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The Conserved Splicing Factor SUA Controls Alternative Splicing of the Developmental Regulator *ABI3* in *Arabidopsis* [W] [OA]

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***ABSCISIC ACID INSENSITIVE3* (*ABI3*) is a major regulator of seed maturation in *Arabidopsis thaliana*. We detected two *ABI3* transcripts, *ABI3*-α and *ABI3*-β, which encode full-length and truncated proteins, respectively. Alternative splicing of *ABI3* is developmentally regulated, and the *ABI3*-β transcript accumulates at the end of seed maturation. The two *ABI3* transcripts differ by the presence of a cryptic intron in *ABI3*-α, which is spliced out in *ABI3*-β. The *suppressor of abi3-5* (*sua*) mutant consistently restores wild-type seed features in the frameshift mutant *abi3-5* but does not suppress other *abi3* mutant alleles. SUA is a conserved splicing factor, homologous to the human protein RBM5, and reduces splicing of the cryptic *ABI3* intron, leading to a decrease in *ABI3*-β transcript. In the *abi3-5* mutant, *ABI3*-β codes for a functional ABI3 protein due to frameshift restoration.**

INTRODUCTION

Seeds are essential for the spread and survival of most plant species and constitute a major food source. Seed features like desiccation tolerance, dormancy, and the accumulation of storage proteins are established during seed maturation. In *Arabidopsis thaliana*, the phytohormone abscisic acid (ABA) controls seed maturation and dormancy by preventing germination and reserve mobilization. ABA signaling at this stage is concomitant with the expression of four major regulatory genes of seed maturation with partially redundant functions: *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, *FUSCA3* (*FUS3*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*) (Kroj et al., 2003; To et al., 2006). ABI3 is a main component of the ABA signaling pathway and is highly conserved among plant species. The ABI3 protein contains four functional domains (Giraudat et al., 1992; Suzuki et al., 1997). The A1 domain is an acidic transcriptional activator (McCarty et al., 1991), and B1 can interact with the seed-specific transcription factor ABI5 (Nakamura et al., 2001). B2 and B3 are two basic DNA binding domains responsible for the ABA-dependent activation of seed maturation genes (Suzuki et al., 1997; Ezcurra et al., 2000; Nag et al., 2005).

Several *abi3* mutant alleles were isolated in *Arabidopsis*. One of the most severe is *abi3-5*, which was originally identified by its stay-green seed phenotype. *abi3-5* seeds are insensitive to ABA during germination, are desiccation intolerant, and have reduced longevity, similar to other strong *abi3* alleles (Ooms et al., 1993).

ABI3 transcription is promoted by LEC1, LEC2, FUS3, ABI3 (To et al., 2006), and ABA (Lopez-Molina et al., 2002). During germination, *ABI3* is repressed by the chromatin remodeling factor PICKLE (Perruc et al., 2007) and the ABI3 protein is targeted to 26S proteasome degradation by the ABI3-INTERACTING PROTEIN2 (Zhang et al., 2005). The identification of several splice variants of *ABI3* homologs in monocotyledon and dicotyledon species (McKibbin et al., 2002; Fan et al., 2007; Gagete et al., 2009) implies that alternative splicing also has an important role in controlling *ABI3* expression. However, splicing variants of *ABI3* were not observed in *Arabidopsis*.

Although alternative splicing of mRNA is an important component of posttranscriptional regulation in higher eukaryotes, its relevance and mechanisms in plants are poorly understood. In *Arabidopsis*, ~42% of all transcripts from intron-containing genes are alternatively spliced (Filichkin et al., 2010). Alternative splicing can produce transcripts that encode for proteins with altered or lost function. Furthermore, it can lead to tissue-specific transcripts or affect mRNA stability and turnover via nonsense-mediated decay (McGlincy and Smith, 2008). Splicing is directed by the spliceosome, a dynamic RNA-protein multicomponent machinery that is conserved among eukaryotes. In *Arabidopsis*, only a few splicing-related proteins have been characterized (Lopato et al., 1999; Ali et al., 2007; Tanabe et al., 2007; Zhang and Mount, 2009), and the information on their biochemical function and their targets in relevant developmental and environmental contexts is limited. We identified *SUPPRESSOR OF ABI3-5* (*SUA*) as a novel plant splicing factor that influences seed maturation by controlling alternative splicing of *ABI3*. SUA is an evolutionary conserved protein that suppresses splicing of a cryptic *ABI3* intron. Splicing of this intron leads to a transcript

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that encodes a truncated ABI3 protein in the wild type but a functional protein in the *abi3-5* mutant background.

RESULTS

Isolation of the *abi3-5 sua-1* Double Mutant

Seeds of the *abi3-5 glabra1* (*gl1*) *transparent testa5-1* (*tt5-1*) triple mutant were mutagenized by γ-irradiation to isolate mutants involved in ABI3 signaling. The *gl1* and *tt5-1* mutations are located on both sides of the *ABI3* locus and were used as phenotypic markers to distinguish suppressor mutants from wild-type contaminants. A strong suppressor mutant of *abi3-5* was identified in the M2 generation and named *suppressor of abi3-5* (*sua-1*). The *gl1-1* and *tt5-1* mutations were removed from this line by backcrossing with its wild-type Landsberg *erecta* (L*er*) genetic background and subsequent selection for *abi3-5* and *sua-1* in the progeny. Ripe *abi3-5* seeds are green due to the presence of chlorophyll, but *abi3-5 sua-1* seeds are yellow-brown, similar to the wild type (Figure 1A). In addition, *abi3-5* seeds are nondormant and sensitive to desiccation, which causes reduced longevity. Seeds of *abi3-5 sua-1* are also nondormant, but their longevity is strongly improved and they still germinate nearly 100% after 10 weeks of storage (Figure 1B). Finally, *abi3-5 sua-1* seeds show an increased sensitivity to ABA and cannot germinate on 15 μM ABA, whereas viable *abi3-5* mutant seeds show 100% germination on 30 μM ABA (Figure 1C).

Identification of the *SUA* Gene

Initial mapping indicated that the *sua* mutation is located on chromosome 3. Fine-mapping was performed using an F2 mapping population of ~4000 individuals derived from a cross between the *abi3-5 sua-1* double mutant (in L*er* background) and Columbia (Col-0). The *abi3-5 sua-1* double mutant was identified in this mapping population by its yellow-brown seed color trait in combination with the ability to germinate in the presence of 5 μm ABA. The location of the *sua-1* mutation was narrowed down to a region of 64 kb at the bottom of chromosome 3 between two markers located at 20.056 and 20.120 Mb. This region contains 17 genes and did not show recombination in our mapping

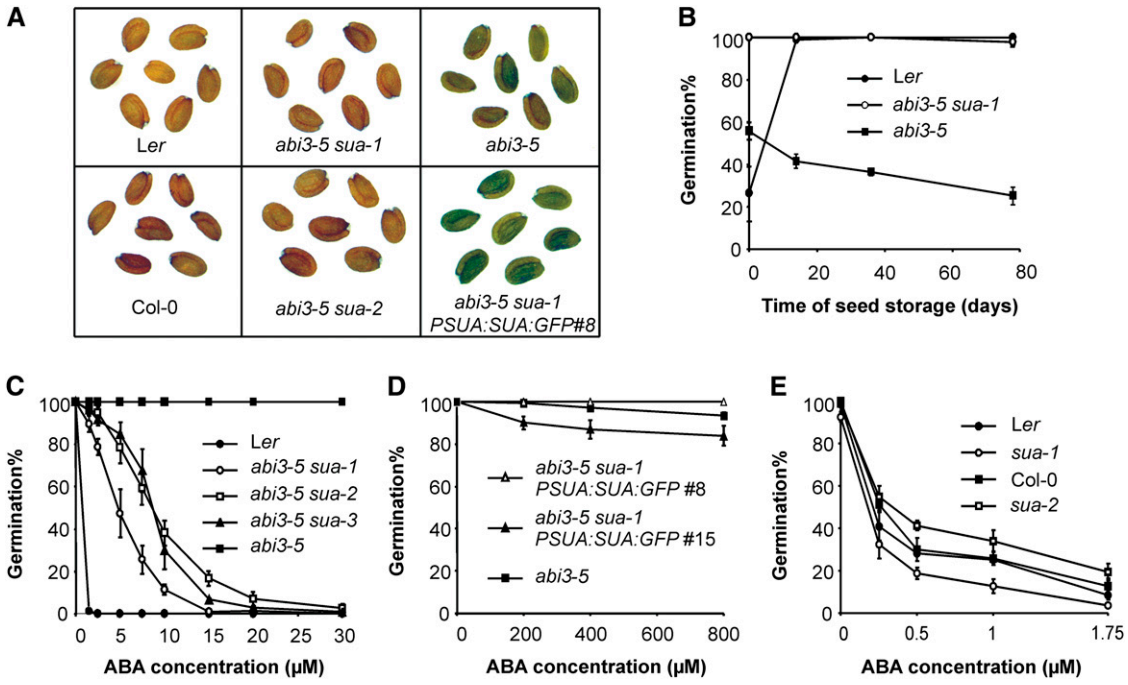


Figure 1. The *sua* Mutation Suppresses *abi3-5* Phenotypes.

(A) Seeds of wild type (L*er* and Col-0), *abi3-5* (in L*er* background), *abi3-5 sua-1* (in L*er* background), *abi3-5 sua-2* (in L*er*/Col-0 background) and *abi3-5 sua-1 PSUA:SUA:GFP* #8 (in L*er* background).

(B) Germination of L*er*, *abi3-5*, and *abi3-5 sua-1* seeds after different periods of dry storage. Harvested seeds were stored at 20°C and 42% relative humidity. Percentages are means (±SE) of three biological replicates.

(C) Germination of L*er*, *abi3-5*, *abi3-5 sua-1*, *abi3-5 sua-2*, and *abi3-5 sua-3* seeds, imbibed at different ABA concentrations. Seeds were 1 week after-ripened and stratified 4 d. Percentages are means (±SE) of four biological replicates.

(D) Germination of 1-week-old *abi3-5*, *abi3-5 sua-1 PSUA:SUA:GFP* #8, and *abi3-5 sua-1 PSUA:SUA:GFP* #15 seeds at different ABA concentrations. Percentages are means (±SE) of three biological replicates.

(E) Germination of L*er*, *sua-1*, Col-0, and *sua-2* seeds, imbibed at different ABA concentrations. Seeds were 6 months after-ripened and 4 d stratified. Percentages are means (±SE) of four biological replicates.

population. Comparison of sequenced candidate genes with sequences in The Arabidopsis Information Resource (Garcia-Hernandez et al., 2002) revealed a 47-bp deletion in the 15th exon of At3G54230 in the *abi3-5 sua-1* double mutant (Figure 2A).

The identity of At3G54230 as the *SUA* gene was confirmed by complementation of the *sua-1* mutant in the *abi3-5* background. A construct containing the *SUA* cDNA, expressed from a 2711-bp putative *SUA* promoter and fused with a C-terminal green fluorescent protein (*GFP*) tag (*PSUA:SUA:GFP*), was used to transform *abi3-5 sua-1* plants. Two independent T2 transformants, containing a single insertion event, both complemented *sua-1* and showed the *abi3-5* phenotype. One of these transformants, *abi3-5 sua-1 PSUA:SUA:GFP* #8, even showed an enhanced *abi3-5* phenotype, yielding seeds with a more intense green color and stronger ABA insensitivity (Figures 1A and 1D).

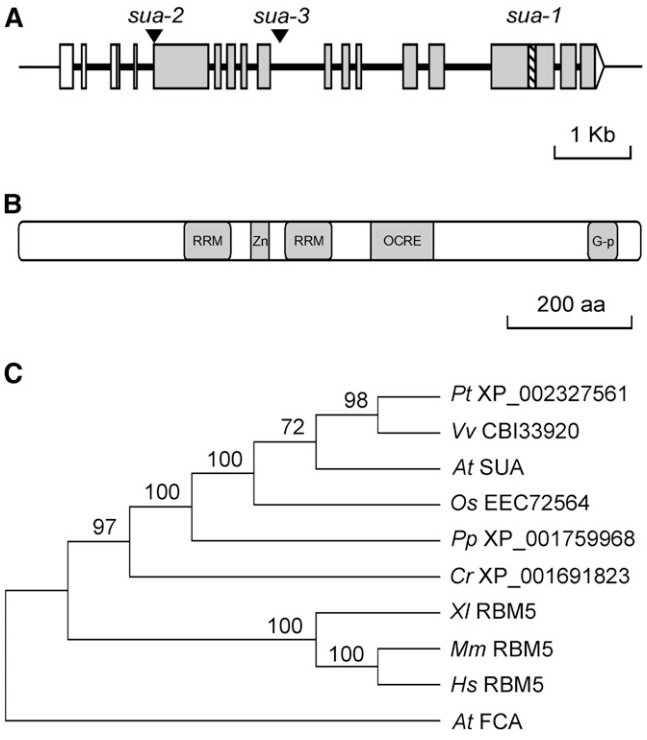


Figure 2. Genetic Structure, Domain Organization, and Phylogenetic Relationships of *SUA*.

(A) Schematic structure of the *SUA* gene. Triangles indicate the T-DNA insertion sites of *sua-2* and *sua-3*, and the dashed region represents the 47-bp deletion of the *sua-1* allele. UTRs are shown in white, exons in gray, and introns as thick lines.

(B) Domain structure of the SUA protein. aa, amino acids; RRM, RNA recognition motif; Zn, zinc finger; OCRE, octamer repeat; G-p, Gly patch.

(C) Phylogram of SUA and its closest related proteins. FCA is an RNA binding protein that was added to the tree to emphasize the similarity between SUA and its homologs in evolutionary distant species. *Populus trichocarpa* (*Pt*), *Vitis vinifera* (*Vv*), *Oryza sativa* (*Os*), *Physcomitrella patens* (*Pp*), *Chlamydomonas reinhardtii* (*Cr*), *Xenopus laevis* (*Xl*), *Mus musculus* (*Mm*), and *Homo sapiens* (*Hs*). Bootstrap values are shown when higher than 50.

Additional mutant alleles of *SUA* in the Col-0 background (*sua-2* and *sua-3*) were obtained from the Salk insertion mutant collection and from the GABI-Kat collection. These lines contain T-DNA insertions in the fourth and the ninth intron and were named *sua-2* and *sua-3*, respectively (Figure 2A). Both alleles lack full-length *SUA* expression and were crossed with *abi3-5*. The double mutants *abi3-5 sua-2* and *abi3-5 sua-3* were selected in the resulting F2, and all of them showed suppression of the *abi3-5* phenotypes, similar to *abi3-5 sua-1* (Figures 1A and 1C).

The *sua* single mutants did not have any obvious visual phenotype. Detailed analysis revealed that *sua-1* seeds are more susceptible to ABA germination inhibition compared with wild-type L*er*. By contrast, *sua-2* seeds germinated better than wild-type Col-0 in the presence of ABA (Figure 1E).

SUA Encodes an RNA Binding Protein Located in the Nucleus and Expressed in All Plant Tissues

SUA encodes a protein with a conserved domain architecture that suggests a function in RNA metabolism. SUA contains two RNA recognition motifs surrounding a Zinc finger domain, an octamer repeat domain, and a Gly-rich domain close to the carboxy end (Figure 2B). The *Arabidopsis* genome does not contain a second gene with this combination of domains. SUA homologs, however, can be found throughout the eukaryotic kingdom (Figure 2C). SUA has 45% sequence similarity with the human RNA Binding Motif Protein 5 (RBM5), which was originally identified as a putative tumor suppressor gene that is part of a small gene family (Edamatsu et al., 2000).

Publicly available microarray data (Zimmermann et al., 2004) show ubiquitous *SUA* expression in *Arabidopsis*, with a moderate enrichment in seeds. Quantitative real-time RT-PCR analysis confirmed that the relative abundance of *SUA* transcripts is comparable in most *Arabidopsis* tissues, but highest in siliques toward the end of seed maturation (Figure 3A). The subcellular localization of the SUA protein was studied using the *PSUA:SUA:GFP* lines. A GFP signal was detected in the nucleus of vegetative and reproductive tissues (Figure 3B). The SUA_GFP chimeric protein showed diverse patterns. Speckles of different size were observed in some nuclei, but fluorescence was diffuse and rather weak in others (Figure 3B). We did not observe a correlation between the SUA_GFP fluorescence pattern and tissue or developmental stages.

SUA Interacts with the Prespliceosomal Component U2AF^{65}

RBM5, the human homolog of SUA, is a member of the prespliceosomal complex (Behzadnia et al., 2007) and interacts with U2AF^{65} in vivo (Bonnal et al., 2008). U2AF^{65} is the larger subunit of the conserved pre-mRNA splicing factor U2AF. It guides splice site selection during the formation of the spliceosomal complex (Zamore et al., 1992; Sickmier et al., 2006). In a yeast two-hybrid GAL4 assay, we detected interaction between SUA and *Arabidopsis* U2AF^{65} (BAH19725) (Domon et al., 1998; Figure 4A). To confirm the SUA-U2AF^{65} interaction in planta, we performed a fluorescence resonance energy transfer/fluorescence lifetime imaging (FRET/FLIM) assay. *Arabidopsis* leaf protoplasts were cotransfected with two vectors for the

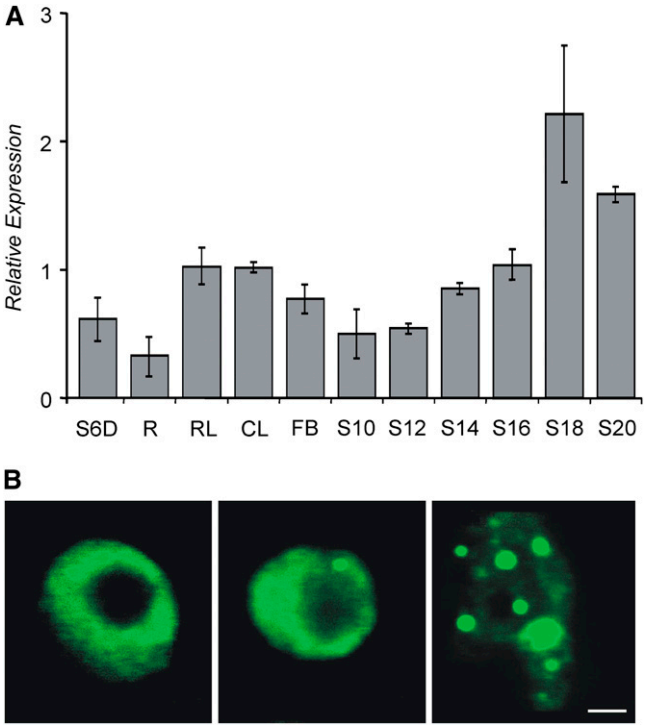


Figure 3. *SUA* Is Expressed in All Tissues and Its Protein Is Localized in the Nucleus.

(A) Quantitative real-time RT-PCR analysis of *SUA* expression in different tissues. *SUA* mRNA levels are normalized to *ACTIN8* mRNA levels. S6D, seedlings 6 d after germination; R, roots; RL, rosette leaves; CL, cauline leaves; FB, flower buds; S10 to S20, siliques 10, 12, 14, 16, 18, and 20 d after pollination. Data are from two independent biological replicates. Error bars represent SE.

(B) Confocal analysis of subcellular localization of SUA:GFP in developing embryo tissue from transgenic *abi3-5 sua* plants containing the *PSUA:SUA:GFP* construct. Three nuclei with different GFP patterns are shown. Bar = 2 μM.

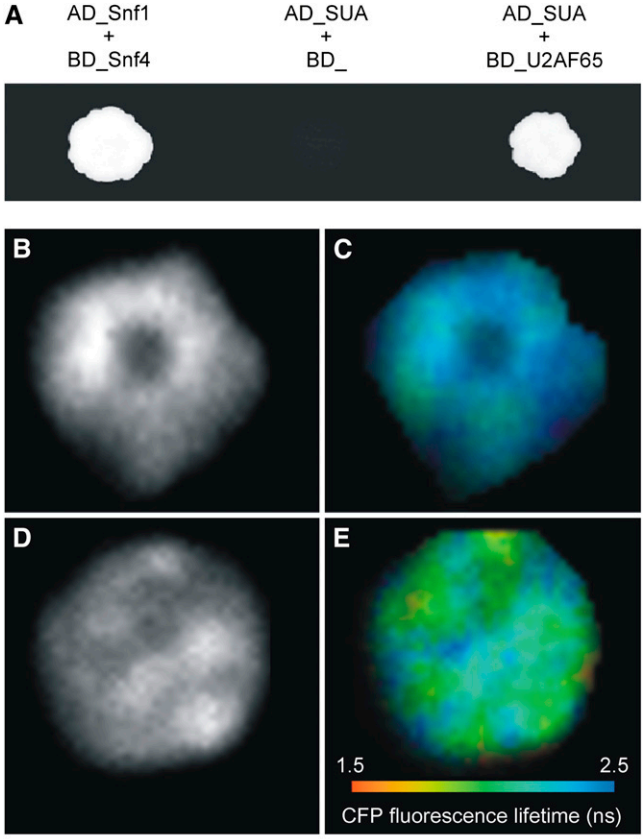
overexpression of SUA_YFP (yellow fluorescent protein) and U2AF^{65}_CFP chimerical proteins. FRET/FLIM analysis of protoplasts coexpressing SUA_YFP and U2AF^{65}_CFP (cyan fluorescent protein) showed a significant reduction of the mean CFP fluorescence lifetime compared with those expressing the U2AF^{65}-CFP alone (Figures 4B to 4F), confirming interaction of both proteins in planta.

The Suppression of *abi3-5* by *sua-1* Is Allele Specific

The *abi3-5* mutant is one of the strongest *abi3* alleles, which all show reduced seed dormancy and decreased sensitivity to ABA during germination (Bies-Etheve et al., 1999). Seeds of the *abi3-4* and *abi3-6* mutants are nondormant, highly insensitive to ABA, and show reduced longevity and a high chlorophyll content similar to *abi3-5*. To study the suppression effect of the *sua-1* mutant on different *abi3* mutant alleles, double mutants were constructed. The ABA-insensitive *abi3-4* and *abi3-6* alleles, as well as the weak *abi3-1* and *abi3-7* alleles (Figure 5A), were combined with *sua-1*, *sua-2*, and *sua-3*. Surprisingly, none of these combinations showed any suppression phenotype, indicating that the suppression of *abi3-5* by *sua* mutants is allele specific (Figure 5B).

Detection of Functional ABI3 Protein in the *abi3-5 sua-1* Double Mutant

The *abi3-5* mutation causes a frameshift leading to a premature stop codon after 34 erroneous codons. The *abi3-4* mutant has a



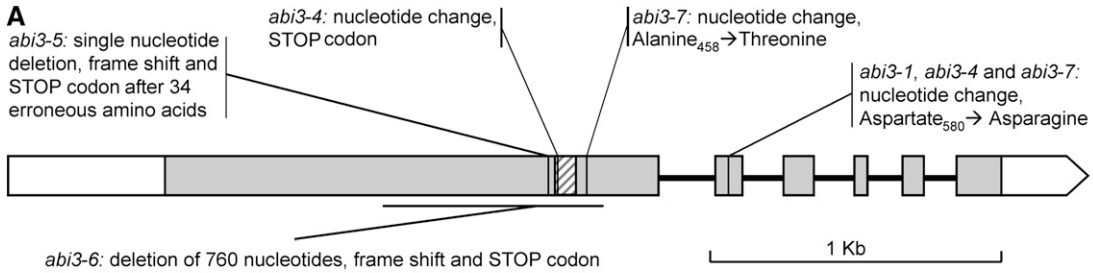
F Donor	Acceptor	Average ns ± SD	N
U2AF^{65}-CFP	-	2,42 ± 0,04	12
U2AF^{65}-CFP	SUA-YFP	2,19 ± 0,14	12

Figure 4. SUA Interacts with U2AF^{65}.

(A) Interaction between SUA and U2AF^{65} detected with the yeast two-hybrid assay. Cotransformed yeast strains were grown on SD-L-W-H with 5 mM 3AT. Snf1 and Snf4 are yeast proteins that strongly interact (Jiang and Carlson, 1997).

(B) to **(E)** Interaction between SUA and U2AF^{65} based on FRET measured by FLIM. FLIM analysis of protoplasts transiently expressing U2AF^{65}-CFP (**[B]** and **[C]**) and coexpressing U2AF^{65}-CFP and SUA-YFP (**[D]** and **[E]**). Intensity channel (**[B]** and **[D]**) and false color code (**[C]** and **[E]**). The absence of interaction results in a long lifetime, visible as a dark-blue color. Interaction leads to a reduction in donor lifetime, visible as a shift toward orange. A representative protoplast nucleus is shown.

(F) Average CFP fluorescence lifetime values for the FRET/FLIM analysis. N, number of nuclei analyzed.



B

	abi3-1	*abi3-4*	*abi3-5*	*abi3-6*	*abi3-7*
sua-1	x	x	✓	x	x
sua-2	ND	ND	✓	x	ND
sua-3	ND	ND	✓	x	ND

C

…GGCCACCACCACCACAGTCTGGTCCCATGCCACATCAACAATT
CCCCATGCCGCCAACCTCGCAgtataatcaatttggagatccaacaggtttcaatg
gatacaacatgaatccgtaccaatatccttatgttcctgcagACAAATGAGAGATCA…

Figure 5. *sua* Is an Allele-Specific Suppressor of the *abi3-5* Allele.

(A) Schematic structure of the *ABI3* gene. The locations and nature of the *abi3-1*, *abi3-4*, *abi3-5*, *abi3-6*, and *abi3-7* mutations are indicated. UTRs are shown in white, exons in gray, and introns as thick lines. The box with diagonal stripes represents the cryptic intron.

(B) Table showing the suppression of the *abi3* phenotype in different combinations of *sua* and *abi3* mutant alleles. A check mark indicates *abi3* suppression; the "x" indicates absence of *abi3* suppression. ND, not determined.

(C) Sequence of the *ABI3* cryptic intron and surrounding region. The asterisk indicates the single base pair deleted in *abi3-5*, and the subsequent stop codon is underlined. The cryptic intron is shown in lowercase letters.

single nucleotide mutation that causes a stop codon at approximately the same position (Bies-Etheve et al., 1999; Figure 5A). Therefore, *abi3-4* and *abi3-5* produce *ABI3* transcripts that translate into truncated ABI3 proteins with similar sizes. Nevertheless, the phenotype of the *abi3-5* mutant is strongly suppressed by *sua*, whereas that of *abi3-4* is not. To understand this discrepancy, we analyzed the ABI3 protein in dry seeds of L*er*, *sua-1*, *abi3-4*, *abi3-5*, and the double mutants *abi3-4 sua-1* and *abi3-5 sua-1* by immunoblotting. A specific antibody, targeted to the amino end of ABI3, was used for detection. The ABI3 protein (720 amino acids) migrates as a 116-kD polypeptide (Parcy et al., 1997). We detected two bands of approximately this size for the ABI3 protein in L*er* and *sua-1* seeds. One of these two bands probably represents a modified version of ABI3. A truncated ABI3 protein corresponding to a 428–amino acid polypeptide and migrating as a 70-kD band, was observed in the *abi3-5* mutant (Figure 6). A similar sized (416 amino acids) highly abundant ABI3 protein was found in *abi3-4* and *abi3-4 sua-1*. The high abundance of the truncated ABI3 protein in *abi3-4* seeds was previously observed by Parcy et al. (1997). In the *abi3-5 sua-1* double mutant, two weak bands of comparable size to full-length ABI3 were detected, along with the smaller truncated abi3-5 mutant protein (Figure 6). The presence of full-length ABI3 protein in *abi3-5 sua-1* seeds was consistent with all the observed suppression phenotypes and predicts the presence of an *ABI3* transcript with a restored reading frame that has lost the *abi3-5* premature stop codon.

Identification of a Novel *ABI3* Splice Variant

The *abi3-5* transcripts were analyzed in detail by RT-PCR and sequencing. In the *abi3-5 sua-1* double mutant, we identified, besides the expected full-length *abi3-5* transcript, an alternatively spliced novel *abi3-5* transcript that lacks a cryptic intron of 77 nucleotides. This cryptic intron is located shortly downstream of the *abi3-5* mutation and includes the premature *abi3-5* stop codon (Figure 5C). The combination of the 1-bp *abi3-5* deletion and the removal of the 77-nucleotide cryptic intron results in a transcript that restores the reading frame of *abi3-5* after 21 erroneous and 26 deleted codons. We named this transcript *abi3-5-*β and named the transcript with the retained intron *abi3-5-*α. The translated *abi3-5-*β polypeptide (abi3-5-β) is predicted to be 694 amino acids. This protein contains all four ABI3 protein domains (Figure 6B), and the phenotype of the *abi3-5 sua-1* seeds indicated that abi3-5-β largely retains the ABI3 molecular functions (Figure 1).

The *ABI3-*β transcript only encodes a functional protein in the *abi3-5* mutant background. In the wild type, it causes a frameshift and codes for a truncated protein of 429 amino acids. This predicted truncated polypeptide was immunodetected in the *sua-1* single mutant and also, at lower levels, in wild-type L*er* seed protein extracts. The wild-type ABI3-β protein migrates with a similar speed in the gel as the proteins encoded by *abi3-4* (α and β splicing forms) and *abi3-5* (α splicing form) mutants (Figure 6).

In addition to the accumulation of the *ABI3-*β splice variant, the *sua-1* mutant also shows an overall increase in *ABI3* expression. The amount of *ABI3-*α transcript, coding for full-length *ABI3*, is higher in *sua-1* than in the wild type (Figure 7A). This could explain the increased ABA sensitivity of *sua-1* seeds. Instead, overall *ABI3* expression in *sua-2* seeds is similar to that in wild-type Col-0, but the portion of the transcript coding for full-length ABI3 is reduced, resulting in a decrease of ABA sensitivity (Figure 1E). We tested the possibility that *sua-1* has a gain-of-function

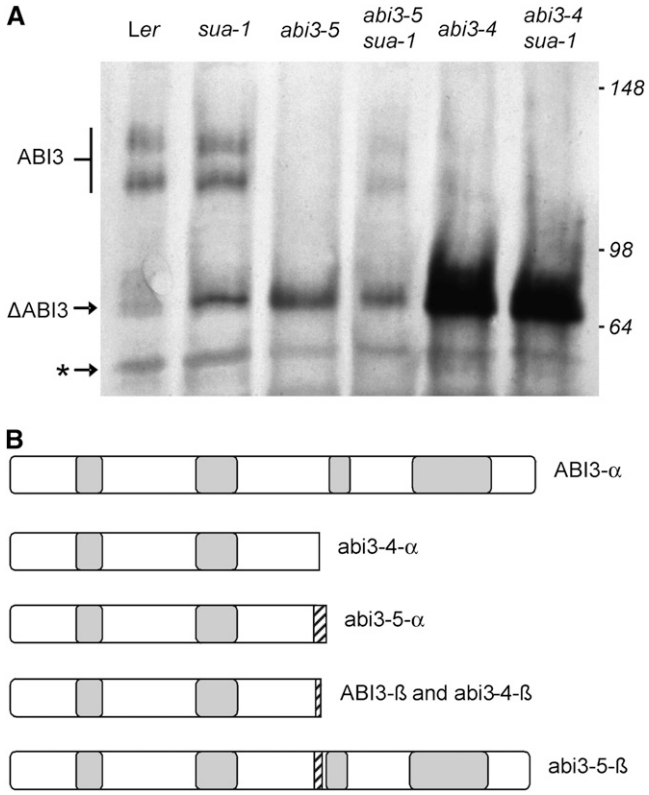


Figure 6. Detection of Full-Length ABI3 Protein in the *abi3-5 sua-1* Double Mutant.

(A) Immunoblot analysis of ABI3 protein. Total protein was extracted from freshly harvested seeds and separated on a Tris-Gly SDS 4 to 12% polyacrylamide gradient gel. The ABI3 protein is identified as a double band of ~116 kD in L*er*, *sua-1*, and *abi3-5 sua-1*. The truncated ABI3 proteins (ΔABI3) produced by *abi3-4*, *abi3-5*, and *abi3-5 sua-1* and the novel splicing variant of *ABI3* are ~70 kD. Asterisk indicates a nonspecific band that is used as loading control. Sizes of the molecular markers (in kilodaltons) are shown next to the blot.

(B) Predicted ABI3 protein isoforms. Gray boxes represent the conserved functional motifs of ABI3 (from left to right: A1, B1, B2, and B3). Boxes with diagonal stripes represent erroneous amino acids stretches.

effect on *ABI3* expression by transforming the *sua-1* mutant allele, expressed from the endogenous *SUA* promoter, into the *sua-2* mutant. Indeed, the obtained transformants showed a higher *ABI3* expression than the *sua-2* mutant and had increased ABA sensitivity (see Supplemental Figure 1 online). This indicates that *sua-1* is a gain-of-function mutant regarding *ABI3* expression.

ABI3 Alternative Splicing Is Developmentally Regulated

The relative abundance of *ABI3-α* and *ABI3-β* transcripts was quantified in wild-type seeds by real-time RT-PCR. Developing siliques 16 d after pollination showed a very low abundance of *ABI3-β* transcript in L*er* and Col-0 (1.53 ± 1.36% and 0.95 ± 0.83%, respectively, of the overall *ABI3* transcripts; Figure 7A). During progressive development of wild-type siliques, the ratio between both *ABI3* transcripts shifted toward *ABI3-β*. At 20 d after pollination, the amount of *ABI3-β* exceeded that of *ABI3-α* (Figure 7B). The observed change in ratio between *ABI3-α* and *ABI3-β* transcripts during seed maturation indicates that alternative splicing of *ABI3* is developmentally regulated.

DISCUSSION

ABI3 Is Regulated by Alternative Splicing

The transcription factor ABI3 regulates seed maturation and influences seed quality. The abundance of ABI3 is tightly regulated at different levels. In addition to complex genetic interactions with *LEC1*, *LEC2*, and *FUS3* at the transcriptional level (To et al., 2006), *ABI3* expression is controlled posttranscriptionally. Alternative splicing of *ABI3* homologs in cereal species (*Triticum aestivum* and *Oryza sativa*) and dicots (*Pisum sativum*) (McKibbin

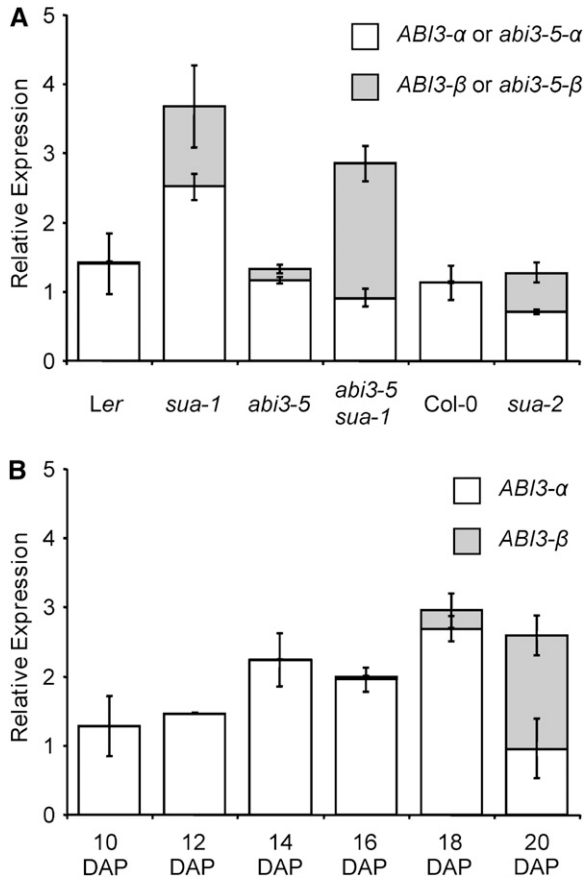


Figure 7. Quantification of *ABI3* Splicing Variants.

Quantitative real-time RT-PCR analysis of *ABI3-α* (white) and *ABI3-β* (gray) expression in L*er*, *sua-1*, *abi3-5*, *abi3-5 sua-1*, Col-0, and *sua-2* **(A)** and in L*er* developing siliques 10 to 20 d after pollination (DAP) **(B)**. For **(A)**, mRNA was extracted from siliques 16 d after pollination. *ABI3* mRNA levels are normalized to *ACTIN8* mRNA levels. Data are from two independent biological replicates. Error bars represent SE.

et al., 2002; Fan et al., 2007; Gagete et al., 2009) generates multiple mis-spliced transcripts that often code for truncated polypeptides. This has been linked to reduced grain quality in rice and wheat (McKibbin et al., 2002; Fan et al., 2007). Here, we show that the *ABI3* gene of *Arabidopsis* is also regulated by alternative splicing. A 77-bp cryptic *ABI3* intron is alternatively spliced, which leads to the occurrence of two transcripts. The *ABI3-α* transcript encodes a full-length ABI3 protein, and the *ABI3-β* transcript encodes a truncated protein that contains two of the four functional domains. Splicing of the cryptic intron of *ABI3* is developmentally regulated, and *ABI3-β* accumulates only at the end of seed maturation. This probably contributes to a fast downregulation of full-length ABI3 in ripe seeds, which is necessary to inhibit the seed maturation program in germinating seeds.

Transcripts with a long 3′ untranslated region (UTR) or with 3′UTR-located introns can be detected and degraded by the nonsense-mediated decay machinery in plants (Kerényi et al., 2008). To distinguish a natural stop codon from a premature stop codon, nonsense-mediated decay requires a second signal that has not been identified yet in plants (van Hoof and Green, 2006). The *ABI3-β* transcript contains a premature stop codon but probably lacks this second signal because it is not affected by nonsense-mediated decay. The protein encoded by the *ABI3-β* transcript contains the A1 acidic transcriptional activation domain and the first basic domain and might still mediate ABA signaling during late seed maturation.

The prevalent model of splicing in *Arabidopsis* is intron definition, in which intronic sequences are recognized by the spliceosomal complex. The features of a canonical plant intron are a consensus 5′ splice site (AG/GU, where GU is the more conserved dinucleotide), a U-rich sequence, and a consensus 3′ splice site (CAG/G where AG is invariant) (Simpson and Filipowicz, 1996; Lorković et al., 2000). *Arabidopsis* exons contain on average 29% U, while introns have on average 42% U (Reddy, 2007). It was shown that U-rich elements can function as splicing signals (Simpson et al., 2004), and short introns and introns with low AU content are more likely to be retained (Wang and Brendel, 2006). The *ABI3* cryptic intron has sequence similarities with canonical plant introns, in particular with the consensus sequences at the two borders (Figure 5C), but it has a U content of only 29%, while the other *ABI3* introns have, on average, 46% U. Because of that, the cryptic *ABI3* intron may not be easily recognized by the spliceosomal complex.

SUA Controls Alternative Splicing of *ABI3*

SUA suppresses splicing of the cryptic *ABI3* intron and thereby influences the ratio between the *ABI3-α* and *ABI3-β* transcripts. Reduced suppression of the cryptic intron in the *sua* mutant leads to an increased amount of *ABI3-β* transcript and decreased levels of the *ABI3-α* transcript. However, a substantial amount of *ABI3-α* transcript could still be detected in the *sua* mutant. Other splicing factors probably act redundantly with SUA in the suppression of the cryptic *ABI3* intron.

Alternative splicing in plants is regulated by tissue-specific developmental cues and stresses and might provide a means for optimal adaptation to the environment (Ali and Reddy, 2008). Alternative splicing of *ABI3* could also be regulated by specific environmental conditions. In this respect, it is interesting to note that publicly available microarray data show an upregulation of *SUA* expression by senescence (Zimmermann et al., 2004). The water content of seeds strongly decreases during the maturation phase until ~7% in mature seed (Baud et al., 2002). This process is comparable to senescence and also coincides with increased *SUA* mRNA levels (Figure 3A). Higher SUA abundance will favor cryptic intron retention and increase the full-length ABI3 protein levels during seed maturation. Consistent with that, our experiments showed a correlation between increased levels of *SUA* transcript and a reduction in *ABI3-β* levels in transgenic plants. The *abi3-5 sua-1 PSUA:SUA:GFP* #8 line, for instance, showed increased levels of *SUA* transcript and reduced amounts of *abi3-5-β*, resulting in an enhanced *abi3-5* phenotype (see Supplemental Figure 2 online; Figures 1A and 1D). SUA-mediated alternative splicing of *ABI3* could represent a system to fine-tune seed maturation. However, in wild-type plants, the *ABI3-β* transcript accumulates at the end of seed maturation when *SUA* is still substantially expressed. Possibly, the SUA protein is not active or degraded at the end of seed maturation. Alternatively, other factors could counteract the role of SUA in retention of the cryptic intron at this time.

The *sua-1* single mutant showed increased ABA sensitivity during germination. This is probably caused by upregulation of *ABI3* expression, which does not occur in *sua-2*. This difference between *sua-1* (in a L*er* background) and *sua-2* (in a Col-0 background) could be explained by natural genetic variation between L*er* and Col-0 that modifies the *sua* mutant phenotype. However, *sua-2* plants transformed with *sua-1* also showed an increased *ABI3* expression and enhanced ABA sensitivity. Therefore, it is more likely that *sua-1* is a gain-of-function allele, which is translated into a truncated protein. This predicted polypeptide includes the RNA recognition motifs and the Zn finger motif but lacks the G patch domain at the C terminus. The nonfunctional mutant sua-1 protein might compete for substrates with other proteins of the mRNA splicing machinery and could therefore function as a dominant-negative allele.

The *abi3-5* mutant still contains a small amount of *abi3-5-β* transcript, which encodes a functional ABI3 protein. Consequently, *abi3-5* is not a complete loss-of-function mutant. The *sua* mutant can suppress *abi3-5* because it enhances the amount of *abi3-5-β* transcript.

SUA Has a Conserved Role in Splicing

The conserved domain architecture of SUA and its role in the suppression of the cryptic *ABI3* intron indicate a function in mRNA processing. Moreover, the speckled fluorescence patterns observed in nuclei expressing the chimeric *SUA:GFP* gene are similar to those obtained with Ser/Arg-rich GFP proteins, which are involved in RNA metabolism in plants (Lorković and Barta, 2004). The SUA protein has two RNA recognition motifs, which are also found in many eukaryotic RNA processing proteins (Burd and Dreyfuss, 1994). Based on its functional motifs, SUA could bind directly to specific RNA targets. However, SUA might also interact with the mRNA targets indirectly and be part of the spliceosome, which is composed of ~300 proteins in *Arabidopsis* (Reddy, 2007).

The SUA protein shares a high sequence similarity with the human tumor suppressor RBM5, which regulates alternative splicing of the apoptosis genes Fas and c-FLIP (Bonnal et al., 2008). Previous work on RBM5 showed that this protein can interfere with the progression of spliceosomal assembly after formation of the prespliceosomal complex. Assuming that conservation of domain architecture indicates conservation of function, it is plausible that SUA prevents excision of the cryptic *ABI3* intron after formation of the prespliceosomal complex. The confirmed interaction of SUA with the spliceosomal factor U2AF^{65} by yeast two-hybrid assay and in vivo suggests that SUA, similarly to RBM5, plays a role in early spliceosome formation.

Mutations in *SUA* have little impact on the visual plant phenotype, similar to other *Arabidopsis* ABA signaling mutants that affect conserved, single copy genes with a role in RNA processing. The *aba-hypersensitive1* (*abh1*) and *supersensitive to aba and drought1* (*sad1*) mutants, for instance, show mild phenotypes and altered expression of only a few genes (Fedoroff, 2002). These mutants show hypersensitivity to ABA at germination. ABH1 functions as the large subunit of an *Arabidopsis* mRNA cap binding complex (Hugouvieux et al., 2001), and SAD1 is an ortholog of the yeast subunit of the U6 small nuclear RNP complex, which is involved in nuclear RNA processing (Bouveret et al., 2000). The lack of strong phenotypes in these mutants could be due to redundancy with nonhomologous genes that share the same function. It is also possible that genes like *SUA* play an essential role under specific, not yet identified, environmental conditions.

With the isolation of *SUA*, we identified a splicing factor in plants that has a function in seed maturation. This function can be entirely explained by the influence of SUA on alternative splicing of *ABI3*. However, a conserved gene like *SUA* is likely to have a broader role, which could be revealed by the identification of additional genes whose splicing is controlled by SUA.

METHODS

Plant Materials and Growth Conditions

The mutants *abi3-5* (Ooms et al., 1993; Bies-Etheve et al., 1999), *abi3-4* (Giraudat et al., 1992), *abi3-1*, *abi3-7* (Ooms et al., 1993; Bies-Etheve et al., 1999), *gl1* (Oppenheimer et al., 1991), and *tt5* (Shirley et al., 1995) are all in the L*er* accession background, and *abi3-6* (Nambara et al., 1994) is in the Col background. All mutants were obtained from the research groups in which they were isolated. The *sua-1* mutant is in the L*er* background. The *sua-2* (SALK T-DNA insertion line, SALK_054379; Alonso et al., 2003) and *sua-3* (GABI-KAT T-DNA insertion line, GK-815C12; Rosso et al., 2003) mutants are in the Col background and were obtained from the Nottingham Arabidopsis Stock Centre. All plants were grown on soil containing a mixture of substrate and vermiculite (3:1) in a greenhouse where the temperature was maintained close to 20°C; 16 h of light was provided daily.

Germination Tests

About 100 to 150 seeds from each batch were sown into 6-cm-wide plastic Petri dishes on round filter papers (Macherey and Nagel) soaked with 580 μL demineralized water. The longevity of seeds was determined after a stratification treatment, consisting of 5 d incubation at 4°C in the dark. Seeds were subsequently put into an incubator, in long-day conditions (16 h light at 25°C, followed by 8 h darkness at 20°C). After 6 d of incubation, the germinated seeds were counted. For dormancy experiments, seeds were processed in the same way, without stratification treatment. In the experiments for assessing the ABA hormone sensitivity, seeds were stratified and incubated as described above on filter papers soaked with solutions of different ABA concentrations. ABA (Sigma-Aldrich) was dissolved in methanol and diluted with water.

Mapping

Simple sequence length polymorphisms and cleaved amplified polymorphic sequences were used as polymorphic PCR markers in the mapping experiment (see Supplemental Table 1 online). Most of the primers were designed to flank short polymorphic sequences referring to the Monsanto Landsberg-Columbia polymorphisms database (https://www.Arabidopsis.org/cgi-bin/cereon/).

RNA Extraction and Quantitative Real-Time RT-PCR

mRNA was extracted from developing *Arabidopsis thaliana* siliques with the Magnetic mRNA Isolation Kit (New England Biolabs). First-strand cDNA was synthesized using the Superscript II Reverse Transcriptase (Invitrogen) and oligo dT(16) primers, starting from 200 ng of mRNA. Quantitative real-time RT-PCR reactions were performed with the SYBR Green Master Mix (Applied Biosystems) with the following primer sets for *ABI3-α* (5′-CCGGGTTTTGGATACATGC-3′, 5′-CGGATTCATGTTGTATCCATTG-3′), *ABI3-β* (5′-ATGCCGCCAACCTCGCAGAC-3′, 5′-ACAGGTTTCTCCGATTTGGG-3′), *ACT8* (5′-CTCAGGTATTGCAGACCGTATGAG-3′, 5′-CTGGACCTGCTTCATCATACTCTG-3′), *SUA* (5′-CAGCAATTGCATCAGAGAAGAG-3′, 5′-ACGTTGTATTTGGGTTTCATGG-3′), and *sua-1* (5′-TGACAATCCACCTACAGTTTCG-3′, 5′-CACCCAAAGTTTCTCCCACTC-3′) and analyzed on a Mastercycler realplex2 system (Eppendorf). Quantification of *ACT8*, *ABI3-α*, *ABI3-β*, *SUA*, and *sua-1* was determined by the standard curve method. For each of the two biological replicates, the amounts of target transcripts were normalized to the value of *ACT8*. For the quantification of each target, the values of two biological replicates were averaged.

Plant Transformation

The binary vectors carrying *PSUA:SUA_GFP* and *PSUA:sua-1* were prepared using standard molecular cloning techniques in combination with the Gateway technology (Invitrogen). *PSUA* consisted of 2711-bp genomic sequence upstream of the *SUA* start codon. The *SUA* and *sua-1* cDNAs comprised the sequences from the start codon to the first stop codon. The constructs were first transformed in *Escherichia coli* strain DH5α (Hanahan, 1983) and subsequently in *Agrobacterium tumefaciens* strain GV3101, carrying the helper plasmid pMP90RK (Koncz and Schell 1986). Transgenic plants were produced through the floral dip method as described by Clough and Bent (1998).

Protein Extraction and Immunoblot

Twenty milligrams of dry seeds were weighed and ground in liquid nitrogen and then extracted with 200 μL of a buffer containing 8 M Urea, 0.2% Triton X-100, 0.2% Sarkosyl, and 100 mM Tris-Cl, pH 7.5. After separation on a Tris-glycine SDS 4 to 12% polyacrylamide gradient gel (Anamed), according to Laemmli (1970), the proteins were blotted on a polyvinylidene fluoride membrane (Millipore) through semidry electrotransfer for 75 min at 2.8 mA/cm^2. The immunological reactions of primary and secondary antibodies with the immobilized target proteins were done in 10 to 15 mL of a buffer containing 50 mM Tris-Cl, 150 mM NaCl, 0.25% Tween 20, and 5% skim milk. The primary polyclonal antibody was

targeted to the amino end of the ABI3 protein and was kindly provided by Kazumi Nakabayashi and Eiji Nambara (RIKEN Plant Science Center, Yokohama, Japan). The secondary antibody was an alkaline phosphatase–conjugated anti rabbit-IgG. The blots were developed in a substrate buffer with 0.12 mM nitro blue tetrazolium, 0.15 mM 5-bromo-4-chloro-3-indolylphosphate, and 4 mM $MgCl_2$ in 0.1 M Tris-Cl buffer, pH 9.5.

Phylogenetic Analysis

Protein sequences with the highest similarity to SUA were chosen after blasting its full-length protein sequence with the database of the National Center for Biotechnology Information. An unrooted phylogenetic analysis was conducted using *MEGA* version 4 (Tamura et al., 2007), using the alignment shown in Supplemental Data Set 1 online. Bootstrap test of phylogeny was performed using the neighbor-joining method from 1000 replications for each branch.

Yeast Two-Hybrid Analysis

SUA and *U2AF*^{65} (AT4G36690) coding sequences were cloned from L*er* into pDONR 207 and pENTR/D-TOPO (Invitrogen), respectively, and then in the pACT2-gateway (GAL4 AD fusion) and pAS2-gateway (GAL4 BD fusion) vectors (modified from Clontech). Yeast two-hybrid assays were performed in yeast strain PJ69-4A (James et al., 1996) that was grown at 30°C. Yeast transformation was performed using a LiAc/SS carrier DNA/PEG method as described by Gietz et al. (1997). Cotransformed colonies were selected on synthetic dropout medium (SD) lacking Leu (L) and Trp (W). Interaction tests were performed on SD lacking L, W, and His (H) and on increasing concentrations of 3-aminotriazole (5, 10, and 20 mM). Interaction between SUA and U2AF^{65} was detected when SUA was expressed fused to GAL4 AD and U2AF^{65} to GAL4 BD. SUA fused to GAL4 BD could activate the transcription of the reporter gene alone.

FRET/FLIM Analysis

SUA and *U2AF*^{65} in entry clones were recombined into pENSG-YFP-gateway and pENSG-CFP-gateway destination vectors, respectively (Wenkel et al., 2006), producing YFP/CFP N-terminal fusions under control of the 35S promoter. FLIM was performed as described by Kwaaitaal et al. (2010). *Arabidopsis* protoplasts (from Col) were obtained from rosette leaves of 4-week-old plants grown under short-day conditions (8 h light/16 h darkness) and were transfected using 20 to 30 μg of pENSG_SUA_YFP and pENSG_U2AF^{65}_CFP plasmids. Before imaging, they were incubated for 16 h in the light at 25°C.

Analysis of nuclear fluorescence was performed by confocal laser scanning microscopy using an LSM510 META confocal microscope (Zeiss). The histograms were analyzed with the SPC-Image 2.9.1 software (Becker and Hickl). Samples of cells expressing U2AF^{65}-CFP alone were fitted with a single-exponential model to estimate the donor lifetime. For the analysis of protoplasts coexpressing U2AF^{65}-CFP and SUA-YFP, a double exponential model was used. The second component was fixed to the average lifetime of the donor to the value found for U2AF^{65}-CFP (2.5 ns) (Tonaco et al., 2006). About 15 nuclei per transformation were analyzed for two biological replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers GU735482 and ADE44117 (At_SUA cds and protein), XP_002327561 (Pt_SUA homolog), CBI33920 (Vv_SUA homolog), EEC72564 (Os_SUA homolog), XP_001759968 (Pp_SUA homolog), XP_001691823 (Cr_SUA homolog), NP_001090434 (Xl_RBM5), AAH43058 (Mm_RBM5), NP_005769 (Hs_RBM5), and NP_849543 (At_FCA).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of *sua-2* Plants Transformed with *PSUA:sua-1* (#A2, #A12, and #A14).

Supplemental Figure 2. Analysis of *abi3-5 sua-1* Plants Transformed with *PSUA:SUA:GFP* (#8 and #15).

Supplemental Table 1. Polymorphic Markers Used for Fine Mapping the *sua-1* Mutation.

Supplemental Data Set 1. Text File of Alignment Used to Generate Figure 2C.

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