Regulatory Networks in Seeds Integrating Developmental, Abscisic Acid, Sugar, and Light Signaling[1]

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Progression through embryogenesis and the transition to germination is subject to regulation by many transcription factors, including those encoded by the Arabidopsis *LEC1* (*LEAFY COTYLEDON1*), *FUS3* (*FUSCA3*), and abscisic acid-insensitive (ABI) *ABI3*, *ABI4*, and *ABI5* loci. To determine whether the *ABI4*, *ABI5*, *LEC1*, and *FUS3* loci interact or act independently, we analyzed *abi fus3* and *abi lec1* double mutants. Our results show that both *ABI4* and *ABI5* interact genetically with both *LEC1* and *FUS3* in controlling pigment accumulation, suppression of vivipary, germination sensitivity to abscisic acid, gene expression during mid- and late embryogenesis, sugar metabolism, sensitivity to sugar, and etiolated growth. However, the relative strengths of the observed interactions vary among responses and may even be antagonistic. Furthermore, the interactions reveal cryptic effects of individual loci that are not detectable by analyses of single mutants. Despite these strong genetic interactions, but consistent with the disparities in peak expression of these loci, none of the ABI transcription factors appear to interact directly with either FUS3 or LEC1 in a yeast (*Saccharomyces cerevisiae*) two-hybrid assay system.

Angiosperm embryo development can be divided into three phases: morphogenesis, cell enlargement, and desiccation (for review, see Rock and Quatrano, 1995). Cell division and histodifferentiation are completed during the morphogenesis phase, leading to an embryo with all structures formed. This is followed by a growth phase during which the embryo fills the seed sac, accumulating storage reserves that can be used later by the germinating seedling before the onset of photosynthetic activity. During the final phase, embryos develop desiccation tolerance, dehydrate, and enter developmental arrest, possibly becoming dormant.

Progression through embryo development to seed maturity and the transition to germination is coordinated by the interactions of stage-specific developmental regulators and the competing effects of hormonal signals such as abscisic acid (ABA), GAs, and ethylene (for review, see Finkelstein et al., 2002). In addition, metabolites such as sugars may act as developmental signals regulating seed maturation (for review, see Wobus and Weber, 1999). The most critical hormone promoting embryo maturation and preventing germination is ABA, which reaches its peak concentration midway through embryogenesis. Severely ABA-deficient mutants of some species, e.g. maize (*Zea mays*), produce viviparous seeds (Robertson, 1955); this effect can be phenocopied by

transgene-driven production of antibodies directed against ABA (Phillips et al., 1997). The Arabidopsis *ABI* (*ABA-INSENSITIVE*) loci were initially identified on the basis of the ABA-resistant germination of mutants at these loci (Koornneef et al., 1984; Finkelstein, 1994). *ABI3*, which is an ortholog of the maize *VP1* (*VIVIPAROUS1*) locus, has the most pleiotropic effects on seed maturation, regulating sensitivity to ABA inhibition of germination, expression of some seed-specific genes, acquisition of desiccation tolerance, and dormancy (Giraudat et al., 1992; Parcy et al., 1994). However, severe *abi3* mutants differ from *vp1* mutants in that they are not viviparous but produce desiccation-intolerant green seeds (Nambara et al., 1992; Ooms et al., 1993). In addition to altering ABA sensitivity of germination, the other *ABI* loci regulate subsets of these responses: *ABI1* and *ABI2* regulate dormancy, but the monogenic mutants have not been found to disrupt embryonic gene expression (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Parcy and Giraudat, 1997). In contrast, *ABI4* and *ABI5* do not regulate dormancy, but do control some embryonic gene expression and also regulate some seedling responses to ABA and sugars (Finkelstein, 1994; Finkelstein et al., 1998; Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000a; Huijser et al., 2000; Laby et al., 2000; Söderman et al., 2000; Lopez-Molina et al., 2001; Rook et al., 2001). *ABI3*, *ABI4*, and *ABI5* encode transcription factors and appear to act combinatorially to control embryonic gene expression and seed sensitivity to ABA (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000a; Söderman et al., 2000). Recent yeast (*Saccharomyces cerevisiae*) two-hybrid studies have shown that ABI3 and ABI5, and their rice (*Oryza sativa*) homologs OsVP1 and TRAB1, can interact di-

rectly (Hobo et al., 1999; Nakamura et al., 2001) and presumably form part of a regulatory complex in plants.

Unlike the *ABI* loci, the *LEC1* (*LEAFY COTYLEDON1*) and *FUS3* (*FUSCA3*) loci were identified on the basis of developmental defects reflecting a failure to temporally separate embryonic and vegetative differentiation (Keith et al., 1994; Meinke et al., 1994; West et al., 1994). In addition to producing cotyledons with leaf-like characteristics such as trichomes, starch accumulation, and anthocyanin accumulation, the latter giving a purple color to the seed, both *lec1* and *fus3* are desiccation intolerant and occasionally viviparous. Although both *fus3* and *lec1* embryos have defects in the expression of some maturation-specific genes (Nambara et al., 2000; Vicient et al., 2000), only *LEC1* affects ABA sensitivity of germination (Parcy et al., 1997).

ABI4, aside from its role in seed development and germination, participates in a sugar signal transduction pathway. Additional *abi4* alleles have been isolated by screens including Suc insensitivity (*sis5* and *sun6*) (Huijser et al., 2000; Laby et al., 2000) or Glc insensitivity (*gin6*) (Arenas-Huertero et al., 2000) in early seedling growth because *ABI4* is required for Glc-induced developmental arrest at this stage. The other *abi* mutants have been tested for their sugar insensitivity and *abi5* was shown to be mildly Glc resistant (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000), but *abi5* mutants have never been isolated by any screens for sugar insensitivity. Although *abi1-1*, *abi2-1*, and *abi3-1* display a Glc-sensitive phenotype, overexpression of *ABI3*, *ABI4*, or *ABI5* confers sugar hypersensitivity (Finkelstein et al., 2002). From these observations, it appears that the ABA-mediated Glc signaling pathway belongs to a branch in which ABI4 and, to a lesser extent, ABI5 and ABI3, participate as signaling molecules.

The phenotypes of the monogenic mutants indicate that these loci control overlapping responses, but they do not show whether these loci interact or act independently. Previous digenic mutant studies have shown synergistic effects of mutations in *ABI3*, *FUS3*, and *LEC1*, resulting in production of highly pigmented viviparous seeds (Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1997). Studies of the molecular basis of this synergism have shown that ABI3 protein accumulation is reduced in the double mutants (Parcy et al., 1997). Both *LEC1* and *FUS3* have now been cloned and found to encode transcription factors (Lotan et al., 1998; Luerssen et al., 1998; Reidt et al., 2000). *LEC1* encodes a CCAAT box-binding factor HAP3 subunit and *FUS3* encodes a transcription factor with a conserved VP1/ABI3-like B3 domain. In this paper, we report the construction and characterization of four digenic mutants combining mutations in either *ABI4* or *ABI5* with those in *FUS3* or *LEC1*. Seed of digenic mutants was compared with that of wild-type and monogenic parents in terms of

pigment content (chlorophyll and anthocyanin), embryonic gene expression, and sensitivity to ABA for inhibition of germination. Because ABI4 and ABI5 appear to play a role in sugar response and the digenic mutants exhibit some characteristics of wild-type seedlings grown on high sugar, we also assayed sugar sensitivity of germination and seedling growth, and accumulation of soluble sugars and starch. All tested combinations appear to reflect genetic interactions, but the strength of the interaction varies with the loci involved and the response. Although some strong genetic interactions were observed between the *ABI*s and both *FUS3* and *LEC1*, none of these appeared to reflect direct physical interactions detectable by a yeast two-hybrid assay system, consistent with previous observations that peak expression of these loci occurs at disparate periods of embryogenesis (Lotan et al., 1998; Luerssen et al., 1998; Finkelstein and Lynch, 2000a; Söderman et al., 2000; Brocard et al., 2002).

RESULTS

Previous studies have shown that Arabidopsis seed development is subject to control by the ABA-insensitive loci *ABI3*, *ABI4*, and *ABI5*, as well as by the developmental regulators *FUS3* and *LEC1*. To determine whether *ABI4* or *ABI5* interacts genetically with either *FUS3* or *LEC1*, we constructed double mutants combining each *abi* mutation with either a *fus3* or *lec1* mutation. The alleles used were *abi4-1*, *abi5-1*, *fus3-3*, and *lec1-1*. The *abi4-1* mutation is a frame shift that results in production of a truncated protein that includes the presumed DNA-binding domain (BD; Finkelstein et al., 1998), but lacks any transcription activation function and confers ABA resistance similar to that of alleles that also lack the DNA-BD (Söderman et al., 2000). The *abi5-1* allele contains a "nonsense" mutation, resulting in production of a truncated protein lacking the basic Leu zipper (bZIP) domain required for DNA binding and dimerization (Finkelstein and Lynch, 2000a); expression of this allele is also severely reduced, reflecting autoregulation (Brocard et al., 2002). Thus, although not genetic null alleles, both the *abi4-1* and *abi5-1* mutations are probably biochemical null alleles. However, it is still possible that the limited amounts of truncated products might interfere with the activity of other unidentified proteins. The *lec1-1* mutation is a deletion that removes the entire *LEC1* gene and, therefore, is a true null allele (Lotan et al., 1998). The *fus3-3* mutation produces a defective exon/intron boundary within the region encoding the conserved B3 domain, resulting in accumulation of aberrant transcripts that are predicted to not encode a functional FUSCA3 protein (Luerssen et al., 1998).

Germination of Digenic Mutant Seeds Is Highly Resistant to ABA

Previous studies have shown that *abi4-1* and *abi5-1* mutants are 10- and 3-fold, respectively, less sensitive to ABA inhibition of germination than the wild-type (Finkelstein, 1994), whereas *fus3* mutants have normal ABA sensitivity (Keith et al., 1994; Parcy et al., 1997), and *lec1* mutants have been described as having either normal (Meinke et al., 1994; West et al., 1994) or roughly 10-fold reduced (Parcy et al., 1997) ABA sensitivity for this response. The discrepancies in results with the *lec1* mutants could reflect differences in the alleles tested, assay media, and criteria used for scoring ABA resistance. Resistance was reported only for germination and cotyledon expansion of *lec1-1* incubated on media that included Suc (Parcy et al., 1997), a condition subsequently shown to reduce sensitivity to exogenous ABA (Garciarrubio et al., 1997; Finkelstein and Lynch, 2000b). To determine whether the *fus3* or *lec1* mutations enhanced the ABA resistance of the *abi* mutants, early desiccation stage seeds were excised and cultured on media containing a range of ABA concentrations, but no sugars. All genotypes were pre-incubated for 3 d at 4°C to eliminate any effects of residual dormancy on germination potential. Under these assay conditions, the *fus3* mutants have an essentially wild-type sensitivity to ABA, *lec1* seeds are resistant to only 3 to 10 μM ABA, and the monogenic *abi* mutants are resistant to only 3 to 30 μM ABA. In contrast, the *abi fus3* double mutants and most of the *abi4 lec1* mutant seeds were capable of germinating on media containing up to 300 μM ABA (Fig. 1A), reflecting strong synergistic effects. Although *abi5 lec1* and its monogenic parents showed similar responses to low ABA (reaching only 30%–40% germination after 1 week), only the digenic mutant could germinate in the presence of 300 μM ABA, albeit at a lower frequency than the other digenics, suggesting a weaker interaction.

Furthermore, although germination of the monogenic *abi* mutants on low concentrations of ABA is usually first observed 2 to 4 d post-stratification, the *abi fus3* digenic mutants begin to germinate on 100 μM ABA within 30 min even before stratification (Fig. 1B). This germination behavior reflects a complete loss of dormancy as well as enhanced resistance to ABA. The *abi4 fus3* digenic mutants were most resistant to ABA, reaching 50% germination within 5 h on 100 μM ABA; the *abi5 fus3* and *abi4 lec1* mutants required several days to reach this level of germination. The *abi5 lec1* digenic mutants germinated even more slowly, reaching less than 10% germination after 1 week on 100 μM ABA (Fig. 1A). Surprisingly, the observed degree of resistance did not correlate with the frequency of vivipary in these lines; the double mutants carrying the *abi5* mutation were more predisposed toward vivipary than those carrying the *abi4* mutation, despite being less resistant to inhibition of germination by exogenous ABA (Finkel-
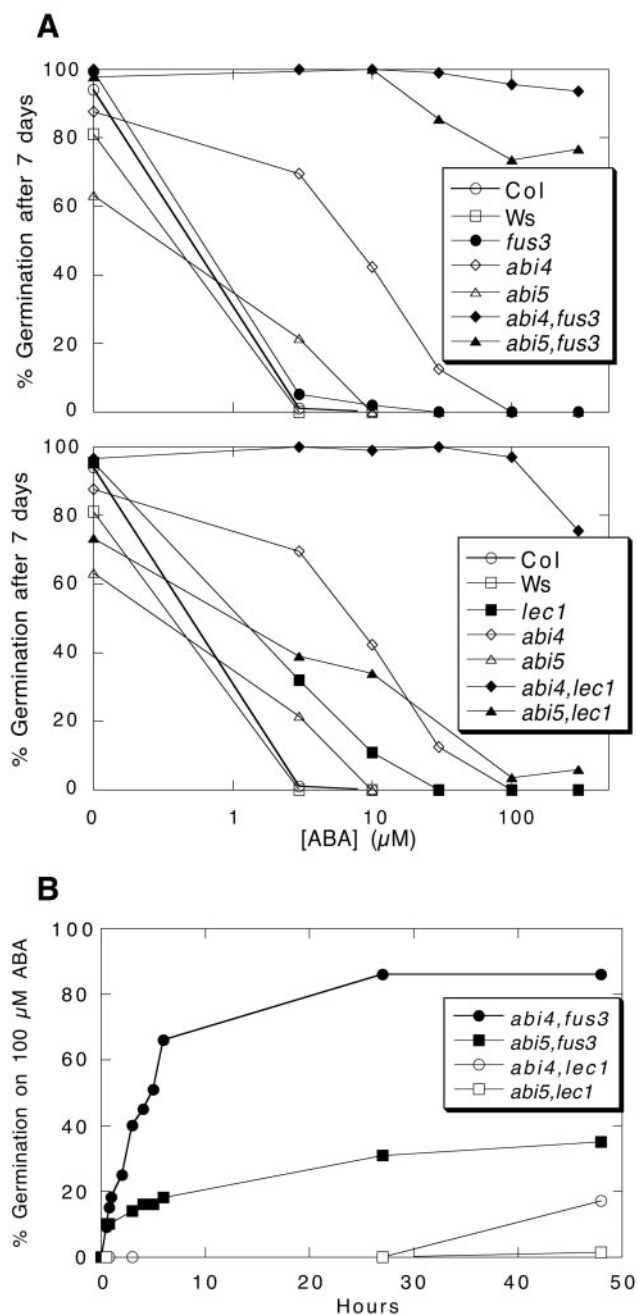

Figure 1. Sensitivity of early desiccation stage mono- and digenic *abi, fus3,* and *lec1* mutants to inhibition of germination by ABA. A, Germination was scored after 7 d of incubation in continuous light on media with indicated concentrations of ABA. Top, Combinations involving *fus3* compared with monogenic parents; bottom, combinations involving *lec1* compared with monogenic parents. B, Kinetics of digenic mutant germination on 100 μM ABA. Graphs show mean values of at least two independent experiments for each genotype.

stein et al., 2002). The degrees of ABA resistance or vivipary were also poor indicators of subsequent seedling growth; many of the mono- or digenic *lec1* lines did not grow well on minimal media, possibly reflecting their poor root growth, but inclusion of Glc

or Suc greatly improved their growth (data not shown).

Pigment Accumulation in Mutant Embryos

Arabidopsis embryos are completely green from torpedo stage until the onset of desiccation, when they lose color as a result of chlorophyll breakdown (Meinke, 1994). However, mutants that fail to complete the maturation process, such as *fus3*, *lec1*, and the severe alleles of *abi3*, also fail to lose chlorophyll at this stage (Nambara et al., 1992; Ooms et al., 1993; Keith et al., 1994; Meinke et al., 1994). In contrast to chlorophyll, anthocyanin does not accumulate to significant levels in Arabidopsis embryonic tissues until after germination. The precocious accumulation of anthocyanin in the *leafy cotyledon* class of mutants (e.g. *fus3* and *lec1*) is part of the basis for describing these as heterochronic mutants (Keith et al., 1994; Meinke et al., 1994).

As previously described for the *abi3 fus3* and *abi3 lec1* digenic mutants (Keith et al., 1994; Parcy et al., 1997), the *abi4* and *abi5* combinations with *fus3* and *lec1* were first recognized as highly pigmented seeds among the segregating F$_2$ progeny. To quantify the effects on pigment accumulation before the onset of vivipary, early desiccation stage seeds were excised and used for extraction of chlorophyll or anthocyanin (Fig. 2). The large SDs reflect the fact that chlorophyll decreases rapidly, whereas anthocyanin increases rapidly, at this stage in seed development. As a consequence, substantial variation can be observed within a single silique.

The amount of chlorophyll in monogenic mutant immature seeds was similar to that in the corresponding wild-type lines (Fig. 2A). Despite the lack of effect of the single mutations, chlorophyll accumulation was significantly enhanced in all of the double mutants except *abi5 lec1*. Although mean anthocyanin levels in *fus3* and *lec1* increased 1.5- to 3-fold relative to their corresponding wild-type lines (Fig. 2B), there was no statistically significant difference in anthocyanin content between any of the monogenic mutants and their wild-type progenitors at this stage. Although the average anthocyanin content of *abi5 lec1* seeds was slightly higher than that in *lec1* seeds at this stage, this was also not a statistically significant difference. In contrast, the anthocyanin levels in the double mutants revealed a strong synergistic interaction (from 10–16-fold higher than the highest of the corresponding monogenic parents) between both *abi* mutations and *fus3* and between *abi4* and *lec1*.

Embryonic Gene Expression

Mutations in *ABI4* and *ABI5* have been shown previously to have minor effects on gene expression during embryogenesis, indicating that these loci are required for only a subset of the *ABI3*-regulated
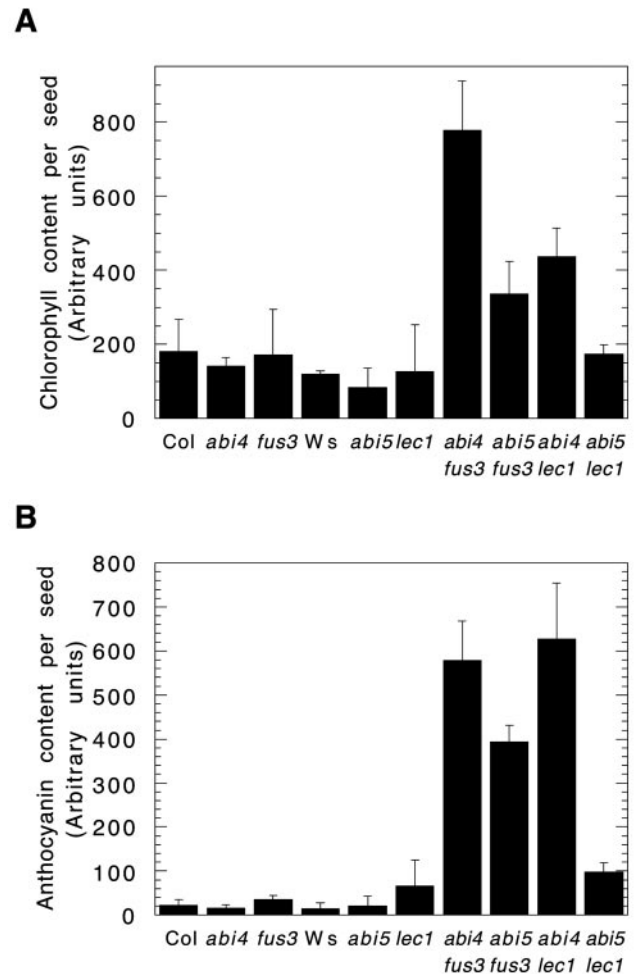

Figure 2. Pigment accumulation in early desiccation stage mono- and digenic *abi*, *fus3*, and *lec1* seeds. Chlorophyll and anthocyanin content are expressed in arbitrary units and normalized to the number of seeds used in each sample. Values are the mean of two to 11 measurements with samples of 23 to 46 seeds each. Bars indicate SDs. A, Chlorophyll content. B, Anthocyanin content.

genes (Finkelstein and Lynch, 2000a; Söderman et al., 2000). In contrast, *fus3* and *lec1* mutations result in severely reduced embryonic gene expression (Keith et al., 1994; Parcy et al., 1997; Nambara et al., 2000; Vicient et al., 2000). For example, genes for the storage proteins *At2S3* and *CRC*, and the lipid body protein oleosin (*PAP147*), show essentially no change in expression in the *abi4* or *abi5* mutants, whereas their transcript accumulation in midembryogenesis is severely reduced in the *fus3* and *lec1* mutants (Fig. 3A). In late embryogenesis, transcript levels for the late embryogenesis-abundant (*LEA*) genes *AtEm1* and *AtEm6* are greatly reduced in *abi5* mutants and are even lower in *lec1* mutants (Fig. 3B). However, this has previously been shown to reflect delayed expression in the *lec1* mutants such that *AtEm1* transcripts reach at least wild-type levels in dry *lec1* seeds (Vicient et al., 2000), possibly because of a stress response preceding death of these desiccation-
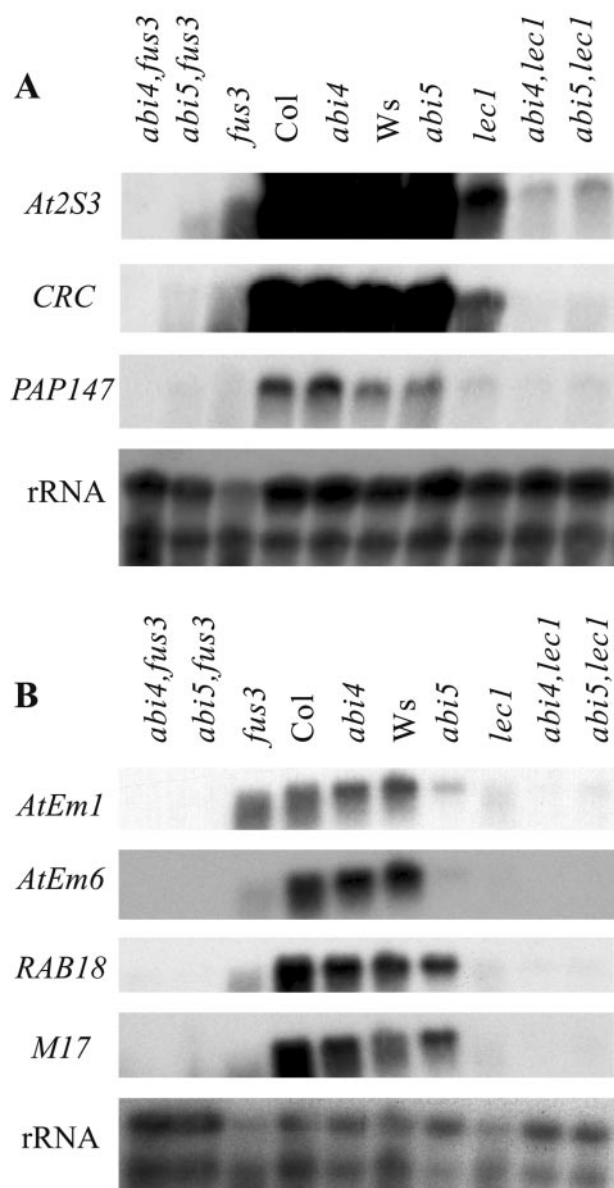
Figure 3. Embryonic gene expression in mono- and digenic *abi*, *fus3*, and *lec1* mutants. RNA was extracted from immature siliques, then analyzed by RNA gel blots hybridized to cloned probes for the indicated transcripts. A, Maturation stage siliques (8–11 DPA). B, Late embryogenesis stage siliques (17–21 DPA). Filters in A and B contain 5 and 2.4 μg of total RNA, respectively.

intolerant seeds. In contrast, although *AtEm6* transcripts accumulate to at least wild-type levels in *abi4* mutants at late embryogenesis (Söderman et al., 2000), they do not increase further by seed maturity, resulting in slightly lower than wild-type levels in dry seeds (Finkelstein, 1994). *AtEm6* expression is also reduced in *fus3* mutants, but *AtEm1* expression increases in *fus3* seeds (Fig. 3B), as previously documented for *AtEm1* promoter activity (Vicient et al., 2000). The expression of *RAB18* and *M17* is severely reduced in *fus3* and *lec1* mutants, and near normal in *abi4* mutants, but *M17* expression is slightly in-

creased in *abi5* mutant seeds at desiccation stage. These results also confirm previous observations (Finkelstein and Lynch, 2000a; Nambara et al., 2000; Söderman et al., 2000; Vicient et al., 2000).

Comparison of transcript accumulation at mid- and late embryogenesis in mono- and digenic mutant seeds suggests that the *abi* mutations slightly enhance the effects of the *fus3* and *lec1* mutations with respect to expression of some storage reserve (*At2S3*, *cruciferin C*, and *oleosin*; Fig. 3A) and *LEA* (*AtEm1*, *AtEm6*, and *RAB18*; Fig. 3B) genes. With respect to *M17* expression, the decreased expression due to *fus3* or *lec1* appears epistatic to the increase observed in the *abi5* mutant. In contrast, the decreased expression of *AtEm1* because of the *abi5* mutation is epistatic to the increase observed in the *fus3* mutant. These complex double mutant phenotypes suggest that different target genes are regulated by varying combinations of transcription factors.

Previous studies have demonstrated cross regulation of *ABI3*, *ABI4*, and *ABI5* transcript accumulation and/or promoter activity (Söderman et al., 2000), as well as synergistic effects of *LEC1* or *FUS3* and *ABI3* on ABI3 protein accumulation (Parcy et al., 1997). To determine whether the observed genetic interactions might reflect cross regulation of *ABI4*, *ABI5*, *FUS3*, and *LEC1* expression, we assayed their transcript accumulation in the various mutant backgrounds (Fig. 4; data not shown). We had shown previously that *ABI4* expression was near normal in *abi5*, *fus3*, and *lec1* seeds (Söderman et al., 2000). Although *ABI4* transcript levels vary slightly among seed lots, these levels show no significant change in the digenic mutants relative to the monogenic parents (data not shown). In contrast, *ABI5* expression is reduced in *abi5*, *lec1*, and all digenic mutant seed. However, this reduction is probably not a trivial reflection of the failure of these seeds to reach maturity, when *ABI5* transcript levels are highest, because monogenic *fus3* seeds express *ABI5* at or above wild-type levels despite failing to complete maturation. *FUS3* expression is significantly reduced only in the *lec1* mutants, and *LEC1* expression is eliminated in all the *lec1* mono- and digenic mutants, reflecting the fact that the *lec1-1* allele is a deletion. These results show cross regulation of *ABI5* and *FUS3* by *LEC1*, and are consistent with the previously described autoregulation of *ABI5* (Finkelstein and Lynch, 2000a; Brocard et al., 2002). The major peak of *LEC1* expression occurs during the 1st week of embryogenesis (Lotan et al., 1998), preceding the major peak of *ABI5* transcript accumulation by approximately 2 weeks (Finkelstein and Lynch, 2000a; Brocard et al., 2002), suggesting that LEC1 regulates *ABI5* expression indirectly.

Regardless of whether cross regulation is observed, the strong synergy among some of these mutations cannot be explained simply by altered expression or stability of the ABI gene products alone; the *abi4*, *abi5*, and *lec1* alleles used in this study are biochemical
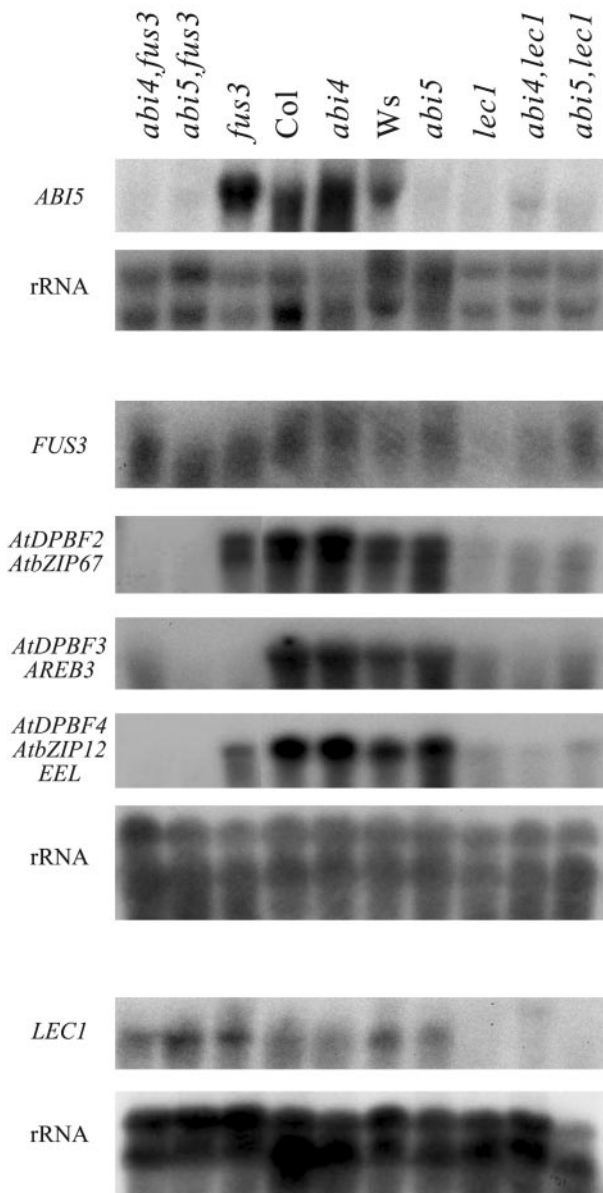
Figure 4. *ABI5*, *FUS3*, *LEC1*, and *ABI5* homologs expression in mono- and digenic *abi*, *fus3*, and *lec1* mutants. RNA was extracted from dry seeds or immature siliques, then analyzed by RNA gel blots hybridized to cloned probes for the indicated transcripts. *ABI5* expression was assayed in dry seeds (5 μg of total RNA per lane), *FUS3* and the *ABI5* homologs were assayed at maturation stage (8–11 DPA; 10 or 7 μg of total RNA per lane for *FUS3* or the *ABI5* homologs, respectively), and *LEC1* was assayed in 1- to 5-DPA siliques (20 μg of total RNA per lane). *ABI5* homologs tested were *AtDPBF2/AtbZIP67*, *AtDPBF3/AREB3*, *AtDPBF4/EEL/AtbZIP12*, *AtDPBF5/ABF3*, and *ABF4/AREB2*; *AtDPBF5/ABF3* and *ABF4/AREB2* transcripts were not detected in any genotype at this stage. The heterogeneity of transcript sizes observed for *FUS3* and *AtbZIP67/AtDPBF2* have been reported previously (Luerssen et al., 1998; Bensmihen et al., 2002). Each hybridization was performed with a fresh blot; the rRNA control depicted is from rehybridization of one representative blot.

nulls and their intrinsic defects cannot be enhanced. However, this synergy might reflect additional cross regulation of other factors that interact with the ABI transcription factors. For example, several other members of the ABI5 subfamily of bZIPs form heterodimers with ABI5 (Kim et al., 2002) and display varying patterns of cross regulation by overexpression of several of the *ABI*s in vegetative tissue (Brocard et al., 2002). Most of these are expressed most abundantly in early to midembryogenesis (Bensmihen et al., 2002), so we compared their transcript accumulation at midembryogenesis (Fig. 4). All members of this bZIP family with detectable expression at this stage were expressed normally in the *abi* monogenic mutants, but these transcript levels were much lower in the *lec1* monogenic and all digenic mutants. Expression of these bZIP genes was more variable in *fus3* mutants, ranging from slightly to strongly underexpressed (Fig. 4; data not shown). In addition to the possible synergistic regulation of these bZIPs by the *ABI* and *FUS3* genes, the combined loss of the ABI factors and these potentially interacting bZIP factors could result in significantly enhanced signaling defects in the digenic mutants.

Interactions Affecting Sugar Response and Accumulation of Sugars

Stunted growth and increased anthocyanin accumulation are characteristic of wild-type plants grown on high concentrations of sugar (>250 mM). Therefore the high anthocyanin content of *abi4 lec1* or *abi fus3* digenic mutant embryos and seedlings is reminiscent of the effects of sugar on seedling growth. Mutations in *ABI4* and *ABI5* result in strong and weak sugar-resistant phenotypes, respectively (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000), whereas overexpression of either gene confers hypersensitivity to sugar (Brocard et al., 2002; Finkelstein et al., 2002). To determine whether the *LEC1* or *FUS3* loci affect sugar metabolism and/or response, and whether they interact with the *ABI* loci in this regard, we compared sugar sensitivity and accumulation of soluble sugars and starch in wild-type, monogenic, and digenic mutant seeds.

Sugar sensitivity was assayed by scoring growth (i.e. germination and production of true leaves) of seedlings derived from early desiccation stage embryos, after incubation on Glc concentrations ranging from 0% to 6% (w/v; up to 333 mM Glc) under either continuous light or dark conditions after stratification in dim light. Although over one-half of the seeds of all genotypes except Columbia (Col) and *abi4* germinated on up to 4% (w/v) Glc in light (Fig. 5A), subsequent seedling growth was severely reduced by 4% (w/v) Glc in all of the wild-type and monogenic lines such that true leaves were seldom seen until after 1 week (Figs. 5B and 6, A and B). The monogenic *fus3* and *lec1* mutants appear to display stage-specific
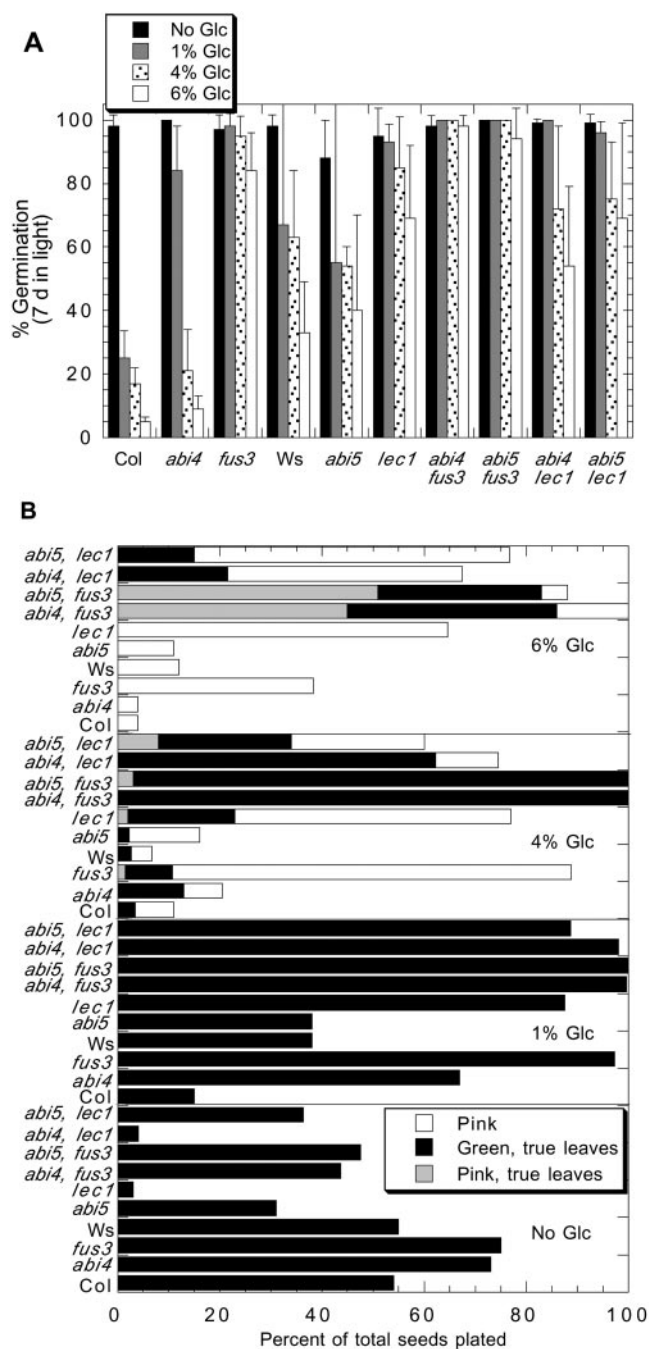
Figure 5. Glc effects on germination and growth in mono- and digenic *abi*, *fus3*, and *lec1* mutants. Seeds were excised at early desiccation stage and cultured on minimal media supplemented with 0%, 1%, 4%, or 6% (w/v) Glc. Germination (A) and seedling color and production of true leaves (B) were scored after 7 d of incubation in continuous light. Graphs show mean values of at least two independent experiments for each genotype. Bars indicate SD for germination data.

defects in sugar response: They germinate at a higher frequency than wild type on media containing high Glc, but appear hypersensitive to Glc effects on seedling growth, producing many stunted pink seedlings

on 4% (w/v) Glc (Fig. 5). Although stunted, the *fus3* seedlings do not arrest growth, some producing dark-pink plants with true leaves (Fig. 6C). In contrast, the *abi fus3* and, to a lesser extent, *abi lec1* digenic mutants, germinate rapidly and maintain substantial growth on high Glc with some plants remaining green and producing true leaves even on 6% (w/v) Glc (Fig. 5). Thus, the digenic mutants appear resistant to germination inhibition, induction of anthocyanin accumulation, and repression of growth by high Glc even though the Glc response of the monogenic *abi* mutants resembles that in wild-type seedlings at this stage (Fig. 5). Similar to results previously described for dry seeds (Laby et al., 2000), sorbitol was less inhibitory of germination in early desiccation stage wild-type seeds than an equimolar concentration of Glc (>90% versus <20% germination for seeds exposed to 222 mM sorbitol and Glc, respectively), consistent with the view that the inhibitory effects of Glc are not simply because of osmotic stress. However, germination of the *fus3* mono- and digenic mutant seeds was even more resistant to sorbitol, reaching 100% even on 333 mM sorbitol, indicating that the Glc-resistant germination of these genotypes is accompanied by increased resistance to osmotic stress. In contrast to their relative effects on germination, sorbitol was slightly more inhibitory of seedling growth (i.e. production of true leaves) than Glc, for all genotypes except Col wild type and *abi4* (data not shown). The inhibition of growth imposed by sorbitol was especially pronounced in the *fus3* and *lec1* mono- and digenic mutants, possibly because at least some of the Glc serves a nutritional function (compare growth on 0% [w/v] versus 1% [w/v] Glc; Fig. 5B). Consistent with this possibility, supplementing the sorbitol with 1% (w/v) Glc substantially improved seedling growth of these genotypes (data not shown). These results indicate that the highly Glc-resistant seedling growth of the *abi fus3* digenic mutants might also be partially because of resistance to osmotic stress. In contrast to Glc, no concentration of sorbitol tested induced anthocyanin accumulation in any genotype, indicating that the observed hypersensitivity to Glc for this response in the *fus3* and *lec1* mutants was not because of osmotic stress.

Dark-grown plants germinated at lower frequencies in all but the *fus3* mono- and digenic lines (Fig. 7), possibly reflecting some residual photodormancy in the other genotypes. The wild-type and monogenic mutant seedlings are all etiolated in the dark, but the length of Col and *abi4* seedlings is quite variable, probably reflecting delayed germination of some individuals. Among germinated dark-grown plants, the anthocyanin accumulation characteristic of the sugar sensitive phenotype was not observed. However, these plants still respond to high sugar by suppressing the extreme hypocotyl elongation characteristic of etiolated growth. In contrast, low sugar (1% [w/v] Glc) slightly promotes elongation of most ge-

Figure 6. Morphology of mono- and digenic *abi*, *fus3*, and *lec1* mutant seedlings. A through D, Seedlings grown for 8 d in continuous light on medium with 4% (w/v) Glc. A, Wild-type Wassilewskija (Ws); B, *abi5*; C, *fus3*; D, *abi5 fus3*. E through J, Seedlings grown for 9 d in the dark on medium with 1% (w/v) Glc. E, Wild-type Ws; F, *abi4*; G, *fus3*; H, *abi4 fus3*; I, *lec1*; J, *abi4 lec1*; K, *abi5 lec1*.

notypes, but the *lec1* mono- and all digenic seedlings are still significantly shorter than the majority of wild-type and monogenic *abi* seedlings exposed to 1% (w/v) Glc (Table I). The *fus3* seedlings are also shorter than the majority of wild-type seedlings, but the variability within the wild-type set prevents this observation from being a statistically significant difference. Surprisingly, all of the digenic lines have

expanded leaves and cotyledons in the dark, resembling de-etiolated mutants (Fig. 6, H, J, and K; data not shown).

Comparison of soluble sugar accumulation in dry seeds showed that Suc levels approximately doubled in all of the *fus3* or *lec1* mono- or digenic seeds except those of *abi5 fus3* (Fig. 8A). In contrast, *abi5 fus3* seeds had significantly increased levels of Fru, which was near the limit of detection in the wild-type and monogenic lines and only slightly increased in the other digenic mutants. More dramatic differences were observed in comparing starch accumulation; as previously described (Keith et al., 1994; Meinke et al., 1994), *fus3* or *lec1* seeds accumulate starch, whereas wild-type have almost no detectable starch. Although monogenic *abi* mutant seeds also lack starch, double mutant seeds contain 7- to 11-fold or 1.5- to 2-fold more starch than the corresponding monogenic *fus3* or *lec1* seeds, respectively (Fig. 8B). Because *fus3* and *lec1* mono- or digenic seeds fail to complete the desiccation phase of embryo development, we also assayed sugar and starch accumulation in early desiccation stage seeds of all genotypes. These seeds contain more starch than dry seeds, but similar trends were observed in comparisons among genotypes (data not shown). It is not clear whether the increased starch accumulation reflects increased synthesis, a failure to hydrolyze starch as normally occurs during Arabidopsis seed maturation, or a combination of these effects. However, it does not result in decreased accumulation of Suc (Fig. 8A), the other major storage form for fixed carbon.
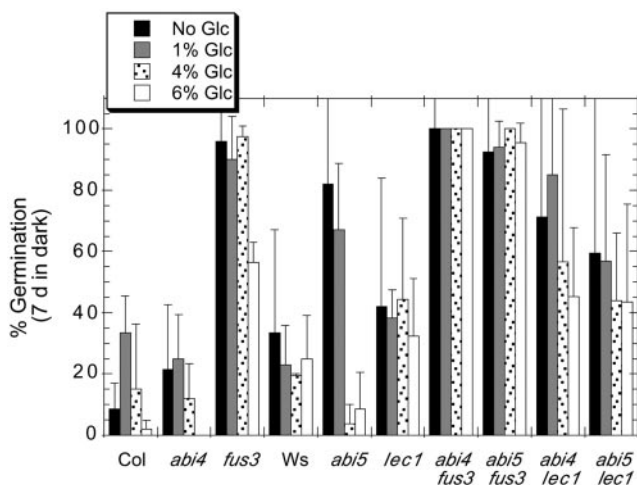

Figure 7. Germination of mono- and digenic *abi*, *fus3*, and *lec1* mutants in darkness. Seeds were excised at early desiccation stage and cultured on minimal media supplemented with 0%, 1%, 4%, or 6% (w/v) Glc. Germination was scored after 7 d of incubation in the dark. Graph shows mean values ± SD of at least two independent experiments for each genotype.

Table I. *Elongation of dark-grown seedlings*

Seeds of the indicated genotypes were excised at early desiccation stage and placed on minimal media with or without 1% (w/v) Glc, incubated for 3 d at 4°C, then for 9 d at 22°C in darkness before measuring hypocotyl lengths. Values shown are mean ± SD of lengths of five to 14 seedlings.

Genotype	Elongation on 1% (w/v) Glc	
	Hypocotyl length	Length on minimal media
	mm	*%*
Col	18.1 ± 6.7	78.9
abi4	14.1 ± 4.0	96.6
fus3	12.6 ± 3.7	137
Ws	22.8 ± 1.3	110
abi5	23.5 ± 1.45	94.3
lec1	4.5 ± 2.3[a]	109
abi4, fus3	8.3 ± 2.1[a]	152
abi5,fus3	7.8 ± 2.2[a]	136
abi4, lec1	1.8 ± 0.5[a]	134
abi5, lec1	6.6 ± 2.2[a]	254

[a]Significantly different from wild type ($P < 0.05$, based on Student's *t* test, unequal variance assumed).

Test of Physical Interactions between ABI, FUS, and LEC Gene Products

Our studies show that *ABI4* and *ABI5* interact genetically with *FUS3* and *LEC1*. Although the severity of the digenic mutant phenotypes vary among responses, all appear to reflect interactions disrupting some subset of the following processes: suppression of vivipary, germination on ABA, pigment accumulation, embryonic gene expression, and sugar sensing and/or metabolism. Similar results have been obtained previously for *ABI3*, *FUS3*, and *LEC1* (Keith et al., 1994; Meinke et al., 1994; Parcy et al., 1997), although the earlier studies did not examine sugar sensing or metabolism. All five of these loci encode transcriptional regulators that might participate in a regulatory complex. To determine if the observed genetic interactions reflect direct physical interactions, we used a yeast two-hybrid assay system with GAL4-DNA-BD fusions to ABI3, ABI4, and ABI5 as "bait" constructs. These constructs used truncations of ABI3 and ABI4 because the full-length proteins are strong transcriptional activators in yeast (Söderman et al., 2000; data not shown). The ABI4, FUS3, and LEC1 proteins were fused to the transcription activation domain (AD) of GAL4. The AD vector and an AD-ABI5 fusion were included as negative and positive controls, respectively. Physical interactions would be reflected by trans-activation of the GAL4-responsive *lacZ* reporter gene. Although these bait constructs do produce functional products (Nakamura et al., 2001; data not shown), no strong interactions (i.e. resulting in greater than 2-fold enhancement of *lacZ* expression) were observed between FUS3 or LEC1 and any of the ABIs (Fig. 9). The high intrinsic activating function of the BD-ABI4 fusion makes it difficult to determine whether the statisti-

cally significant, but less than 2-fold, enhancement of *lacZ* activation by the AD-FUS3 construct is biologically significant. The even higher intrinsic activation because of a BD-FUS3 fusion precluded attempting the reciprocal experiment (data not shown). Although AD-LEC1 produced a 2-fold increase in trans-activation by BD-ABI5, no interaction was detected between BD-LEC1 and AD-ABI5 (data not shown). These results also show apparent homodimerization of ABI3, consistent with the previously documented cooperative DNA-binding activity of the B3 domain (Suzuki et al., 1997), as well as reproducing the previously documented ABI5 homodimerization and ABI5/ABI3 interaction (Nakamura et al., 2001). We also tested for formation of a ternary complex by combining AD-FUS3 and BD-LEC1 with each ABI, but no interactions were observed in these combinations (data not shown).
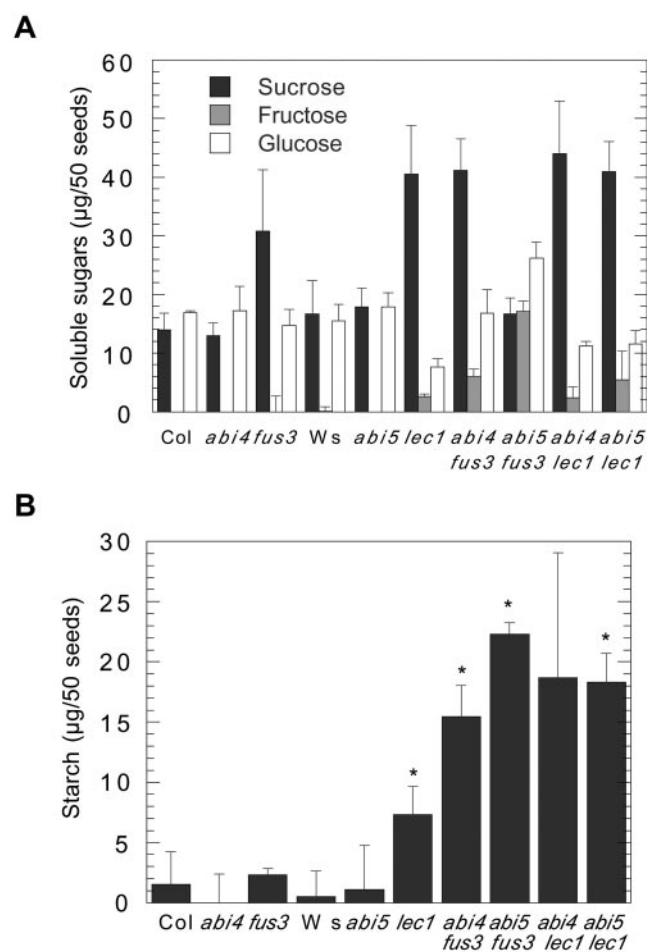

Figure 8. Soluble sugar and starch accumulation in mono- and digenic *abi*, *fus3*, and *lec1* mutant seeds. Suc, Glc, and Fru (A) and starch (B) were assayed enzymatically in extracts from 50 dry seeds of the indicated genotypes. Graph shows mean values ± SD of three samples of 50 seeds each. Asterisks indicate starch contents significantly different from wild type for the monogenic mutants or from the corresponding monogenics for the digenic mutants ($P \ll 0.05$, based on Student's *t* test, unequal variance assumed).
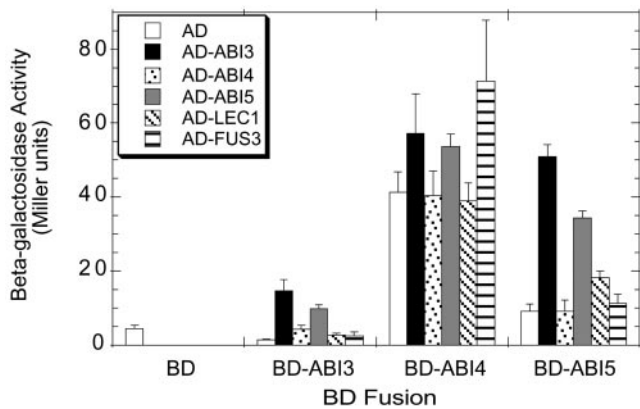
Figure 9. Yeast two-hybrid assays of interactions between ABI transcription factors and LEC1 or FUS3. β-galactosidase activity of yeast harboring plasmids encoding the GAL4-AD (AD) or the indicated GAL4-AD fusions in combination with either GAL4-BD (BD) or the indicated GAL4-BD fusions. The BD-ABI3 and BD-ABI4 fusions encode truncated forms of these ABI proteins to reduce their intrinsic activation function (see "Materials and Methods" for details). Values are the means ± SD of assays on at least three independent transformants.

DISCUSSION

Regulatory Networks in Seed Development and Germination

FUS3 and *LEC1* expression peak during the morphogenesis phase of embryo development, but these genes affect processes throughout seed development, regulating the establishment of body plan, suppression of vivipary, reserve accumulation, expression of some *LEA* genes, and induction of desiccation tolerance (Lotan et al., 1998; Luerssen et al., 1998; Raz et al., 2001). Mutations in these genes result in seed lethality unless embryos are rescued before desiccation. Although *ABI3*, *ABI4*, and *ABI5* genes are also expressed early in embryogenesis, expression of *ABI4* and *ABI5* peaks at seed maturity, consistent with a greater role in controlling the transition from embryogenesis to germination (Parcy et al., 1994; Söderman et al., 2000; Brocard et al., 2002). Comparison of severe loss of function alleles for these *ABI* loci has shown that *ABI3* is most critical for seed maturation and sensitivity to ABA inhibition of germination, but that *ABI4* and *ABI5* may be more important in regulating seedling establishment, particularly under stress conditions. Unlike the *abi* and *lec1* mutants, *fus3* mutants do not display ABA-resistant germination.

Previous experiments showed that *ABI3*, *FUS3*, and *LEC1* act synergistically to control multiple processes during seed development, including promotion of chlorophyll breakdown, suppression of anthocyanin accumulation, and control of sensitivity to ABA for germination inhibition (Parcy et al., 1997). Similarly, *ABI1* and, to a lesser extent, *ABI2* appear to act synergistically with *FUS3*; *abi1 fus3* and *abi2 fus3* double mutant seeds have increased vivipary and are redder than their respective monogenic parents, but the pig-

ment levels were not quantified (Keith et al., 1994). We report herein the digenic analyses with *FUS3* or *LEC1* and *ABI4* or *ABI5*.

Synergistic effects on ABA-resistant germination and pigment levels were observed for the *abi4 fus3*, *abi5 fus3*, and *abi4 lec1* mutants, but the defects of the monogenic parents were at most slightly enhanced in *abi5 lec1* mutants. All double mutants tested had synergistic effects on starch accumulation. Comparison of embryonic gene expression showed that most of the transcripts assayed were greatly reduced in *fus3* and *lec1* mutants such that it was difficult to determine whether any further suppression occurred in the double mutants. However, the observation that the monogenic *abi* mutants have little or no effect on storage protein gene expression, yet further decrease storage protein gene expression in the digenic mutants, is consistent with a synergistic interaction. The observations that *fus3* and *lec1* appear epistatic to *abi5* with respect to effects on *M17* expression, yet *abi5* appears epistatic to *fus3* with respect to *AtEm1* expression, are not readily explained by simple hierarchical genetic interactions. Surprisingly, although sugar induction of anthocyanin accumulation in precociously germinating desiccation stage seeds was not affected in *abi* mutants and was enhanced in *lec1* and *fus3* mutants, the digenic mutants were resistant to this sugar-induced response: The fraction of seedlings with anthocyanin accumulation was consistently lower in the digenics than in the monogenic lines grown on any given Glc concentration. One possible explanation for this is that in the digenic lines the heterochronic *lec1* or *fus3* mutations change the developmental context such that the *abi* mutations function as they would during normal germination, when they confer sugar-resistant growth (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). In this regard, the *abi* mutations appear to have an epistatic effect on sugar induction of anthocyanin accumulation. These results are all suggestive of genetic interactions among these genes, but the nature of the interaction varies depending on the affected response.

To date, all of the *ABI* transcription factors have been shown to interact genetically with *FUS3* and *LEC1*. Possible explanations for these results include cross regulation of expression and/or direct physical interactions. Although all the *ABI* and *LEC* class transcription factors analyzed in these studies are expressed to varying degrees throughout embryo development, they do not display the same developmental profile of accumulation. *LEC1* expression is most abundant early in embryogenesis (Lotan et al., 1998) and *FUS3* transcripts peak at midembryogenesis (Luerssen et al., 1998), whereas *ABI4* and *ABI5* transcripts peak at seed maturity (Finkelstein and Lynch, 2000a; Söderman et al., 2000). The recently cloned *LEC2* gene also encodes a B3 domain transcription factor, is expressed from early to midembryogenesis

(Stone et al., 2001), and might also interact genetically with these *ABI* loci, but this hypothesis has not yet been tested. The major functions of the *LEC* class genes appear to be maintenance of an embryonic state, whereas the *ABI*s promote embryo maturation. The observed effects of the mutations on transcript accumulation are consistent with sequential or hierarchical *LEC1*-dependent activation of *FUS3* and *ABI5*. Consistent with an indirect effect of LEC1 on *ABI5* expression, a LEC1-GAL4AD fusion failed to significantly enhance expression of ABI5 promoter-lacZ fusions in yeast (data not shown). An alternate explanation for *LEC1*-dependent *FUS3* and *ABI5* expression is that the *lec1* mutants might either reduce or lose the stages when these genes are normally expressed. Consistent with this hypothesis, the observed restoration of *FUS3* expression in the *abi lec1* mutants at midembryogenesis (Fig. 4) might reflect an *abi*-dependent failure to accelerate toward maturation phase, when *FUS3* transcript levels normally decrease. Expression of several of the ABI5-homologous bZIPs is also dependent on *LEC1*, and to a lesser extent *FUS3* and the *ABI*s, but the lack of characterized mutants for most of these loci makes their role less clear. Surprisingly, one of these (AtbZIP12/EEL/AtDPBF4) has recently been shown to act antagonistically to ABI5 with respect to *AtEm* expression, possibly by competing for binding to the same sites within the promoter (Bensmihen et al., 2002). Similar to the reported phenotype of *abi5 eel* digenic mutants, the *fus3* digenics and all the *lec1* mutant lines have reduced *AtEm* expression, consistent with their decrease in both *ABI5* and *AtbZIP12/EEL/AtDPBF4* expression. In contrast, the slight decrease in *AtbZIP12/EEL/AtDPBF4* expression in *fus3* mutants may be sufficient for the observed increase in *AtEm1* expression, yet does not enhance *AtEm6* expression. Unlike *ABI5* and its homologs, *ABI4* expression appears to be unaffected by the *LEAFY COTYLEDON* class mutants. Thus, of all the regulatory loci tested, expression of *ABI5* and/or its homologs are most closely correlated with changes in marker gene expression.

In addition to the observed cross regulation of regulatory gene expression, levels of active protein for these regulators might be subject to posttranscriptional controls, as have been described for ABI5 in seedlings (Lopez-Molina et al., 2001). *ABI3* transcript levels in *abi3*, *fus3*, and *lec1* mono- and digenic mutants did not correlate with severity of the mutant phenotype, yet comparison of ABI3 protein levels in these lines demonstrated that *FUS3* and *LEC1* act synergistically with *ABI3* to control ABI3 accumulation (Parcy et al., 1997). Surprisingly, *fus3* and *abi3-4* monogenic mutants show the opposite effect: enhanced ABI3 accumulation. This complexity of regulation is similar to our observations of *FUS3* expression in mono- versus digenic mutants. One possible explanation for the synergistic effects on ABI3 pro-

tein accumulation is that ABI3 might be stabilized by direct interaction with FUS3 or LEC1, both of which also encode transcription factors. However, on the basis of yeast two-hybrid assays with the *ABI* and *LEAFY COTYLEDON* class gene products, ABI3 appears to interact directly only with ABI5 and itself (Nakamura et al., 2001; Fig. 9). Perhaps it is not surprising that FUS3 and ABI5 do not appear to interact directly because the interaction between ABI3 and ABI5 requires the B1 domain of ABI3 (Nakamura et al., 2001) and FUS3 lacks a B1 domain, even though both ABI3 and FUS3 are members of the B3 domain family of transcription factors (Luerssen et al., 1998). The failure to detect interactions among the remaining transcription factors might reflect a simple lack of physical interactions, a requirement for modification (e.g. phosphorylation) that does not occur in yeast, deletion of a domain required for interaction (to reduce intrinsic activation by the BD fusion), or a requirement for additional factors. As a consequence, the lack of interaction in yeast does not exclude the possibility that these factors participate in a regulatory complex in plants. However, the discrepancies in developmental timing of peak expression for most of these regulators argue against direct interactions as a major mechanism of the observed genetic interactions.

The ABI/LEC Class Network Integrates Responses to Developmental, Chemical, and Abiotic Signals

Physiological and genetic studies have demonstrated both antagonistic and similar effects of ABA and sugar in embryogenesis and germination (for review, see Finkelstein and Gibson, 2002). The midembryogenesis transition from growth by cell division to growth by enlargement is correlated with a decrease in Glc and an increase in endogenous ABA. This transition suppresses vivipary and is also dependent on *FUS3* and *LEC1* function (Raz et al., 2001), but not *ABI4* or *ABI5*. Exogenous ABA inhibits germination at seed maturity, but this effect can be suppressed by low levels of Glc or Suc, demonstrating another antagonistic interaction between sugar and ABA (Finkelstein and Lynch, 2000b). However, the developmental arrest and intense anthocyanin accumulation induced by exposure to high concentrations of sugar during germination of mature seeds is partially dependent on ABA and the *ABI* transcription factors (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). Although only *ABI4* has been identified genetically by sugar-sensing screens to date, under- and/or overexpression of *ABI5* and *ABI3* also modify sugar sensitivity (Finkelstein et al., 2002).

Comparison of sugar metabolism and response to sugar or osmotic stress demonstrated that all were disrupted in *fus3*, *lec1*, and digenic mutant seeds. Furthermore, sugar sensitivity appears to be regu-

lated differently before and after seeds reach maturity. For example, although highly resistant to Glc after seed maturity, *abi4* mutants are only weakly resistant to Glc inhibition of germination and growth before seed maturity. In contrast, the *fus3* and *lec1* mutants confer sugar/osmotic stress-resistant germination, but hypersensitivity to sugar-induced anthocyanin synthesis at this stage; their lack of desiccation tolerance precludes testing their sensitivity at the dry seed stage. The growth defects of the *fus3* and *lec1* mutant embryos might be enhanced by the combination of hypersensitivity to sugar and doubled endogenous levels of Suc. However, the digenic mutants are highly resistant to sugar/osmotic effects on germination and growth, as well as to sugar-specific induction of anthocyanin synthesis, despite having increased endogenous levels of Suc (or Fru, in the case of *abi5 fus3*) and exhibiting intense pigmentation as developing embryos. The degree of sugar/osmotic resistance in the digenic mutants correlates with their ABA resistance. One possible explanation for this result is that the digenic mutants have undergone a phase transition that permits extremely rapid germination and escape from the brief developmental window (up to approximately 36 h post-stratification) of sensitivity to high concentrations of sugar (Gibson et al., 2001), whereas the monogenic *fus3* and *lec1* mutants precociously enter this sugar-sensitive phase but do not escape it.

Our results also show that the extreme anthocyanin accumulation characteristic of seedlings grown on high concentrations of sugar is a synergistic effect of sugar and light signaling. Anthocyanin accumulation is a well-characterized response to UV or high-intensity light stress (for review, see Mol et al., 1996) and moderate anthocyanin accumulation in response to low sugar (1% [w/v] Suc) has been demonstrated to be phytochrome dependent (Montgomery et al., 1999). However, neither moderate light nor a high sugar concentration alone is sufficient to induce extreme anthocyanin accumulation. The *fus3* and *lec1* mutants are characterized by the red color of their embryos and their hypersensitivity to sugar-induced anthocyanin accumulation, both consistent with a role for these loci in repressing this light- and sugar-induced response. Although *fus3* differs from the other *FUSCA* loci in that it has not yet appeared in a screen for "de-etiolated" or "constitutively photomorphogenic" growth, we found that the *abi fus3* digenic mutants had a mild de-etiolated phenotype on low concentrations of sugar. Similarly, although *lec1* mutant seedlings tend to be stunted under all conditions, cotyledon and true leaf expansion in the dark is observed only in the *abi lec1* digenic mutants. These results suggest that the *ABI* and *LEC* class loci interact in some aspects of light and sugar response as well as ABA and seed developmental responses. Consistent with this hypothesis, attempts to combine *lec1* with a constitutively photomorphogenic mutant,

cop1, resulted in embryo lethality at torpedo stage (Meinke et al., 1994). This early lethality might reflect a genetic interaction between *lec1* and *cop1* or an additive effect resulting in heterochronic onset of seedling lethality, as previously suggested (Meinke et al., 1994).

Recent studies of *ABI3* have also demonstrated a role in some aspects of light response such as plastid differentiation (Rohde et al., 2000). Furthermore, *ABI3* has been shown to interact genetically with *DET1* in regulating germination, plastid differentiation, anthocyanin accumulation, floral determination, and internode elongation (Kurup et al., 2000; Rohde et al., 2000). However, as described herein for *ABI4*, *ABI5*, *FUS3*, or *LEC1*, the interactions are complex, ranging from synergistic effects on germination to antagonistic effects on plastid differentiation (Rohde et al., 2000).

Summary

The *ABA INSENSITIVE* and *LEAFY COTYLEDON* class transcription factors regulate overlapping events in seed development. Comparison of digenic mutants shows that, for most responses in late embryogenesis, the most severe defects are observed in *abi4 fus3*, with progressively less severe defects in *abi5 fus3*, *abi4 lec1*, and *abi5 lec1*. Genetic interactions ranging from synergistic to antagonistic have been documented for each combination of mutations, and have revealed a variety of cryptic effects of these mutations. Although *FUS3* and *LEC1* appear to interact with all of the *ABI* transcription factors, previous analyses of *fus3 lec1* digenic mutants led to the conclusion that these loci participate in distinct regulatory pathways (West et al., 1994). The complexity of these interactions is more consistent with combinatorial controls than a hierarchical signaling pathway, but some cross regulation of transcript or protein accumulation has also been described. However, few of these regulatory proteins appear to physically interact in yeast two-hybrid assays. Finally, although initially identified as regulators of ABA response and/or seed development, all of these loci also appear to function to varying degrees in mediating response to light, sugar, and osmotic stress. In fact, the characteristic reddish color of the *leafy cotyledon* class mutant embryos and seedlings may be explained by their combination of increased endogenous soluble sugars and hypersensitivity to light-dependent, sugar-induced anthocyanin synthesis.

MATERIALS AND METHODS

Plant Material

The *abi4-1* and *abi5-1* mutant lines were isolated from the Col and Ws backgrounds, respectively, as described by Finkelstein (1994). The *fus3-3* and *lec1-1* mutant lines were isolated from the Col and Ws backgrounds, respectively, as described by Keith et al. (1994) and West et al. (1994).

For RNA isolation from siliques, plants were grown in soil in continuous light at 22°C. Siliques were harvested in pools corresponding to four developmental stages: early embryogenesis (1–5 DPA), maturation (8–11 DPA), late embryogenesis (17–21 DPA), and dry seed (>21 DPA). The siliques were weighed, flash frozen in liquid nitrogen, and stored at −70°C until extraction. Dry seed were stored at room temperature.

Double Mutant Construction

To construct double mutants, *abi* plants were crossed with either *fus3* or *lec1* plants. The *fus3* and *lec1* mutations result in desiccation-intolerant seeds, so individuals homozygous for either of these mutations must be rescued by excision and culture of early desiccation stage seeds. For each cross, the F_2 progeny clearly segregated a novel phenotypic class of highly pigmented seeds (approximately 1/16 in this generation). These individuals were rescued and found to display phenotypic markers of the *fus3* and *lec1* lines (i.e. a linked *gl* mutation and trichomes on the cotyledons, respectively). The *abi* lines used for the crosses with *fus3* carried the linked *er* and *py* markers, such that *abi* individuals displayed the *erecta* growth habit and thiamine auxotrophy. In addition, DNA polymorphisms corresponding to each *abi* mutation could be scored by cleaved-amplified polymorphic sequence reactions, permitting direct confirmation of the *abi* genotype. For *abi4-1*, an 895-bp fragment with an NlaIV polymorphism was amplified with the following primers: 5′-CCCATAATAATCCTCAATCC-3′ and 5′-AAATCCCAAATACTCCCC-3′. For *abi5-1*, an 826-bp fragment with an *Ava*II polymorphism was amplified with the following primers: 5′-CAATCAACAAGCAGCAG-3′ and 5′-TCTCTCCACTACTTTCTCCAC-3′. Amplification conditions followed standard protocols (Konieczny and Ausubel, 1993), using 50°C annealing for *ABI4* and 60°C annealing for *ABI5*. Additional double mutant lines were obtained by selecting *abi* F_2 segregants by requiring germination on 3 μM ABA (mixed isomers, Sigma, St. Louis), then screening the F_3 progeny for highly pigmented seeds (approximately 25% in this generation). Double mutant lines were maintained by excising and culturing early desiccation stage seeds in each succeeding generation.

Germination Assays

Germination assays were performed with early desiccation stage seeds (20–70 seeds per treatment). Siliques were harvested and surface sterilized in 70% (v/v) ethanol, then seeds were excised and placed on minimal medium (Haughn and Somerville, 1986) containing 0.7% (w/v) agar supplemented with different concentrations of ABA, Glc, and/or sorbitol. The dishes were incubated for 3 d at 4°C to break any residual dormancy, then transferred to 22°C in continuous light (50–70 μE m^{-2} s^{-1}); germination was scored after 7 d.

Quantification of Chlorophyll and Anthocyanin Pigments

Early desiccation stage seeds were excised from the siliques, counted, and stored at −70°C. Immature seeds were ground at 4°C in 400 μL of either 80% (v/v) aqueous acetone (chlorophyll) or 1% (v/v) HCl in 60% (v/v) methanol (anthocyanin) as described by Parcy et al. (1997). The absorption spectrum was recorded between either 500 and 700 nm (chlorophyll) or 400 and 650 nm (anthocyanin). The quantity of pigments was measured as the value above baseline at the absorption maximum (663.5 nm for chlorophyll and 533.5 nm for anthocyanin), then normalized to the number of seeds used in each sample.

RNA Gel-Blot Analysis

RNA was isolated from immature siliques as previously described (Söderman et al., 2000). Dry seed RNA preps were based on a modified procedure of Vicient and Delseny (1999) as previously described (Söderman et al., 2000). RNA concentrations were estimated based on A_{260} and 280 nm.

Total RNA (2.4–20 μg per lane) was size fractionated on MOPS-formaldehyde gels (Sambrook et al., 1989), then transferred to nylon membranes (Osmonics Inc., Westborough, MA) using 20× sodium chloride/sodium phosphate/EDTA as blotting buffer. RNA was bound to the filters by UV cross-linking (120 mJ cm^{-2} at 254 nm). Uniformity of loading and transfer were assayed qualitatively by hybridization to an rDNA probe. The *ABI5* probe was a PCR-amplified genomic fragment excluding most of the conserved bZIP domain. The *ABI4* probe was an *Eco*RI fragment from a cDNA clone encompassing all but the first two and final codons of the coding sequence. Transcripts from *FUS3* and *LEC1* were detected by hybridization to cDNA clones. Hybridization probes for the *AtDPBF* transcripts were full-length cDNA clones described by Kim et al. (2002). Transcripts from *CRC*, *PAP147* (oleosin), *At2S*, *M17*, *AtEm1*, *AtEm6*, and *RAB18* were detected by hybridization to cDNA clones labeled by random priming to a specific activity of 10^8 cpm μg^{-1}, as described by Söderman et al. (2000). At least two independent RNA samples were analyzed for each genotype, stage, and probe tested.

Two-Hybrid Assays

Translational fusions between *ABI3*, *ABI4*, *ABI5*, *LEC1*, and *FUS3* genes and the *GAL4* activation and DNA-BDs were constructed in the pGAD-C(x) and pGBD-C(x) vectors, respectively (James et al., 1996). The GAL4-BD-ABI4 construct encoded a slightly truncated form of ABI4 (amino acids 3–287) because a full-length ABI4 fusion provides very strong transcription activation function in the absence of any AD fusion (Söderman et al., 2000). Similarly, the ABI3 fusion contains only the C-terminal basic domains of ABI3 (amino acids 216–670) because the N-terminal acidic domains provide a strong transcription activation function. The BD-ABI5 construct encoded all but the first eight amino acids of ABI5, thus including all conserved domains. The different fragments for the BD and AD fusions involving ABI genes were cloned as previously described by Nakamura et al. (2001). The newly constructed AD fusions encoded full-length LEC1 or all but the first 22 amino acids of FUS3, thereby including all conserved domains. All gene fusions were transformed into yeast (*Saccharomyces cerevisiae*) as previously described by Nakamura et al. (2001).

Quantification of Soluble Sugars and Starch

Starch and soluble sugar (Suc, Fru, and Glc) levels in samples of 50 dry seeds were determined as previously described Chia et al. (2000). After extraction of soluble sugars, the extract was divided into three fractions for parallel determinations of Suc, Fru, and Glc levels. Suc was digested with 400 units of invertase and 1 unit of phosphoglucoisomerase, followed by measurement of released Glc by the infinity Glc reagent (Sigma). Fru and Glc levels were determined by digestion of extracted soluble sugars with, respectively, 1 unit phosphoglucoisomerase or no enzyme, followed by measurement of released Glc by the infinity Glc reagent. Control experiments indicated that the Glc reagent was not contaminated with phosphoglucoisomerase, such that the measured Glc levels did not include any contribution from endogenous Fru levels (data not shown).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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