*. We performed whole-mount β-glucuronidase (GUS) staining because this allowed direct visualization of the staining patterns in a large number of embryos. For the *FUS3* gene, we used a *FUS3:GUS* reporter construct and analyzed its expression in mature embryos (10 d after pollination [DAP]) (Figure 1A). As demonstrated previously, *FUS3* expression is detectable throughout the various parts of the wild-type embryo (root tip, embryo axis, and cotyledons) (Kroj et al., 2003; Tsuchiya et al., 2004). It was recently shown that this expression mainly originates from the protoderm of the embryo (Tsuchiya et al., 2004). Introduction of the *FUS3:GUS* reporter transgene in *abi3*, *fus3*, and *abi3 fus3* mutants showed that the spatial expression pattern of *FUS3* is not altered in these backgrounds (Figure 1A). As previously demonstrated, *FUS3* expression is absent from sectors of *lec2* mutant cotyledons (Figure 1A; Kroj et al., 2003). Strong alterations of *FUS3* expression were revealed by crossing the *lec2* mutant with *abi3* or *fus3*. In the *lec2 abi3* mutant, *FUS3:GUS* expression was restricted to the root meristem; in *fus3 lec2*, it was confined to the embryo axis; and in the *fus3 lec2 abi3* triple mutant, *FUS3:GUS* expression was completely lost (Figure 1A). Comparison of *fus3 lec2 abi3* with *fus3 lec2* shows that *ABI3* regulates *FUS3* in the embryo axis. Comparison of *lec2 abi3* with *lec2* shows that *ABI3* regulates *FUS3* expression in the embryo axis and in the cotyledons. Similarly, the effect of the *fus3* mutation in the *lec2* mutant (by comparing *lec2* with *lec2 fus3*) or in the *lec2 abi3* background (by comparing *lec2 abi3* with *lec2 abi3 fus3*) shows that *FUS3* positively regulates its own expression in the root meristem and the cotyledons. Finally, the effect of the *lec2* mutation in the *fus3*, *abi3*, or *fus3 abi3* backgrounds shows that *LEC2* regulates *FUS3* expression throughout the embryo.

We tried to confirm some of these expression patterns using in situ hybridization. As opposed to the signal obtained with the highly expressed *Cruciferin C* (*CRC*) storage protein probe (Figure 2A), the *FUS3* probe in the wild-type background gave a very weak and uniform signal (data not shown) and was not restricted to the epidermis as previously reported (Tsuchiya et al., 2004). We concluded that our experimental conditions were not sufficient to detect the weak level of *FUS3* expression. However, based on in situ hybridization (Tsuchiya et al., 2004), mutant complementation (Gazzarrini et al., 2004), and functional evidence (Kroj et al., 2003), *FUS3* expression appears to be faithfully reproduced by the sensitive *FUS3:GUS* reporter. Furthermore, this construct offers the unique opportunity to monitor *FUS3* expression in the *fus3* mutant backgrounds (*fus3*, *fus3 abi3*, *fus3 lec2*, and *fus3 abi3 lec2*) and to establish *FUS3* autoregulation.

ABI3 Expression Is Regulated by *LEC2*, *ABI3*, and *FUS3*

We also investigated *ABI3* expression using the *ABI3:GUS* construct. As previously reported (Parcy et al., 1994; Lara et al., 2003), *ABI3* expression was detected throughout the embryo axis and cotyledons in wild-type seeds (Figure 1B). The root meristem staining was usually weak. In the *lec2* mutant, the *ABI3* expression pattern was modified in the cotyledons where variable regions were devoid of staining (Figure 1B). These regions varied in shape and size as described for *AT2S3*, *CRC*, and *FUS3* expression in *lec2* (Figure 2A; Kroj et al., 2003). We concluded that *LEC2* also controls *ABI3* expression. *ABI3:GUS* expression was not reduced in *abi3* or *fus3* single mutants but appeared weaker in the periphery of the cotyledons of the *abi3 fus3* double

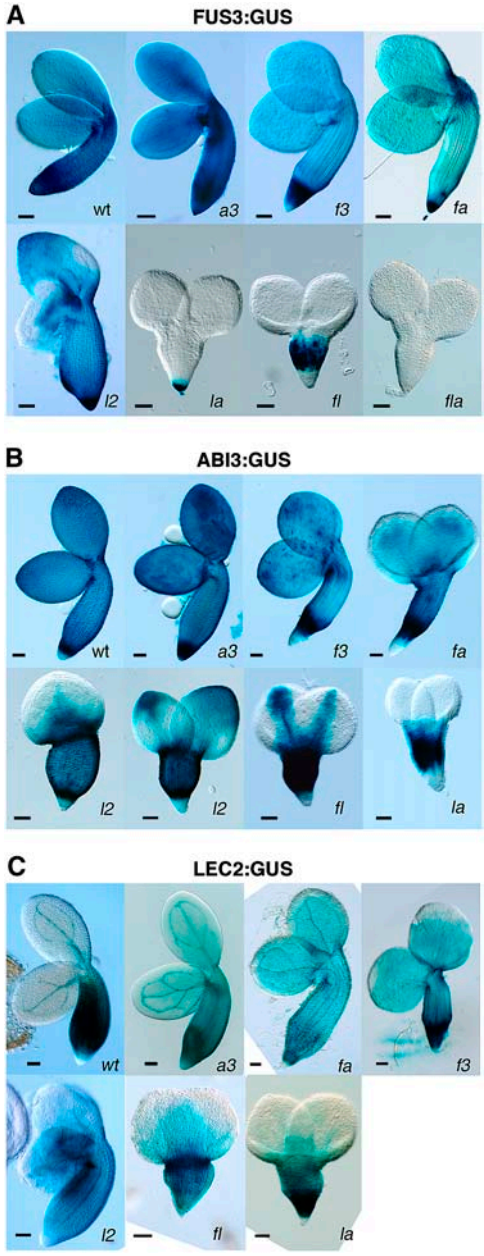

Figure 1. ABI3:GUS, FUS3:GUS, and LEC2:GUS Activities in Wild-Type and Mutant Embryos.

Expression patterns of *FUS3:GUS* **(A)**, *ABI3:GUS* **(B)**, and LEC2:GUS **(C)** in wild-type, *abi3* (*a3*), *fus3* (*f3*), *lec2* (*l2*), *fus3 abi3* (*fa*), *lec2 abi3* (*la*), *fus3 lec2* (*fl*), and *fus3 lec2 abi3* (*fla*) 10-DAP embryos and LEC2:GUS in 14-DAP embryos. The size and shape of the sectors with reduced *ABI3* or *FUS3* expression in *lec2* mutant embryos are extremely variable: two examples of phenotypes are shown for *ABI3:GUS* and one for *FUS3:GUS*. *LEC2:GUS* persistent expression is also variable and not always as pronounced as shown here for *lec2*, *fus3*, or *fus3 abi3*. Bars = 50 μm.

mutant, suggesting that *ABI3* might also be regulated by *FUS3* and by *ABI3* itself. The effect of *abi3* and *fus3* mutations was very apparent in the *lec2* mutant backgrounds. *ABI3:GUS* expression was absent from the lateral parts of the cotyledons of the *fus3 lec2* mutants. The strong phenotypic variability observed in the *lec2* mutant was abolished in *fus3 lec2*, and the pattern shown in Figure 1B was systematically observed, thereby demonstrating that *FUS3* is involved in *ABI3* regulation. In the *lec2 abi3* double mutant, *ABI3:GUS* expression was completely absent from the cotyledons, confirming that *ABI3* positively regulates its own expression in cotyledons. We complemented the analysis of *ABI3:GUS* transgenic plants by monitoring *ABI3* mRNA expression by in situ hybridization (Figures 2B and 2C). In wild-type embryos, we confirmed that *ABI3* expression was uniform throughout the cotyledons and embryo axis (Figures 2B and 2C). In *lec2* embryos, *ABI3* mRNA was absent from variable sectors of the cotyledons, exactly as shown by the *ABI3:GUS* constructs, thereby indicating that the *ABI3:GUS* construct faithfully reproduces the *ABI3* mRNA pattern.

We also analyzed *LEC2:GUS* expression patterns in all single and double mutant backgrounds. In these experiments, we never observed any decrease in LEC2:GUS staining at any developmental stages examined (from the globular stage to desiccating

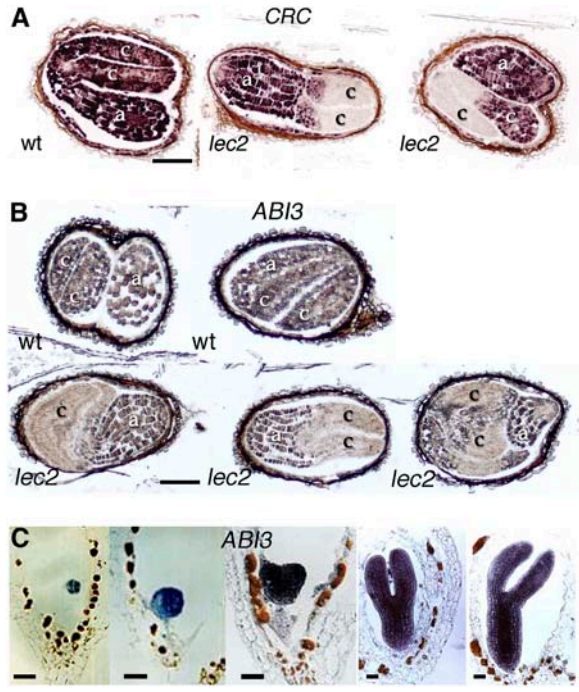

Figure 2. Analysis of *ABI3* and *CRC* Expression by in Situ Hybridization in Developing Seeds.

(A) *CRC* storage protein gene expression was analyzed in wild-type and *lec2* backgrounds.
(B) *ABI3* expression analyzed in wild-type and *lec2* 10-DAP embryos. Note the presence of variable sectors devoid of staining.
(C) Expression of *ABI3* in early stages of embryo development.
a, embryo axis; c, cotyledons. Bars = 100 μm in **(A)** and **(B)** and 20 μm in **(C)**.

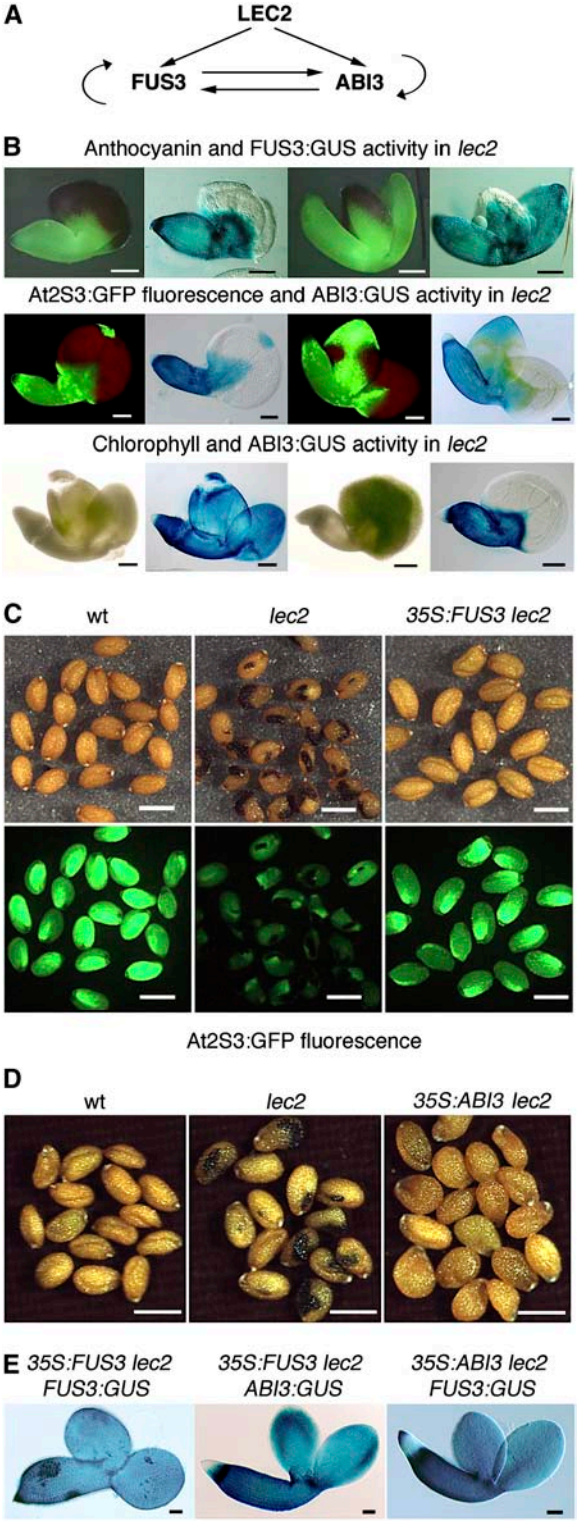

Figure 3. Network between B3 Transcription Factors (*LEC2*, *ABI3*, and *FUS3*).

(A) Positive interactions between *ABI3*, *FUS3*, and *LEC2* as deduced from Figure 1.

(B) Spatial coincidence of the domains lacking *ABI3* or *FUS3* expression and the phenotypic traits in *lec2*, such as anthocyanin accumulation or reduced AT2S3:GFP fluorescence at 10 DAP or absence of chlorophyll degradation at 14 to 15 DAP.

(C) Rescue of the *lec2* mutant phenotypes by constitutive expression of *FUS3*. The same groups of 15-DAP seeds are shown under white or blue illumination to observe the seed shape and color (top panels) and the AT2S3:GFP fluorescence (bottom panels). Dark spots on the *lec2* seeds result from the combined presence of anthocyanin and chlorophyll.

(D) Rescue of the *lec2* mutant phenotypes by constitutive expression of *ABI3.* Anthocyanin and chlorophyll accumulations in the *lec2* mutant are suppressed by the *35S:ABI3* transgene.

(E) Rescue of ABI3:GUS and FUS3:GUS expression in 10-DAP *lec2* embryos by *ABI3* or *FUS3* constitutive expression. The regions lacking *ABI3* or *FUS3* promoter activity observed in *lec2* mutant (Figure 1) have disappeared when *ABI3* or *FUS3* are constitutively expressed.

Bars = 100 μm in **(B)**, 500 μm in **(C)** and **(D)**, and 50 μm in **(E)**.

embryos). At late developmental stages, when *LEC2* expression disappears from the wild-type cotyledons (Kroj et al., 2003), we sometimes observed a sustained LEC2:GUS activity in the *lec2*, *fus3*, and *lec2 fus3* mutant backgrounds, suggesting that *LEC2* might be repressed by FUS3 and LEC2 (Figure 1C). Since we did not succeed in detecting *LEC2* expression by in situ hybridization and since no obvious phenotypes are associated with the potentially extended *LEC2* expression, we did not investigate this point further.

Confirmation of the Regulatory Links with Functional Tests

Cross-regulation between ABI3, FUS3, and LEC2, as established from expression analyses in wild-type and mutant backgrounds, is summarized in Figure 3A. These regulatory controls are largely corroborated by phenotypic analysis. Indeed, phenotypes characteristic of *ABI3* (or *FUS3*) loss of function are obvious in several mutant backgrounds with a functional *ABI3* (or *FUS3*) gene. For instance, the appearance of chlorophyll in dry embryos, which is one signature of *ABI3* loss of function, was visible in sectors of *lec2* (Figure 3B) and *fus3 lec2* mutants (data not shown), consistent with the *ABI3:GUS* expression pattern. Similarly, anthocyanin accumulation in cotyledons, a characteristic of *FUS3* loss of function, coincided with the loss of *FUS3:GUS* expression in *lec2* and *lec2 abi3* mutants (Figure 3B; data not shown; Meinke et al., 1994). Also, storage protein gene expression, monitored using the *AT2S3:GFP* (green fluorescent protein) reporter (Kroj et al., 2003), precisely coincided with the domains expressing both *ABI3* and *FUS3* in the *lec2* mutant (Kroj et al., 2003; Figure 3B). In summary, phenotypic data provide evidence for the functional significance of mutual regulation within a network involving *LEC2*, *ABI3*, and *FUS3*.

Our model suggests that part of *LEC2* action might be indirect and that LEC2 controls several aspects of seed maturation, including the prevention of anthocyanin and chlorophyll accumulation, through the positive regulation of *FUS3* and *ABI3* (Figure 3A). If this model is correct, constitutive expression of *FUS3* or *ABI3* should complement some of the defects caused by the *LEC2* loss of function. As shown in Figures 3C and 3E, the

constitutive expression of *FUS3* (directed by the *35S* promoter) into *lec2 AT2S3:GFP* plants allowed a nearly complete phenotypic rescue of *lec2*, including the recovery of uniform *AT2S3:GFP* expression, the absence of anthocyanin and chlorophyll accumulation, and the suppression of ectopic trichomes (Table 2). *35S:FUS3* also restored a uniform *FUS3:GUS* expression (Figure 3E). Only the irregular shape of some of the *lec2* embryo axes was unaffected by *FUS3* expression (Figure 3E). Interestingly, constitutive expression of *FUS3* also complemented defects associated with the lack of *ABI3* (such as chlorophyll accumulation, which is not under FUS3 control), suggesting either that constitutively expressed *FUS3* can fulfill ABI3 functions or that FUS3 might be able to restore uniform *ABI3* expression in the *lec2* mutant. To test the latter possibility, we introduced an *ABI3:GUS* reporter construct into the *35S:FUS3 lec2* background and observed that *ABI3* expression indeed recovered a wild-type expression pattern in cotyledons (Figure 3E). These experiments suggest that many defects present in the *lec2* mutant might indeed be indirectly due to the loss of *FUS3* and *ABI3* expression. They also show that *FUS3* can positively regulate its own expression and that of *ABI3*, thereby confirming the conclusions previously inferred from loss-of-function analysis (Figure 3A).

As for *FUS3*, constitutive expression of *ABI3* in *lec2* suppressed chlorophyll and anthocyanin accumulation and ectopic trichome formation (Table 2) and restored uniform *FUS3:GUS* expression in the *lec2* mutant embryos (Figures 3D and 3E), confirming that *ABI3* positively regulates *FUS3 (*Figure 3A). It is thus likely that *ABI3* or *FUS3* are sufficient to complement most of the *lec2* mutant defects because constitutive expression of one of these two factors is sufficient to restore the uniform expression of the other.

LEC2 Is Involved in *FUS3* Initiation and *ABI3* Maintenance

Cotyledons of *lec2* exhibit variable sectors that are devoid of *ABI3* and *FUS3* expression. To explain this phenomenon, we reasoned that LEC2 might either play an essential role early on, during the initial induction phase of *ABI3* and *FUS3* expression, or alternatively, that it might be required only later to ensure uniform *ABI3* and *FUS3* expression patterns. To distinguish between these two possibilities, we analyzed the expression of *ABI3* and *FUS3* in early stages of embryo development. In wild-type embryos, *ABI3* and *FUS3* expression is first detected at the globular stage. *ABI3* expression starts in the embryo proper at the globular stage (Figures 2C and 4A), while *FUS3* expression initiates in the suspensor and, by the transition stage (between globular and heart stages), covers the whole embryo (Figure 4B; Parcy et al., 1994; Kroj et al., 2003; Tsuchiya et al., 2004). In the *lec2* mutant, *ABI3* expression was the same as that in the wild type in transition stage embryos (Figure 4A), whereas *FUS3* expression was abolished in the embryo proper, though not in the suspensor (Figure 4B). This experiment indicates that initiation of *FUS3* expression depends on LEC2, whereas that of *ABI3* does not. Later on, in the heart and torpedo stages of embryo development, *ABI3* expression started to fade from sectors of the cotyledons (Figure 4A), while *FUS3* expression appeared in patches at variable locations (Figure 4B). By the bent cotyledon stage, *ABI3* and *FUS3* expression coincided, as deduced from the fact that they both shared the same pattern as the *AT2S3:GFP* marker (Figure 3B; Kroj et al., 2003). Therefore, LEC2 is required early for *FUS3* initiation in the embryo proper and later to ensure ABI3 uniform expression (but not for *ABI3* initiation). Since ABI3 regulates *FUS3* in mature cotyledons, we wondered whether it was necessary for the appearance of *FUS3* in patches in heart stage embryos of the *lec2* mutant. We therefore analyzed *FUS3:GUS* expression in *lec2 abi3* heart and torpedo stages and did not detect any FUS3:GUS activity in the embryo axis or cotyledons (Figure 4B), confirming that the late induction of *FUS3* in *lec2* mutants depends on *ABI3*.

LEC1 Controls *ABI3* and *FUS3* Expression in Cotyledons

LEC1 is the fourth master regulator of seed maturation in addition to LEC2, FUS3, and ABI3 and encodes the CBF-A subunit of the CCAAT binding trimeric transcription factor (Lotan et al., 1998). Based on single mutant phenotypes, *LEC1* had been proposed to positively regulate *LEC2* and *FUS3* and to act independently of *ABI3* (Meinke et al., 1994; Vicient et al., 2000a; Kagaya et al., 2005). Recently, LEC1 has been proposed to regulate *ABI3* and *FUS3* expression (Kagaya et al., 2005). However, double mutant analyses showed that *ABI3*, *LEC2*, and *FUS3* were still active in the *lec1* background (West et al., 1994; Parcy et al., 1997; Raz et al., 2001). We examined *ABI3*, *LEC2*, and *FUS3* promoter activity in the *lec1* mutant. We found a drastic local reduction of *FUS3:GUS* activity in cotyledons and root tips and a more moderate one for *ABI3:GUS* (Figure 5). *LEC2* expression did not decrease but showed a moderate increase in late stages as observed in *lec2* and *fus3* mutants (Figure 1). Again, these modifications of reporter gene expression were consistent with local phenotypes. *lec1* mutant embryos showed a marked chlorophyll accumulation at the periphery of the cotyledons, where *ABI3* expression was most reduced (Figure 5). *lec1* cotyledons have reduced ABA sensitivity, consistent with *ABI3* reduced expression (Parcy et al., 1997). Finally, the *lec1* mutant accumulated anthocyanin and showed reduced storage protein expression, consistent with the

Table 2. Constitutive *FUS3* or *ABI3* Expression Suppresses Ectopic Trichome Formation in the *lec2* Mutant

Genotype	Wild Type	*lec2*	*35S:FUS3 lec2* line 19	
Plants with trichomes on cotyledons (%)	0% (92)	69% (202)	0% (84)	
Genotype	Wild type	*lec2*	*35S:ABI3 lec2* line 8	*35S:ABI3 lec2* line 18
Plants with trichomes on cotyledons (%)	0% (45)	37% (111)	0% (57)	0% (85)

Two independent experiments have been performed explaining the variable percentage of *lec2* seedlings bearing ectopic trichomes. The number of seedlings examined is indicated in parentheses.

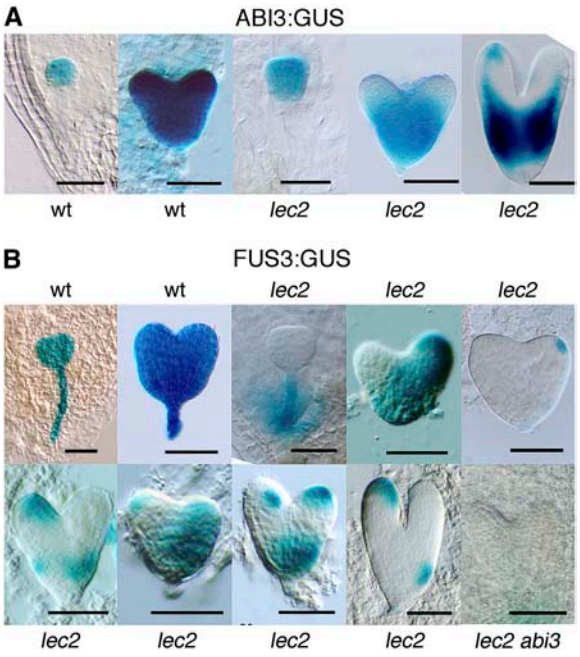

Figure 4. ABI3:GUS and FUS3:GUS Expression during Early Embryo Development.

(A) ABI3:GUS expression during wild-type and *lec2* early embryo development. In the *lec2* embryo, the ABI3:GUS expression pattern is the same as in wild-type embryos at globular stage but reduced in emerging cotyledons from heart stage on.
(B) FUS3:GUS expression during wild-type and *lec2* early development. In the *lec2* embryo, FUS3:GUS expression does not uniformly appear in the globular stage embryo (as in wild-type embryos) but in patches at heart stage. Patches are absent in the *lec2 abi3* double mutant.
Bars = 50 μm.

FUS3 expression pattern (Figure 5). This set of data indicates that LEC1 affects *ABI3* and *FUS3* expression locally (with a major effect on *FUS3* expression in cotyledons) and suggests some of the *lec1*-associated phenotypes might actually be indirectly due to a reduction in the expression of *ABI3* and *FUS3*.

DISCUSSION

A Regulatory Network for Seed Maturation

In this study, we have unraveled the regulatory network linking ABI3, FUS3, LEC1, and LEC2, four major regulators of seed maturation (Figure 6A). In a previous study, we showed that *LEC2* was necessary for uniform *FUS3* expression in parts of the cotyledons (Kroj et al., 2003). We show here that *FUS3* is controlled by a set of local and redundant regulations that vary spatially throughout the embryo and involve ABI3, LEC2, and FUS3 itself. In the root tip, *FUS3* expression is redundantly controlled by *LEC2* and by *FUS3* itself; in the embryo axis, *FUS3* expression is redundantly controlled by *LEC2* and *ABI3*; in the cotyledons, *FUS3* expression is under the control of all three regulators (Figure 6B). In addition to this set of regulatory controls, LEC1 also locally regulates *FUS3* expression in cotyledons. Our data are consistent with recently published analyses showing that LEC2 and LEC1 can induce *FUS3* expression in vegetative tissues (Kagaya et al., 2005; Santos Mendoza et al., 2005) and that *FUS3* expression is reduced in whole-seed mRNA of the *lec1* mutant (Brocard et al., 2002; Kagaya et al., 2005).

We also showed that *ABI3* expression is controlled by LEC1, LEC2, FUS3, and by ABI3 itself in the developing cotyledons. The decreased *ABI3* expression in *fus3 lec2* and *fus3 abi3* double mutants is consistent with previously obtained ABI3 protein level data (Parcy et al., 1997; Kroj et al., 2003). In *lec1* mutants, a reduction of ABI3 expression was not systematically observed (Parcy et al., 1997; Kagaya et al., 2005). As suggested by Kagaya et al. (2005), this discrepancy very likely arose from differences in seed stages examined. In this study, we indeed observed that ABI3:GUS was weaker in *lec1* embryos than in wild-type embryos at 10 DAP (Figure 5) but close to the wild-type expression in desiccating seeds (14 to 15 DAP; data not shown), which is the stage used by Parcy et al. (1997) for protein analysis. Interestingly, *ABI3* regulation differs from that of *FUS3*, as it was not abolished in the embryo axis of the double mutants tested (*abi3 fus3*, *abi3 lec2*, and *fus3 lec2*). This expression therefore appears to be independent of FUS3 and LEC2. Consistent with this hypothesis, a 5′ deletion of the *ABI3* promoter that removed all RY elements (likely binding sites for LEC2 and FUS3) resulted in an ABI3:GUS pattern confined to the embryo axis (F. Parcy, unpublished data).

Several of the features of the regulatory network studied here clearly rendered its analysis difficult. First, the gene regulatory controls in this network act locally, rather than on the whole embryo, thereby complicating genetic analyses that are often based on whole embryo characteristics (such as gene expression). Second, functional redundancies exist (for example, between ABI3 and FUS3 or between FUS3 and LEC2) that, again, are tissue dependent. *FUS3* expression is regulated redundantly by *ABI3* and *LEC2* in the embryo axis but by *LEC2* and *FUS3* in the root tip (Figure 6B). Third, *FUS3* and *ABI3* autoregulate. Hints of such types of regulation were previously provided by the

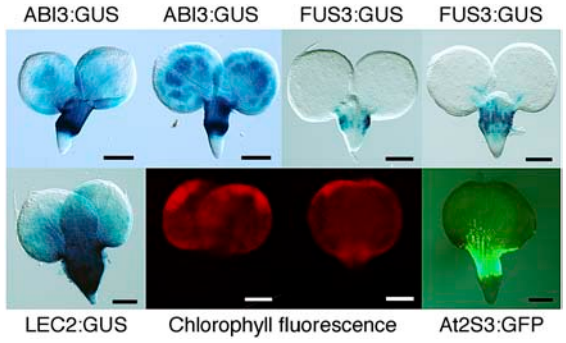

Figure 5. *lec1* Mutation Affects the *ABI3*, *FUS3*, and *LEC2* Expression Pattern.

All embryos are *lec1* mutants and show reduced *ABI3:GUS*, *FUS3:GUS*, and *AT2S3:GFP* expression at 10 DAP and increased chlorophyll fluorescence at 14 to 15 DAP. Chlorophyll was present mainly in the regions where *ABI3:GUS* was reduced. *AT2S3:GFP* persisted in regions expressing *FUS3:GUS*. Bars = 100 μm.

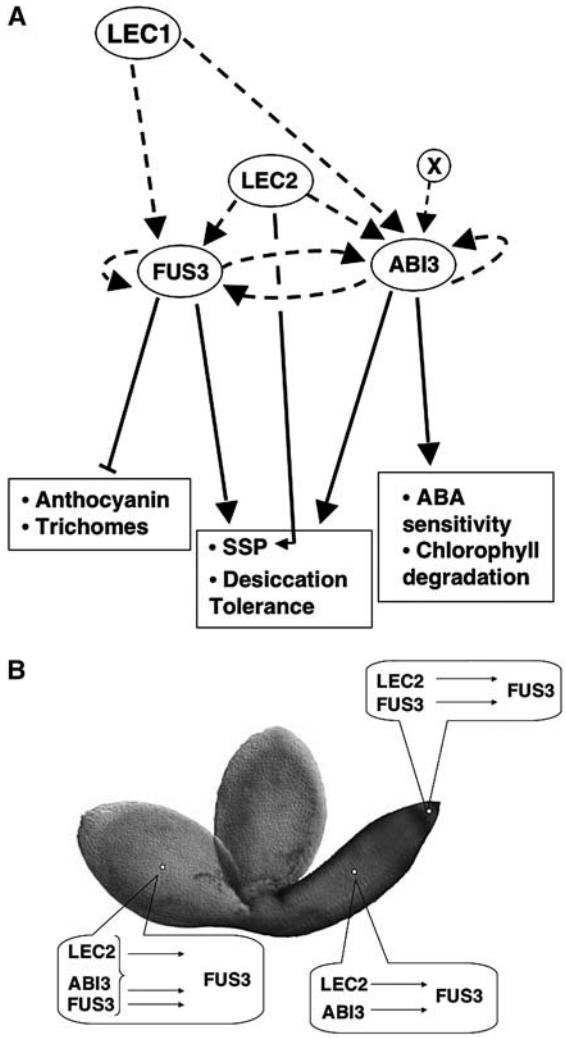

Figure 6. Gene Network Architecture.

(A) Schematic description of the network. Relations between the regulators are depicted as dashed lines, whereas proposed downstream actions are show as solid lines. X represents the unknown factor inducing *ABI3* expression at the globular stage.

(B) Summary of *FUS3* spatial regulation by various combinations of the B3 transcription factors.

analysis of the weak *abi3-1* allele (Parcy et al., 1997) or from the ectopic activation of seed regulators in vegetative tissues (Kagaya et al., 2005; Santos Mendoza et al., 2005). However, reporter constructs have been necessary to firmly establish these cases of autoregulation in strong mutant backgrounds. This network illustrates the intricate nature of regulatory circuitry and the difficulty of interpretation of global analyses of gene expression in whole seedlings or organs.

Functional Implications of the Network

Our results, together with other published data (Kroj et al., 2003; Kagaya et al., 2005), establish that LEC2 and LEC1 act on *ABI3* and *FUS3* expression. Moreover, we observed that *FUS3* or *ABI3* constitutive expression could rescue most aspects of the *lec2* mutant phenotype, suggesting that *ABI3* and *FUS3* act downstream of *LEC2*. However, we should point out that interpretation of this result is not straightforward because *ABI3*, *FUS3*, and *LEC2* encode homologous transcription factors, whose specificity might be altered by constitutive expression. For example, we found that *35S:FUS3* suppressed chlorophyll accumulation, a function known to be normally performed by ABI3. Since we also showed that *35S:FUS3* was capable of restoring *ABI3* uniform expression in *lec2* cotyledons, the simplest explanation is that *FUS3* constitutive expression induces *ABI3*, which suppresses chlorophyll accumulation. However, we cannot exclude that constitutive expression of FUS3 acts on chlorophyll independently of ABI3. One way to test this hypothesis would be to introduce the *35S:FUS3* transgene in a *lec2 abi3* double mutant and observe if chlorophyll accumulation remains. Similarly, it is conceivable that *FUS3* or *ABI3* constitutive expression complement the *lec2* mutant because of the lack of specificity. We do not favor this hypothesis because FUS3 and ABI3 only complement phenotypic defects that are related to their endogenous function (such as chlorophyll breakdown, anthocyanin repression, or SSP gene expression). The abnormal shape of the *lec2* mutant embryo axis is not complemented by *FUS3* or *ABI3* (Figure 3). Also, neither *35S:ABI3* nor *35S:FUS*3 induces the formation of ectopic embryos as *LEC2* does (Stone et al., 2001). These results suggest that ABI3, FUS3, and LEC2 retain some specificity even when overexpressed. We concluded that *LEC2* is upstream of *ABI3* and *FUS3* because (1) *ABI3* and *FUS3* expression are absent from sectors of *lec2* embryos; (2) some mutant phenotypes, typical of *ABI3* and *FUS3* loss of function, appear precisely in these sectors; and (3) these defects can be complemented by constitutive expression of *ABI3* or *FUS3*. However, it is clear that LEC2 does not act only via ABI3 or FUS3. Several complementary studies have shown that LEC2 is capable of activating directly 12S and 2S storage protein genes (Kroj et al., 2003; Santos Mendoza et al., 2005; Braybrook et al., 2006). It is thus likely that, in wild-type plants, LEC2 controls SSP gene expression through two different mechanisms: directly, by binding the SSP promoter, and indirectly, by activating *FUS3* and *ABI3* as indicated in Figure 6A.

Expression analyses of *FUS3* and *ABI3* in *lec1* suggest that LEC1 also might indirectly control many aspects of seed maturation through regulation of *ABI3* and *FUS3* expression. Testing this hypothesis will require analyzing the extent to which the *lec1* mutant phenotype can be complemented by *ABI3* or *FUS3* constitutive expression, as we did for the *lec2* mutant. The question of whether LEC1 participates in ABA sensitivity has previously been a matter of debate (West et al., 1994; Parcy et al., 1997; Brocard et al., 2002). Based on observation of radicle growth, *lec1* has been shown to be ABA sensitive, but *lec1* cotyledon expansion is partially insensitive to ABA (West et al., 1994; Parcy et al., 1997). The observation that *ABI3* expression is predominantly reduced in cotyledons offers a simple explanation for this apparent contradiction: LEC1 might affect ABA sensitivity only indirectly, through ABI3 regulation in the cotyledons. As recently suggested, LEC1 might also control storage protein expression through *ABI3* and *FUS3* control (Kagaya et al., 2005). Testing the

Table 3. Oligonucleotides Used for PCR Genotyping

Mutation/Transgene	Oligonucleotides 5′–3′	Comment
abi3-6	gcttcttcatcaaaccaaa	1.1-kb wild-type band
	cgatgatggagaataacagtggt	0.35-kb mutant band
fus3-3	gattcctcttccaaaaggaactca	*Rsa*I cuts the wild-type band
	ggtcttaagatcgacatggataca	twice and the mutant once
lec2-1	acgtgcagatctccgacaagaa	Wild-type band 0.4 kb
	ttaccaagtattggttcgagaaat	
	cgatggagatgcgatggtataatatgtggccgcaacgat	0.6-kb mutant band
lec1-1	tgtgccgtttcgagttgccttgt	0.75-kb wild-type band
	atggcccggagaagacgattt	0.52-kb mutant band
	tggaccccgttgagtagactgtt	
ABI3:GUS transgene	cgatgatggagaataacagtggt	
	tcacgggttggggtttctac	
FUS3:GUS transgene	gaaacccaaagagatccacc	
	tcacgggttggggtttctac	
LEC2:GUS transgene	tgaatggctattaatggtgtttactct	
	tcacgggttggggtttctac	

direct and indirect target genes of LEC2 and LEC1 will require further experiments, possibly using versions of these transcription factors that can be posttranslationally activated (Kagaya et al., 2005; Santos Mendoza et al., 2005).

The Network Structure and Its Implications: A Confirmation of Theoretical Predictions?

The maturation network presented in Figure 6A resembles modules that are known to drive developmental progression or patterning in other higher eukaryotes (Davidson et al., 2002, 2003; Rudel and Sommer, 2003; Levine and Davidson, 2005). The network analyzed here appears to function to ensure a uniform expression of essential regulators, such as ABI3 and FUS3, during the entire phase of seed maturation in a manner that is independent of their initial activation. Our analysis at early developmental stages indicates that LEC2 induces *FUS3* in the embryo at the globular stage (*FUS3* is thus the earliest known LEC2 target gene), whereas *ABI3* is induced independently of LEC2 (by an unknown factor X depicted in Figure 6A). However, as soon as the heart stage, mutual activation loops between ABI3 and FUS3 play a pivotal role in maintaining their uniform expression patterns. This maintenance might be of particular importance toward the end of seed maturation, when *LEC2* expression decreases, and ABI3 and FUS3 are required to uniformly establish desiccation tolerance.

Interestingly, the structure of the network revealed here provides a possible explanation for the observed phenotypic variability of the *lec2* mutant (Figure 3C; Meinke et al., 1994; Kroj et al., 2003). Our analyses at early stages of embryo development allowed us to trace back the origin of the observed variable *lec2* defects to the heart stage, where *ABI3* expression disappears and *FUS3* expression appears in randomly positioned patches. These expression patterns seem surprising but are easily explained by the structure of the network. Modules with autoregulatory loops have been the focus of intense studies, and theoretical analyses have predicted that small variations (or noise) can be stabilized as a result of bistability generated by the network (Thattai and van Oudenaarden, 2001; Blake et al., 2003; Isaacs et al., 2003). Applying these principles to the situation analyzed here, we can propose the following explanation. In *lec2* mutant embryo development, there might be a critical moment where *ABI3* initial induction is fading. At this moment, in some localized parts of the *lec2* embryo, ABI3 residual expression levels might be sufficient to trigger *FUS3* expression, thereby feeding into the positive loop and leading to stabilization of the expression of both regulators (first stable state). In other parts of the *lec2* embryo, ABI3 may not succeed in inducing *FUS3*, leading to the local loss of expression of both regulators (second stable state). The observed phenotype (embryo regions with both *FUS3* and *ABI3* expression, other regions lacking both) is the exact theoretical outcome of this bistable network (Thattai and van Oudenaarden, 2001; Blake et al., 2003; Isaacs et al., 2003). To our knowledge, our observations are one of the few experimental confirmations of such predictions. Our results therefore not only provide an explanation for a puzzling seed phenotype but also represent an experimental confirmation of theoretical studies of general interest.

Molecular Mechanism and Appearance in Evolution

What could be the molecular mechanisms underlying this network? Since ABI3, FUS3, and LEC2 are B3 transcription factors, and since RY elements are present in the promoters of *FUS3* and *ABI3*, some of the regulatory controls might be direct and involve physical binding of the B3 transcription factor to the *FUS3* and *ABI3* promoters. However, FUS3 has been shown to act from the embryo epidermis and to regulate storage protein gene expression in internal cell layers using ABA as a mobile mediator (Gazzarrini et al., 2004). Thus, this indirect mode of action could also apply to *ABI3* regulation by FUS3. This point will require further analysis.

Finally, it would be interesting to understand when and how this regulatory network was first established over the course of plant evolution. A likely mechanism (Wagner, 2001; Amoutzias et al., 2004; Teichmann and Babu, 2004) for the generation of this complex regulatory network of B3 transcription factors could be the repeated duplication of an ancestral B3 gene with autoregulatory

properties, thereby generating three B3 transcription factors that regulate their own and each other's expression. The existence of *ABI3* orthologs in monocotyledonous species (McCarty et al., 1991), together with the recent discovery of *FUS3* and *LEC2* homologs in cereals (F. Parcy and J. Vicente-Carbajosa, unpublished data), suggests that this network may also function in cereal grains and that it originated before the taxonomic split between the monocotyledons and eudicotyledons. Analysis of components of the network in basal angiosperms or gymnosperms might allow the identification of the ancestral autoregulatory gene.

METHODS

Plant Material

We used the following *Arabidopsis thaliana* strains: *lec1-1* (Meinke, 1992) in Wassilewskija, *AT2S3:GFP* in Columbia-0 (Col-0) (line FP91.54.3) (Kroj et al., 2003), and *lec2-1*, *abi3-6 gl1*, and *fus3-3 gl1* introduced in the *AT2S3:GFP* background (Kroj et al., 2003). Except for *lec1-1* and *abi3 fus3 lec2* triple mutants, all strains used in this study (including those mentioned as wild types in the figures) contained the *AT2S3:GFP* transgene. *FUS3:GUS* (line 3.2 in Col-0), *LEC2:GUS* (line 3.2 in Col-0), and *ABI3:GUS* (line LAG3-4 in Landsberg *erecta*) have been described (Parcy et al., 1994; Kroj et al., 2003).

Generation and Characterization of Transgenic Plants

A double enhanced 35S promoter–NOS terminator cassette (Parcy et al., 1994) and a Gateway cassette (Invitrogen) were successively introduced in the pZP312 vector (pZP derivative conferring Basta resistance in plants built by C. Fankhauser) to generate the pFP108-rfA plasmid in which *FUS3* and *ABI3* cDNAs were recombined according to Invitrogen's recommendations. *Arabidopsis* plants were grown and transformed as described (Kroj et al., 2003). The presence of mutations and transgenes was verified by PCR genotyping as described below.

Nineteen *35S:FUS3* lines in *lec2 AT2S3:GFP* were generated. Visual inspection of the T2 seeds (under white and blue illumination) showed *lec2* phenotypic rescue in 15 of them. Four single-locus lines were followed until the homozygous stage and showed a degree of rescue similar to the line *35S:FUS3* #19, shown in Figure 3 and used for crosses to *lec2 ABI3:GUS* and *lec2 FUS3:GUS*. *35S:ABI3* (pFP111) was introduced directly into the *lec2 FUS3:GUS* background. Among 18 T1 lines generated, six showed apparent rescue of the *lec2* phenotype, and five were followed until homozygous stage and showed a similar degree of rescue as line FP111 #3 shown in Figure 3.

Expression Analysis

The GUS staining and AT2S3:GFP fluorescence analyses were performed as described (Kroj et al., 2003). Individual embryos (at least 20 for each genotype) were observed and stained in rudimentary flow cells to directly compare AT2S3:GFP, GUS activity, and chlorophyll or anthocyanin accumulation as described by Kroj et al. (2003). When needed, anthocyanin was washed in 90% acetone (to allow observation of chlorophyll), and chlorophyll was washed with 95% ethanol (observation of anthocyanin). In situ hybridization was performed as described by Lara et al. (2003) using *CRC*, *ABI3*, or *FUS3* as probes except for Figure 2C (performed as in Vicient et al., 2000b).

Counting of Trichomes on Cotyledons

To prevent ectopic trichomes on cotyledons to desiccate, siliques were harvested when still green (before the first sign of valve dehiscence) and were surface sterilized, and seeds were dissected out and sowed on germination medium (Parcy et al., 1994). Seedlings bearing trichomes were counted 5 to 7 d after stratification. Genotypes used were the following: *AT2S3:GFP* in Col-0 (indicated as wild type in Table 2), *35S:FUS3 lec2 AT2S3:GFP* line 19, *lec2 AT2S3:GFP*, *35S:ABI3 FUS3:GUS lec2* line 8, and *35S:ABI3 FUS3:GUS* line 18. The wild type and the *lec2* mutant are presented twice because experiments were performed in two independent growth conditions.

PCR Genotyping of Mutations and the Transgene

Transgenes and mutations were followed by PCR amplification of genomic DNA using the oligonucleotides listed in Table 3.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At4g27160 (*AT2S3*), At4g28520 (*CRC*), At3g24650 (*ABI3*), At3g26790 (*FUS3*), At1g28300 (*LEC2*), and At1g21970 (*LEC1*).

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