Arabidopsis *FHY3/FAR1* Gene Family and Distinct Roles of Its Members in Light Control of Arabidopsis Development[1]

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FHY3 (far-red elongated hypocotyls 3) and FAR1 (far-red-impaired response) are two homologous proteins essential for phytochrome A controlled far-red responses in Arabidopsis (*Arabidopsis thaliana*). There are 12 additional *FHY3/FAR1*-related genes in the Arabidopsis genome. The predicted sizes of this family of proteins range from 531 amino acids to 851 amino acids, and they share 12.0% to 82.4% amino acid identities over their entire lengths. In addition, most FRS proteins contain one to three coiled-coil domains and one or two putative nuclear localization signals. Semiquantitative reverse transcription-polymerase chain reaction analyses revealed that all *FRS* genes except *FRS10* are expressed in all tissues examined, including rosette leaves, cauline leaves, inflorescence stems, flowers, and siliques. Analyses of gene specific *promoter*::*GUS* fusion reporter gene expression revealed that all *FRS* genes except *FRS1* are expressed in hypocotyls, and their expression in hypocotyl is induced by far-red light treatment. Transient expression of green fluorescent protein tagged FRS fusion proteins in onion (*Allium cepa*) epidermal cells revealed that all FRS proteins are targeted into the nucleus. T-DNA knockout *frs6* and *frs8* mutants flowered early under both long-day and short-day conditions (with much more drastic effects under short-day conditions), suggesting that *FRS6* and *FRS8* regulate flowering time. In addition, *FRS9* RNAi transgenic plants showed a specific hypersensitivity to red light inhibition of hypocotyl elongation and light-regulated gene expression, indicating that *FRS9* is a specific negative regulator of phyB signaling mediating seedling deetiolation. In summary, our results support the notion that *FRS* family members play distinct roles in light control of Arabidopsis development, most likely by regulating nuclear gene expression.

As sessile organisms, plants utilize sophisticated sensory systems to monitor their ambient environments and undergo adaptive growth. Light is one of the major environmental signals that influences many aspects of plant growth and development, including seed germination, seedling deetiolation, phototropism, stomata and chloroplast movement, stem elongation, circadian rhythms, and flowering (Quail, 2002; Wang and Deng, 2003, 2004).

Plants use three major classes of photoreceptors to monitor variations in direction, duration, quantity, and wavelength of light. The cryptochromes and phototropins sense the blue/UV-A region of the spectrum, whereas the phytochromes perceive primarily the red (R) and far-red (FR) wavelengths. Of these photoreceptors, phytochromes are the best characterized. In Arabidopsis (*Arabidopsis thaliana*), phytochromes are encoded by five distinct genes (*PHYA–PHYE*) belonging to a small gene family. The gene products of *PHYB* to *PHYE* have similar functions in regulating light responses under continuous R and white light, with phyB playing a predominant role. phyA is primarily responsible for the very low fluence responses and for the FR light-dependent high-irradiance responses (HIRs), including inhibition of hypocotyl elongation, opening of apical hook, expansion of cotyledons, accumulation of anthocyanin, and FR preconditioned blocking of greening (Whitelam et al., 1993; Deng and Quail, 1999; Neff et al., 2000; Briggs and Olney, 2001).

Molecular genetic studies have identified two loci, *FHY3* and *FAR1*, as two positive regulators specifically for phyA-mediated HIR responses in response to FR light. Their loss-of-function mutants display elongated hypocotyls specifically under continuous FR light (FRc). Although *fhy3* and *far1* mutants display similar defects in hypocotyl elongation and anthocyanin accumulation, *fhy3* has a more pleiotropic effect on phyA signaling. For instance, apical hook and cotyledon opening, and FRc preconditioned block of greening are affected by *fhy3* but not by *far1* (Hudson et al., 1999; Wang and Deng, 2002). Interestingly, *FHY3* and *FAR1* encode two homologous proteins, and overexpression of FAR1 or FHY3 can suppress each other's loss-of-function mutant phenotype (Wang and Deng, 2002). Furthermore, a genome expression study demonstrated that the transgenes *FHY3* and *FAR1* driven by the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter could essentially restore the genome expression profiles abolished by the *far1-2* and *fhy3-1* mutations, respectively (Wang et al., 2002). These observations suggest that FHY3 and FAR1 play both overlapping and distinct roles in phyA signaling.

The Arabidopsis genome project revealed that *FHY3* and *FAR1*, together with *FRS1* to *FRS12* (for *FAR1*-related sequences), comprise a 14-member gene family (Arabidopsis Genome Initiative, 2000). The sequence

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homologies and conserved secondary structure among the family members suggest that this group of proteins could be involved in distinct signaling processes in response to various biotic and abiotic stresses. In addition, FRS-like proteins have also been identified in other plant species, including monocotyledonous plants, indicating that this family of proteins is conserved throughout the evolution of the plant kingdom. Therefore, they may play essential roles unique to plant growth and development (Hudson et al., 1999; Wang and Deng, 2002). In this report, we conducted a detailed molecular analysis of this gene family, their expression patterns, and subcellular localization of the encoded proteins. We also present evidence for differential roles of members of this gene family in mediating distinct photo-responses.

RESULTS

Identification of the *FHY3/FAR1* Gene Family

We used full-length peptide sequences of both FAR1 and FHY3 as the query sequences in our BLAST searches against the Arabidopsis genome sequences at the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/) and identified 12 additional sequences that share signifi-

Table I. *Summary of FHY3/FAR1 gene family*

MW, Molecular weight; NLS, nuclear localization signal.

Gene Name	Locus	Protein ID	Amino Acid	MW	Coiled-Coil Domain	NLS	cDNA Clone
				kD			
FAR1	At4g15090	AAD51282	827	95.4	1	+	RAFL09-68-G16, Hudson et al. (1999)
FHY3	At3g22170	NP_188856	839	96.0	1	+	RAFL21-40-L21, BX825360, BX840685, CK111774, T20465, BE529279, AV528578, AV529810, AV523823, Wang and Deng (2002)
FRS1	At4g19990	NP_193732	687	80.3	2	−	AV555016, this work
FRS2	At2g32250	NP_180784	807	92.0	2	+	RAFL16-06-P03, RAFL07-55-M09, BX820631, BX837725, BX834611, AV565526, BE525295, BE527036, BE525980, this work
FRS3	At2g27110	NP_565636	851	96.3	None	+	RAFL09-09-M18, RAFL15-44-E06, N37149, BX839696, T14215, AV522646, AV528393, AV528316, AV522858, this work
FRS4	At1g76320	NP_177759	670	78.5	1	+	RAFL25-49-E06, BX838539, BX834442, BX837506, AV557793, this work
FRS5	At4g38180	NP_195531	788	90.5	1	+	RAFL09-61-A11, BX834891, this work
FRS6	At1g52520	NP_175661	703	81.6	1	+	RAFL05-20-M15, RAFL21-86-F16, RAFL24-26-K23, RAFL25-17-H18
FRS7	At3g06250	NP_566278	764	87.8	1	+	RAFL09-18-D04, BX835891, BX839909, AV528281
FRS8	At1g80010	NP_178118	696	79.6	None	−	This work
FRS9	At4g38170	NP_195530	531	60.8	3	−	RAFL19-54-J19, BX838072, BX827635, this work
FRS10	At5g28530	AAF88018	685	78.5	1	+	None
FRS11	At1g10240	NP_563865	680	77.7	2	+	RAFL09-33-I16, RAFL16-61-E10
FRS12	At5g18960	NP_197397	788	90.5	1	+	RAFL16-82-P07, BX833504, AV535499, AV535209, AV440408, T14014, this work

cant homologies with both FAR1 and FHY3 throughout most of their lengths (Table I). These homologous genes are distributed on all five chromosomes of Arabidopsis with the exception of *FRS5/FRS9*, which are arranged in tandem on chromosome IV. To carry out functional studies of the additional 12 *FRS* genes, we sought to obtain their full-length cDNA clones. For *FRS6*, *FRS7*, and *FRS11*, full-length cDNAs were available when we initiated this work and were obtained from the Arabidopsis Biological Resources Center (ABRC). For other novel *FRS* genes (*FRS1*, *FRS2*, *FRS3*, *FRS4*, *FRS5*, *FRS8*, *FRS9*, *FRS10*, and *FRS12*), we generated their cDNA clones using reverse transcription (RT)-PCR. For each gene, a pair of primers with added restriction sites at their 5′ ends was designed to cover the longest open reading frame based on genome annotation. We obtained RT-PCR products for all novel *FRS* genes except *FRS10*. These PCR products were subcloned into the pCR-TOPO2.1 vector (Invitrogen, Carlsbad, CA) and validated by sequencing. All of these cDNA clones, with the exception of *FRS1*, are consistent with the annotation of the Arabidopsis genome project and available cDNA or expressed sequence tag sequences. Sequence analysis of our *FRS1* cDNA and other reported cDNA sequences indicated that this gene was misannotated in the number and locations of introns (Fig. 1) and that our cDNA clone was missing approximately 80 amino acids of C-terminal sequences. We performed a separate RT-PCR to generate the missing C-terminal cDNA fragment and ligated it to the original *FRS1* cDNA clone (representing the N-terminal portion) to create a real full-length cDNA clone for *FRS1.* In the case of *FRS10*, we have been unable to obtain RT-PCR products using various tissue sources (cotyledons, hypocotyls, roots, rosette leaves, cauline leaves, inflorescence stems, flowers, and siliques), suggesting that the *FRS10* transcript might be unstable, or expressed at a very low level, or only expressed under certain specific conditions. Therefore, the deduced amino acid sequence of *FRS10* (At5g28530) was used in our molecular analysis (Table I).

The predicted sizes of the FRS family of proteins range from 531 amino acids to 851 amino acids. Interestingly, FHY3 and FAR1 share the highest homology to each other (47.3% amino acid identity and 79.4% similarity), and they comprise a branch of this gene family (Fig. 2). Other FRS proteins share 17.6% to 37.5% amino acid identities with FHY3 over their entire lengths. It is likely that FRS7 and FRS12 are products of a recent gene duplication event (82.4% amino acid identity and 93.3% similarity; Table II). FRS1 and FRS4 are most similar to each other and they also form a branch of the gene family (31.8% amino acid identity and 59.1% similarity). FRS6 and FRS8 share the highest homology and form another branch (42.0% amino acid identity and 74.5% amino acid similarity). In addition, the tandem arrangement and high homology between FRS5 and FRS9 suggest that they are a pair of recently duplicated genes (33.4% identity and 56.7% similarity). All FRS proteins except FRS3 and FRS8 contain one to three putative coiled-coil domains (predicted with the COILs program at http://www.ch.embnet.org/software/COILS_form.html). Further, most of the FRS proteins also possess one or two putative nuclear localization signals (NLSs) of four-residue pattern (predicted with the PSORT program at http://psort.nibb.ac.jp/form.html). The putative NLSs are located either in the C-terminal portion in some FRS proteins (FRS2, FRS7, and FRS12) or in the N-terminal half of other molecules (FRS3, FRS4, FRS5, FRS10, and FRS11). FAR1, FHY3, and FRS6 possess two putative NLSs, with one located in the N-terminal half and the other in the C-terminal half (Fig. 3). Three of the proteins (FRS1, FRS8, and FRS9) lack putative NLSs. It is worth noting that FRS8 possesses neither coiled-coil domains nor NLSs, whereas FRS9 is significantly smaller than the other FRS proteins and contains three putative coiled-coil domains (Fig. 3).

In addition to the coiled-coil domains and putative NLS, Hudson et al. (2003) reported that FAR1, FHY3, and FRS4 (AAF16668) are related to Mutator-like elements (MULEs; Lisch, 2002). Sequence analyses indicate that all predicted FRS polypeptides share significant homology with the transposase domain of type II *MuDR* family transposons such as *Jittery* (AAF66982) and *MuDRA* (S59141) of maize. In addition, all FRS proteins also share similarities with type II *MuDR* family transposons in the putative zinc-binding motif with conserved Cys and His residues (Fig. 4). The zinc-binding motif of transposon is involved in the nicking and transesterification steps of the integration process (Haren et al., 1999). The functional implications for these conserved domains in FRS proteins are currently unknown.

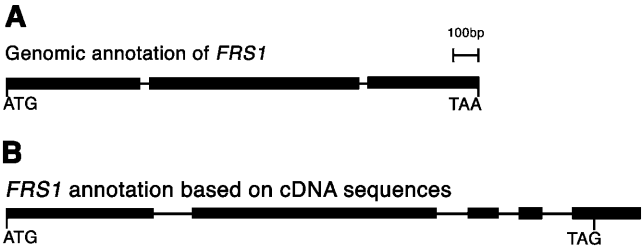

Figure 1. *FRS1* gene structure.

Expression Patterns of the *FRS* Genes

To understand the respective roles of the *FRS* genes in plant development, we conducted a semiquantitative RT-PCR analysis to determine the tissue-specific expression patterns for all *FRS* genes except *FRS10* for which no RT-PCR product was detected. The *FRS* genes with detected RT-PCR products are expressed in all organs examined, including rosette leaves, cauline leaves, inflorescence stems, flowers, and siliques (Fig. 5A).

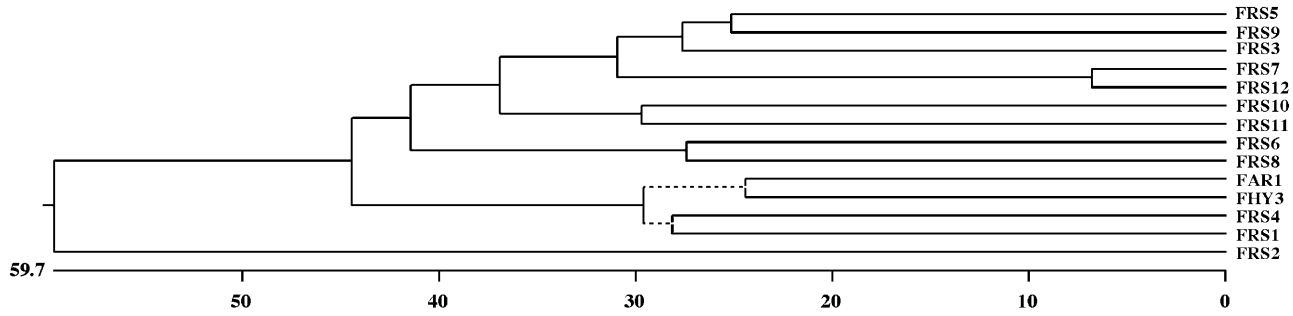

Figure 2. A phylogenetic tree of the *FRS* gene family based on amino acid sequences. The plot was obtained by the Cluster Method of the MegAlign program (DNAstar, Madison, WI).

To extend the observations made with the RT-PCR analyses, we have generated *FRS*::*GUS* reporter lines for each of the *FRS* gene, in which the β-glucuronidase (GUS) gene was fused to each of the *FRS* promoters (*FRS*::*GUS*). Promoter regions (with 5′-untranslated regions included) up to 2 kb from these genes were cloned into the binary vector pCAMBIA3301 (CAMBIA, Australia, http://www.cambia.org/) to replace the 35S promoter and drive the GUS gene expression. These *FRS*::*GUS* reporter gene constructs were introduced into Columbia (Col) wild-type plants via agrobacterium-mediated transformation (Clough and Bent, 1998). Several independent homozygous transformant lines were established for each of the reporter genes. Histochemical staining analyses of GUS activity revealed that all these *FRS* genes except *FRS1* are clearly expressed in hypocotyls. Comparison of dark versus FR light-grown seedlings showed that *FRS* gene expression in hypocotyls is induced by FR light treatment. *FRS5*, *FRS7*, *FRS8*, *FRS10*, *FRS11*, and *FRS12* are also expressed in cotyledons of light-grown seedlings. In addition, *FRS2*, *FRS3*, *FRS4*, *FRS6*, *FRS8*, and *FRS9* are also expressed in roots, whereas *FRS1*, *FRS5*, *FRS10*, *FRS11*, and *FRS12* have barely detectable or no detectable level of expression in this tissue (Fig. 5B). These results are consistent with a potential role of these *FRS* genes in regulating light control of Arabidopsis seedling development. Strikingly, the *FRS10*::*GUS* reporter gene is strongly expressed in hypocotyls and cotyledons, despite the fact that we have not been able to detect *FRS10* transcript from these tissues using RT-PCR (data not shown), suggesting that the native *FRS10* transcript might be highly unstable.

FRS Protein Subcellular Localization

Previously, FAR1 and FHY3 were shown to be targeted to the nucleus of onion (*Allium cepa*) epidermal cells and Arabidopsis cells, respectively (Hudson et al., 1999; Wang and Deng, 2002). To determine the subcellular localization of the 12 additional FRS proteins, we have generated green fluorescent protein (GFP)-FRS fusion protein constructs for each *FRS* gene (with the exception of *FRS10* for which no cDNA clone is available) in the pRTL2-S65TGFP vector that contains the strong CaMV35S promoter to drive transient gene expression (Restrepo et al., 1990; von Arnim et al.,

Table II. *Amino acid identities (above slash) and similarities (below slash) over the alignment lengths between predicted full-length sequences of FRS proteins*

Pair-wise analyses were performed using ClustalW with a gap-opening penalty of 10 and a gap extension penalty of 0.2 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html).

Protein Name	FAR1	FRS1	FRS2	FRS3	FRS4	FRS5	FRS6	FRS7	FRS8	FRS9	FRS10	FRS11	FRS12
FHY3	47.3/79.4	33.2/63.3	37.5/69.6	27.1/61.0	35.6/61.5	28.5/62.7	21.4/54.5	20.1/48.0	23.6/52.7	19.4/45.0	18.3/46.9	17.6/48.1	20.0/47.9
FAR1		34.5/61.7	37.3/70.0	27.6/61.8	37.7/65.7	29.2/64.3	23.9/54.8	20.7/47.6	23.7/54.8	20.3/44.5	19.3/49.0	20.7/54.2	21.3/45.5
FRS1			28.7/60.0	21.4/54.9	31.8/59.1	24.0/54.7	20.5/46.4	12.0/46.5	18.8/46.5	20.1/49.9	12.1/49.6	13.6/50.1	12.2/43.9
FRS2				25.0/59.7	31.9/58.3	26.2/56.4	20.7/48.0	18.6/42.6	20.7/47.8	18.5/45.0	17.0/43.3	16.1/48.4	14.1/49.9
FRS3					23.5/51.5	35.9/63.4	19.6/50.1	23.4/45.3	20.0/50.8	28.9/49.1	18.0/45.2	17.5/47.7	23.2/44.8
FRS4						28.4/62.0	24.4/55.1	22.2/45.8	25.3/55.2	21.6/50.9	19.1/47.6	21.7/54.6	21.8/45.5
FRS5							25.6/58.2	24.6/52.0	24.4/57.1	33.4/56.7	22.7/52.0	23.6/56.0	24.9/51.8
FRS6								23.4/59.0	42.0/74.5	17.7/41.0	22.4/53.0	21.5/57.7	23.6/54.9
FRS7									21.7/56.5	20.2/38.3	20.8/53.1	19.6/52.6	82.4/93.3
FRS8										16.4/40.0	21.2/54.9	23.7/54.6	21.8/53.3
FRS9											12.3/40.6	14.7/39.1	19.3/37.5
FRS10												37.8/71.3	19.9/50.1
FRS11													19.0/51.7

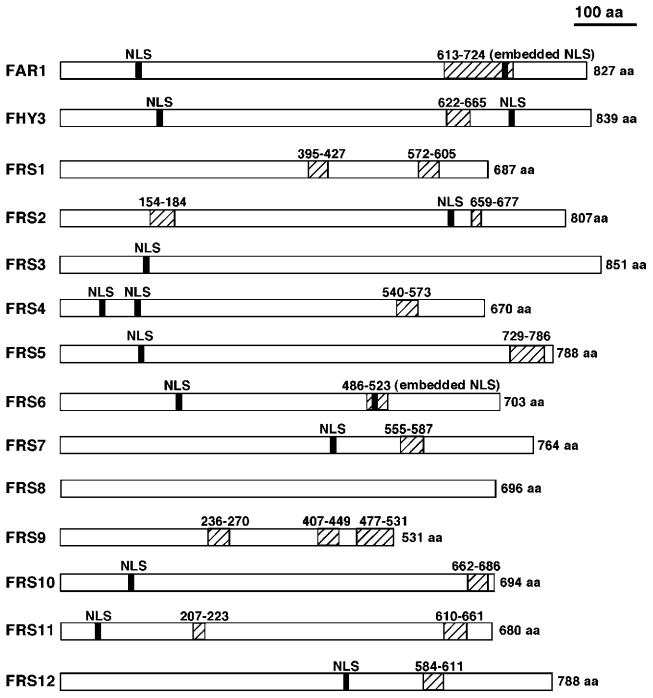

Figure 3. Diagram of predicted molecular structure of FRS proteins. Vertical bars, Putative NLSs. Slashed boxes, Putative coiled-coil domains.

1998). We conducted transient expression assays to determine the fusion protein localization and light regulation in onion epidermal cells. After gene delivery by particle bombardment, onion cells were incubated for 24 to 48 h under both dark and light conditions, and then the GFP localization patterns were examined by microscopy. Consistent with the presence of putative NLSs in FRS2, FRS3, FRS4, FRS5, FRS6, FRS7, FRS11, and FRS12, their GFP-fusion proteins are localized into the nucleus. Most strikingly, we found that FRS1, FRS8, and FRS9 are also targeted into the nucleus despite their lack of predicted NLSs. It should be noted that FRS1 seems to be nuclear enriched with residual amount of cytoplasmic distribution, whereas other FRS proteins seem to be exclusively nuclear localized (Fig. 6). Further, the nuclear localization of these FRS proteins is independent of the light treatment (data not shown).

Identification of T-DNA Insertion Mutants in *FRS6* and *FRS8*

For functional analysis of the *FRS* gene family, we searched the T-DNA insertional pools generated by The Salk Institute Genome Analysis Laboratory (SIGnAL) and The Syngenta Arabidopsis Insertion Library (SAIL, formerly GARLIC) for T-DNA insertion loss/reduction-of-function *frs* mutants (Sessions et al., 2002; Alonso et al., 2003). We used both antibiotic selection and PCR/sequencing to confirm the presence and determine the number of T-DNAs. For PCR confirmation, we amplified the T-DNA flanking region for each T-DNA line using a left-border primer and a gene-specific primer. We have also backcrossed original lines with corresponding wild-type ecotype (Col) to remove other possible background mutations and to reaffirm the cosegregation of the T-DNA insertion with the observed mutant phenotypes (see below).

Homozygous mutant lines for two independent alleles of *frs6*, designated *frs6-1* (Salk_019743) and *frs6-2* (Salk_114017), and two independent alleles of *frs8*, designated *frs8-1* (Salk_122261) and *frs8-2* (Salk_077996), have been identified from the SIGnAL population. In all four lines, T-DNAs were inserted in the predicted coding regions and presumably would cause null mutations (Fig. 7A). RT-PCR analysis showed that accumulation of their corresponding message RNAs was abolished in these mutant lines (Fig. 7B). Photobiology experiments were conducted to determine the effects of altering *FRS6* and *FRS8* gene expression on light control of Arabidopsis development. Although all of these homozygous mutant seedlings deetiolated normally in response to different light conditions (white light, FR, R, blue, and darkness), they all display a slight but reproducible early flowering phenotype (about 1 d earlier) under long-day conditions (LD; 16 h light/8 h darkness; Fig. 8, A and B). Under short-day conditions (SD; 8 h light/16 h darkness), these mutants flowered much earlier (approximately 10 d earlier) than wild-type control plants (Fig. 8, C and D). Consistent with this, both *frs6* and *frs8* mutant plants also possess fewer rosette leaves than wild-type plants at bolting (Fig. 8, B and D), suggesting that *FRS6* and *FRS8* have a similar role in controlling flowering time.

RNAi Silencing of *FRS9*

T-DNA insertion mutants for *FRS9* were not available until recently. Therefore, we attempted to use the RNA interference (RNAi) gene silencing technology to eliminate or reduce mRNA accumulation (Wesley et al., 2001). To achieve gene-specific silencing, two inverted repeat sequences (approximately 350 bp each) of *FRS9* were generated by PCR and inserted into the pHANNIBAL vector (Wesley et al., 2001), and a *Not*I fragment containing this repeat sequence was cloned into the pART27 binary vector (Gleave, 1992). This construct was transformed into Col wild-type Arabidopsis and several homozygous transgenic lines were established. Photobiology experiments showed that multiple homozygous *FRS9* RNAi lines exhibit a hypersensitive response specifically to continuous R light (Fig. 9, A–C). This phenotypic alternation seems to be specifically caused by RNAi silencing of *FRS9*, as *FRS9* mRNA transcript level is undetectable, whereas the expression of *FRS5*, the closest homolog of *FRS9*, is not affected in the RNAi plants (Fig. 9D). To provide molecular evidence for the observed phenotypic abnormality of *FRS9* RNAi plants, northern-blot analyses were conducted to compare the expression of *CAB* and

RBCS, two representative light-responsive genes. After 12 h of R light irradiation, the induction of both *CAB* and *RBCS* is clearly more enhanced in the *FRS9* RNAi transgenic plants than the wild-type control plants (Fig. 9E). Together, these results suggest *FRS9* acts as a negative regulator specific to phyB signaling.

DISCUSSION

Molecular Features of FRS Proteins

The founding members of the *FRS* gene family, *FAR1* and *FHY3*, were independently identified as two essential signal transducers for phyA-mediated FR-HIR responses (Hudson et al., 1999; Wang and Deng 2002). Other FRS proteins share 17.6% to 37.5% amino acid identities with FHY3 over their entire lengths. Similar to FHY3 and FAR1, all other FRS proteins except FRS3 and FRS8 contain one to three putative coiled-coil domains. Further, most of the FRS proteins possess one or two putative NLSs. The overall sequence similarities and the conserved secondary structure suggest that some of the FRS proteins may have overlapping function in mediating light signaling like FHY3 and FAR1. The presence of coiled-coil domain(s) in most FRS proteins suggests that they may form homo- and/or hetero-dimers or interact with diverse partners to control a broad range of signaling processes. Indeed, evidence for homodimerization of FAR1 and FHY3 and their interaction with other proteins has been reported previously (Wang and Deng, 2002; Hudson et al., 2003). Further, overexpressing the C-terminal portion of FHY3 (C473-839, which contains a coiled-coil domain) in a wild-type background causes an apparent loss of responses to FRc light, suggesting a dominant-negative effect most likely caused by hetero-interaction of the partial FHY3 fragment with other FRS proteins through their coiled-coil domains (Wang and Deng, 2002).

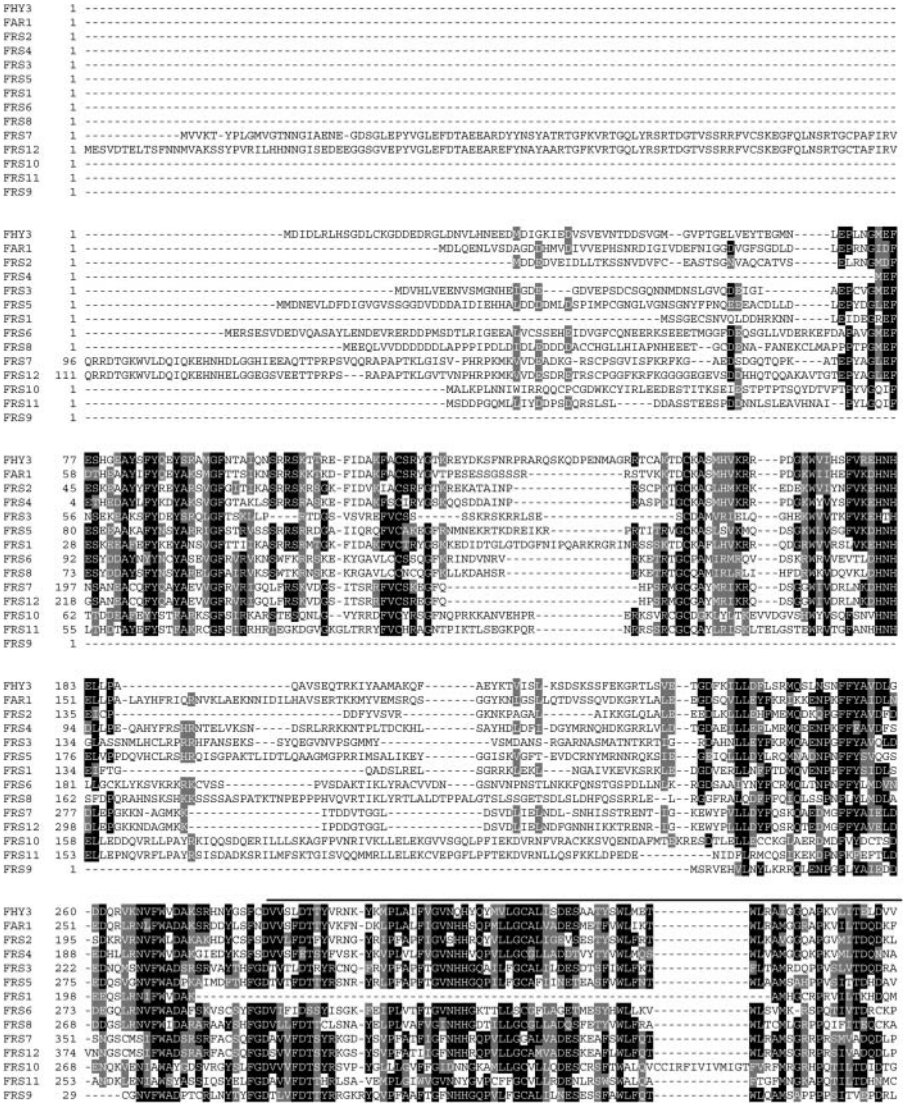

Figure 4. Amino acid sequence alignment between all FRS members. Identical and similar amino acid residues are shaded in black and gray, respectively. The *Mutator* transposase-homologous domain is indicated by a line above the sequences. The asterisks indicate the conserved Cys and His residues of the potential zinc-binding motif.

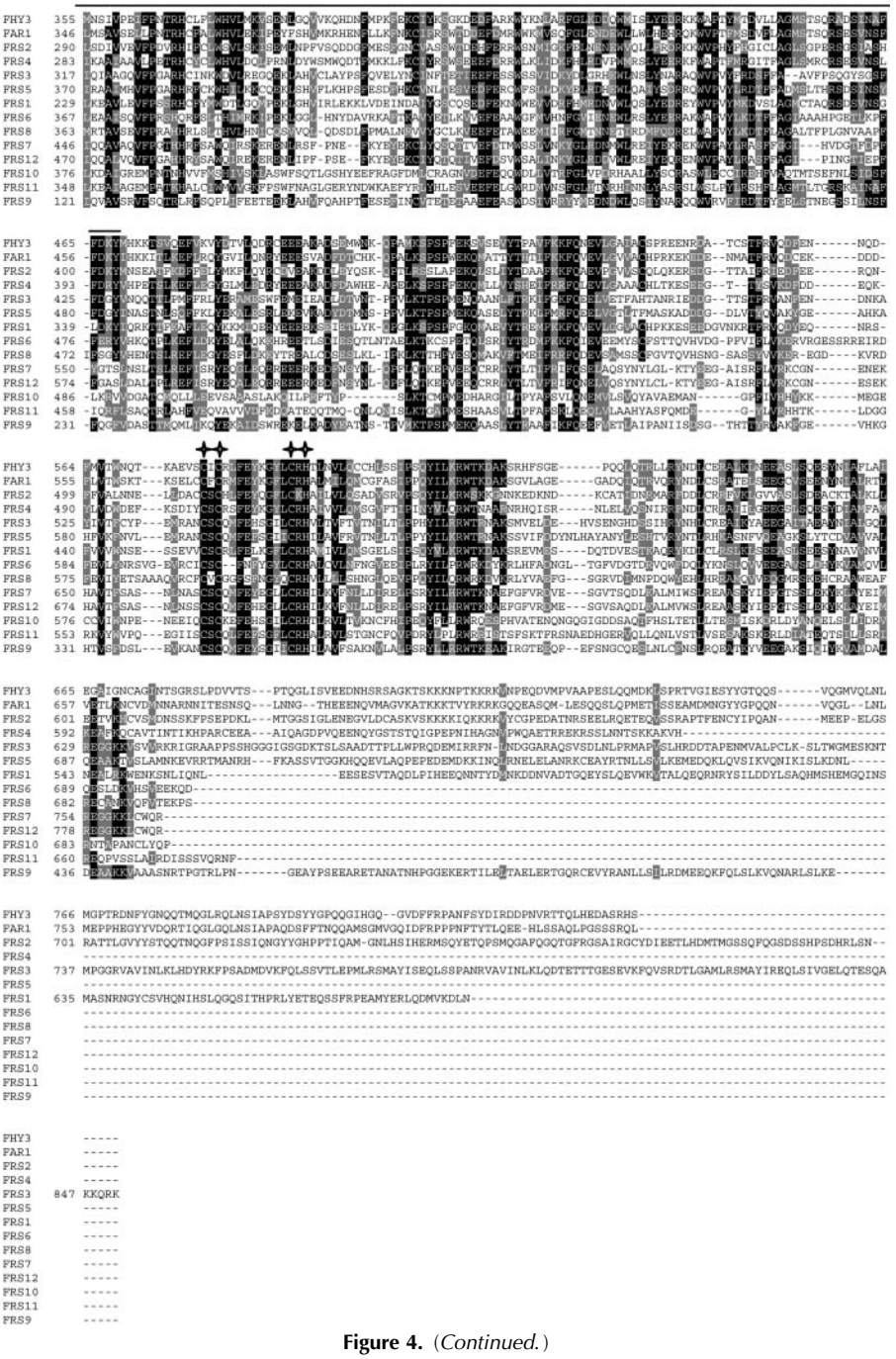

Figure 4. (*Continued.*)

The sequence homologies between FRS proteins and the MULEs suggest that the *FRS* gene family might be derived from a common ancestor of a MULE-type mobile element. However, it is unlikely that they possess transposition capacity, as these genes lack the characteristic terminal inverted repeats and other transposon-associated features (Hudson et al., 2003). The nuclear localization of FAR1 and its demonstrated ability to activate a reporter gene expression in yeast and Arabidopsis has lead to the proposition that FAR1 might act as a transcriptional regulator (Hudson et al., 1999; Hudson et al., 2003). Genome profiling analyses have shown that both FAR1 and FHY3 are required for phyA-mediated gene expression by FR light (Wang et al., 2002). Since all FRS proteins are localized into the nucleus and all share similarities with transposases of the MULE family, these FRS proteins may define a novel class of transcriptional regulators with transposase homology. Transposases of the *Mutator* family are capable of

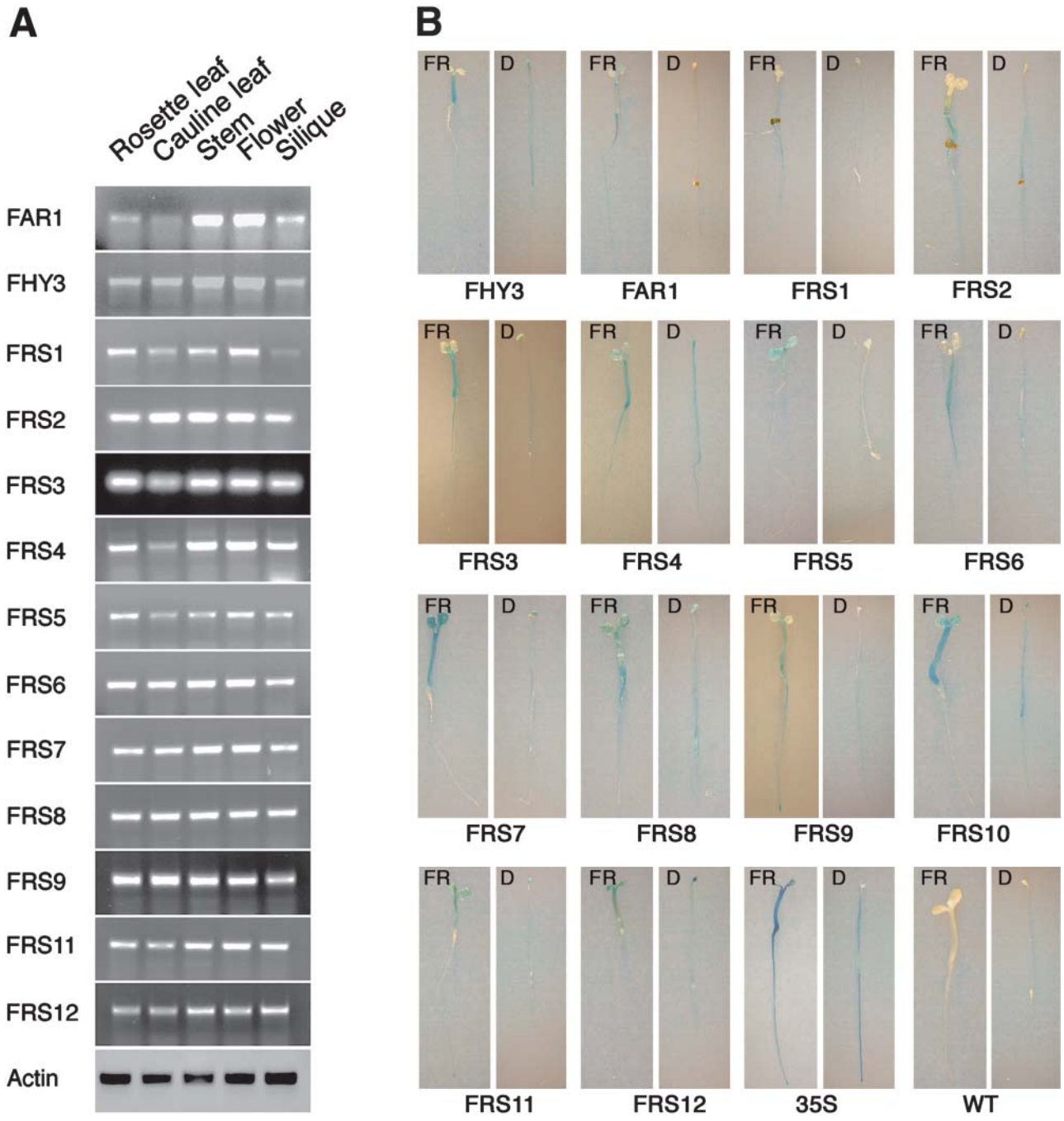

Figure 5. Expression patterns of the *FRS* genes. A, RT-PCR showing that all *FRS* genes (except *FRS10*) are expressed in all organs examined here (rosette leaf, cauline leaf, stem, flower, and silique). B, Histochemial staining of *FRS*::*GUS* reporter gene transgenic lines. The left sections are histochemical staining of FR light-grown plants and the right sections are histochemical staining of dark-grown plants. 35S, transgenic plants harboring 35S promoter driven GUS reporter gene. WT, Wild-type nontransgenic plants (Col ecotype).

regulating the expression of their own genes and the associated genes via direct DNA binding (Barkan and Martienssen, 1991; Benito and Walbot, 1997; Raizada et al., 2001). It will be interesting to determine whether FRS proteins can bind DNA directly and to identify their direct target genes in future studies.

Previously, both FAR1 and FHY3 have been demonstrated to be targeted into the nucleus (Hudson et al., 1999; Wang and Deng, 2002), consistent with the presence of putative NLSs in both proteins. Here we showed that 11 additional FRS proteins are also targeted into the nucleus of onion epidermal cells

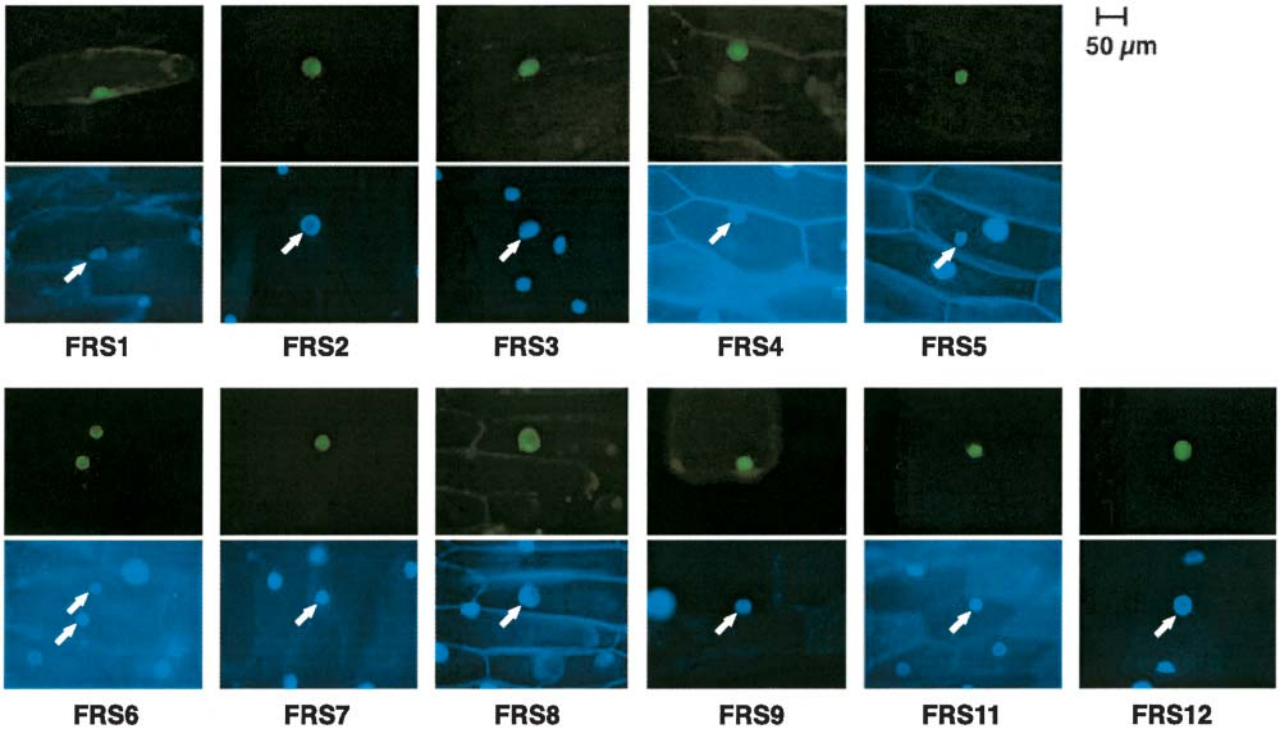

Figure 6. Subcellular localization of GFP-FRS fusion proteins in onion epidermal cells. The top sections show GFP localization of the fusion proteins. The lower sections show DAPI staining of the corresponding images. Arrowheads indicate the positions of nuclei. Bar represents approximately 50 μm.

and this pattern is independent of light control, although it should be noted that FRS1 is only nuclear enriched with clear residual cytoplasmic distribution.

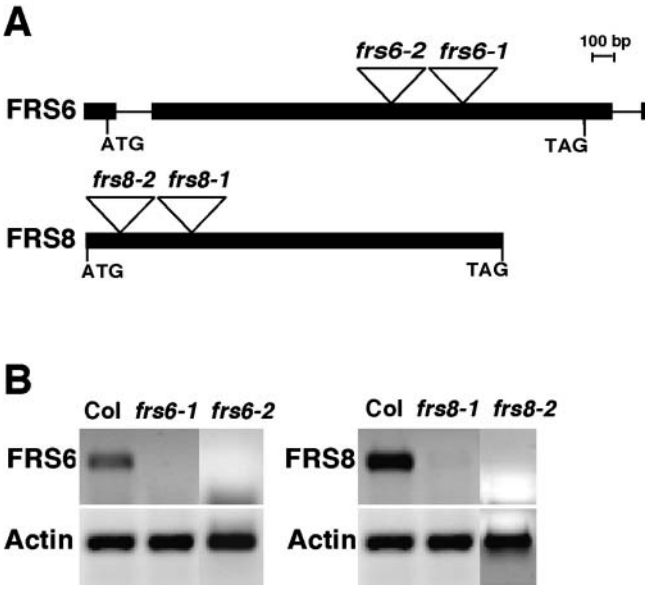

Figure 7. Analyses of *frs6* and *frs8* T-DNA insertion mutants. A, Diagram of the genomic structures of *FRS6* and *FRS8* genes and locations of T-DNA insertions. B, RT-PCR analysis showing that message RNAs of *FRS6* and *FRS8* were abolished in their respective *frs* homozygous mutants.

Strikingly, FRS1, FRS8, and FRS9 are predicted to lack putative NLSs, thus their nuclear localization suggests that they may either use nontypical NLSs for nuclear import or they may interact with their partners (such as other NLS-containing FRS proteins) and be imported into nucleus as protein complexes.

Distinct Roles of FRS Proteins in Mediating Light Control of Arabidopsis Development

Our RT-PCR analyses showed that with the exception of *FRS10*, all *FRS* genes are expressed in all major organs examined, including rosette leaves, cauline leaves, stems, flowers, and siliques. We have not been able to recover RT-PCR products for *FRS10* using various tissue sources, consistent with the fact that no expressed sequence tag clones or any type of cDNA clones have been documented for this gene. However, *FRS10*::*GUS* reporter gene shows strong expression in hypocotyls and cotyledons (Fig. 5B). These observations suggest that the endogenous *FRS10* transcript might be highly unstable. Further studies, such as nuclear run-on assay, are required to clarify this issue. Histochemical staining analyses of *FRS*::*GUS* fusion reporter genes revealed that all these *FRS* genes except *FRS1* are clearly expressed in hypocotyls. Comparison of dark versus FR light-grown seedlings showed that *FRS* gene expression in hypocotyls is induced by FR light treatment. *FRS5*, *FRS7*, *FRS8*, *FRS10*, *FRS11*, and *FRS12* are also expressed in cotyledons of light-grown

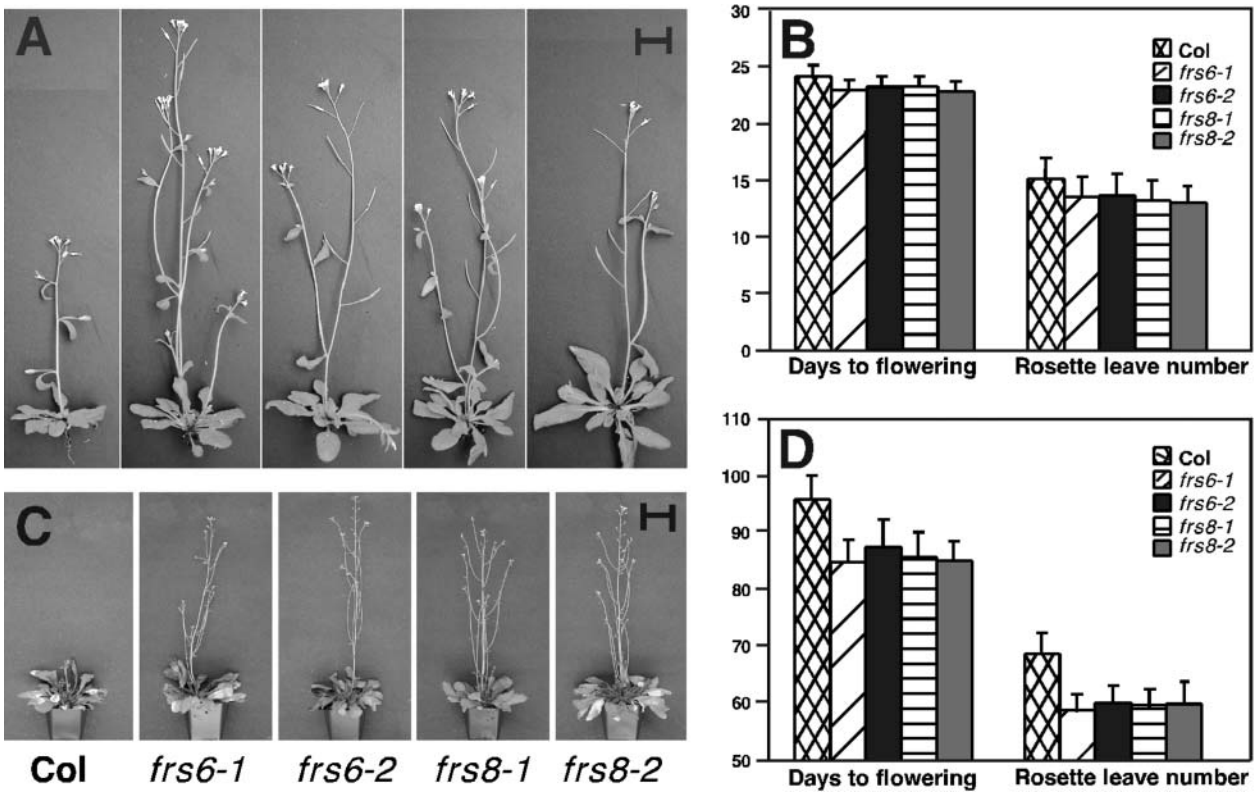

Figure 8. Early flowering phenotype of *frs6* and *frs8* mutants. A and B, *frs6* and *frs8* mutants flower early under LD conditions. C and D, *frs6* and *frs8* mutants flower early under SD conditions. B and D show days to flower and the rosette leaf numbers of various plants at bolting under LD and SD conditions, respectively (based on the opening of first flower). Bar in A represents approximately 1 cm. Bar in C represents approximately 5 cm. Bars in B and D represent SDs.

seedlings (Fig. 5B). These results are consistent with a potential role of these *FRS* genes in regulating light control of Arabidopsis development. Functional analyses of *FRS6*, *FRS8*, and *FRS9* genes supported this notion. *frs6* and *frs8* mutants display an early flowering phenotype under both LD and SD conditions, with more drastic effect under SD conditions. Both phytochromes and cryptochromes play important roles in flowering time regulation (Guo et al., 1998; Somers et al., 1998). Among these photoreceptors, phyA promotes flowering as Arabidopsis *phyA* mutant flowers later than wild-type plants in LD (Johnson et al., 1994; Neff and Chory, 1998). phyB plays an inhibitory role in floral initiation. The Arabidopsis *phyB* mutant flowers earlier than wild-type plants in both LD and SD conditions, with a more pronounced phenotype in SD than in LD conditions (Goto et al., 1991; Mockler et al., 1999; Lin, 2000). The early flowering phenotype of *frs6* and *frs8* mutants under both LD and SD conditions and their more pronounced phenotype in SD conditions suggest that *FRS6* and *FRS8* likely act as positive regulators in phyB signaling pathway controlling flowering time. Interestingly, FRS6 and FRS8 polypeptides have the highest homology to one another and form a separate branch of the *FRS* gene family (42.0% amino acid identity and 74.5% amino acid similarity), supporting the functional similarity of these two genes. On the other hand, the R light hypersensitivity exhibited by the *FRS9* RNAi transgenic plants indicates that *FRS9* is a negative regulator of phyB signaling mediating seedling deetiolation. Together, our results support the notion that *FRS* gene family members play differential roles regulating distinct photo-responses.

It should be noted that there is potential functional redundancy among this family of proteins, or they may have tissue and developmental stage-specific functions, thus masking the detection of an aberrant phenotype for some *frs* single mutants under the limited experimental conditions we tested. Future studies employing multiple alleles, double/higher order mutants, gain-of-function studies, and genome profiling changes caused by altering *FRS* gene expression will most likely reveal more information about the function of these *FRS* genes in light regulation of Arabidopsis development. Further, different members of this gene family could also be involved in signal transduction processes of other biotic or abiotic stresses such as phytohormones. The molecular analysis of this plant-unique gene family has laid a foundation for elucidating their biological functions as well as their biochemical mechanisms of action.

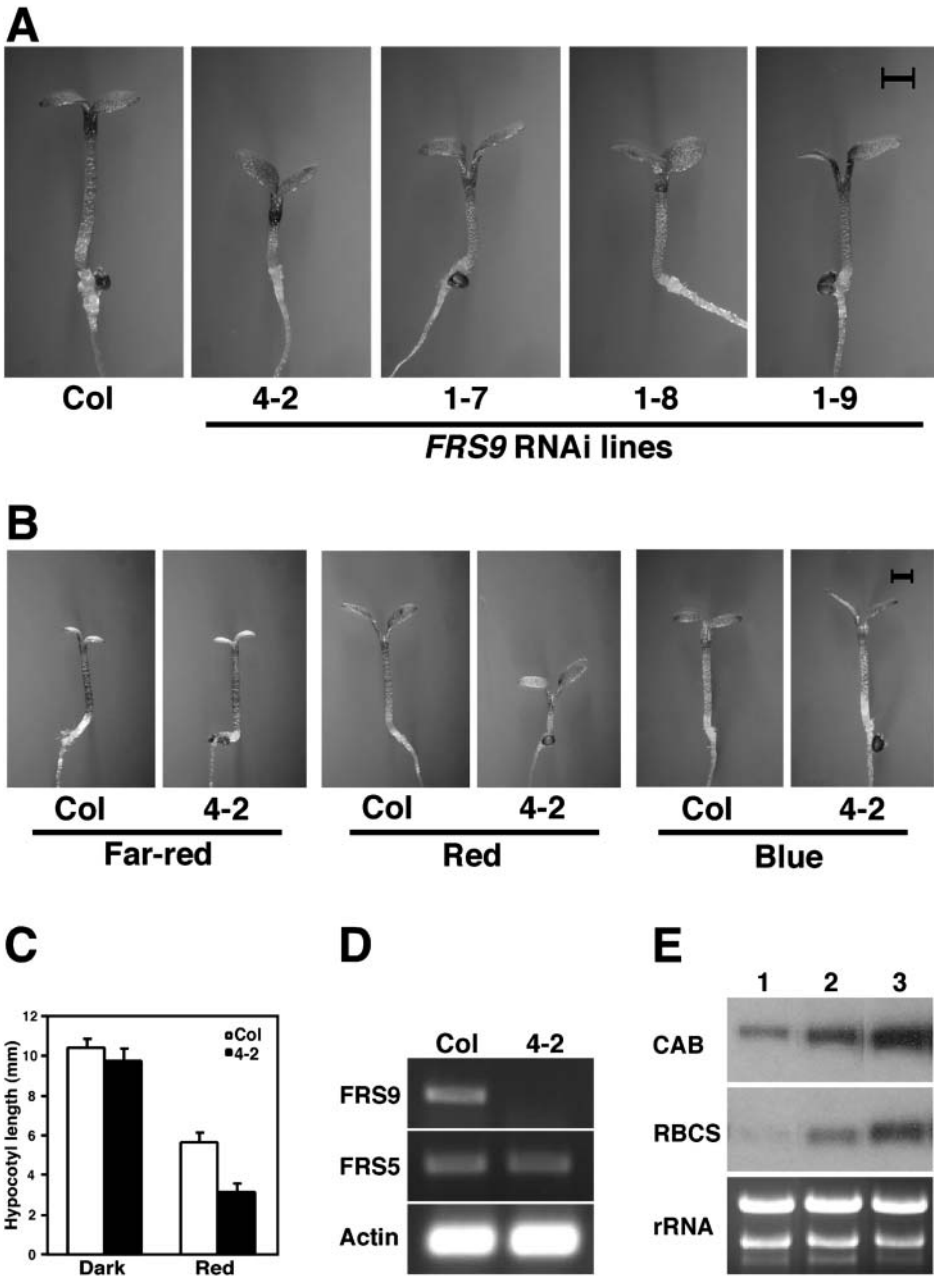

Figure 9. Phenotype of *FRS9* RNAi transgenic plants. A, Four independent RNAi lines of *FRS9* show hypersensitive response to continuous R light. Bar represents approximately 1 mm. B, FRS9 RNAi plants (represented by the transgenic line 4-2) are specifically hypersensitive to R light and display normal responses to FR and blue light. Bar represents approximately 1 mm. C, Quantification of hypocotyl lengths of seedlings grown in continuous darkness or R light for 4 d. D, RT-PCR showing that *FRS9* mRNA accumulation is abolished in *FRS9* RNAi plants (represented by the transgenic line 4-2), whereas accumulation of *FRS5* mRNA is not affected. E, *CAB* and *RBCS* gene expression is enhanced in *FRS9* RNAi plants compared with wild-type plants (grown in darkness for 4 d then transferred to continuous R light for 12 h). Col, Wild-type Col ecotype plants. 1, Col grown in darkness for 5 d; 2 and 3 are Col and *FRS9* RNAi plants (line 4-2) grown in darkness for 4 d then transferred to continuous R light for 12 h.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) materials are of Col ecotype background. T-DNA insertion mutants were obtained from ABRC (Columbus, OH). The seeds were surface-sterilized and sown on 1× Murashige and Skoog media plates with 1% Suc and cold-treated for 3 d at 4°C. Then the plates were exposed to white light for 24 h to stimulate seed germination before being transferred to FRc, R, blue light, or dark conditions for 4 d at 22°C. FR, R, and blue lights were supplied by LED light sources, with irradiance fluence rates of approximately 100, 1,200, and 250 uW/cm^2, respectively (measured with International Light [Newburyport, MA] model IL1400A with sensor model SEL-033/F/W). White light was supplied by cool-white fluorescent lamps. Adult plants were grown in environmental chambers at 22°C and 60% humidity with 16 h of continuous fluorescence light unless otherwise specified.

PCR Genotyping

To identify the T-DNA insertion mutants, we amplified the T-DNA flanking region for each T-DNA line using a vector left-border primer (5′-CGGAACCACCATCAAACAGG-3′) and a gene-specific primer. The insertion site was confirmed by DNA sequencing.

RT-PCR

To generate full-length cDNA clones for *FRS* genes, RNAs were extracted from light-grown Arabidopsis seedlings with the RNeasy Plant Mini kit (Qiagen USA, Valencia, CA). First-stand cDNA was synthesized by StrataScript RT at 42°C for 30 min, and PCR was carried out by PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) with the following program: an initial 95°C 1 min followed by 30 cycles of 95°C 30 s, 60°C 30 s, 68°C 7 min; and a final extension at 68°C for 10 min. Primers were designed to cover the whole open

reading frame of each *FRS* gene with suitable restriction enzyme sites added at their 5′-ends to facilitate downstream cloning efforts. The primer pairs used for RT-PCR were: *FRS1*, 5′-GGTACCGGATCCATGTCGTCAGGAGAGTGTAGC-3′ and 5′-GTCGACACTAGTTTACTTTCCAGACTTCTTGCA-3′; *FRS2*, 5′-CTCGAGGGATCCATGGATGATGAAGATGTAGA-3′ and 5′-GACTCTAGATTAATTGGATAAGCGGTGATC-3′; *FRS3*, 5′-GTCGACCTGGATCCATGGATGTTCATTTGGTGGAAG-3′ and 5′-TCTAGATCAAAAGCGTTGCTTCTTTGC-3′; *FRS4*, 5′-CTCGAGTCAGATCTATGGAGTTCGAGACTCACGAA-3′ and 5′-TCTAGATCACCCAGGGGGATTGTTCTG-3′; *FRS5*, 5′-GTCGACAGATCTATGATGGATAATGAAGTGCTC-3′ and 5′-TCTAGATCACAGATTATCCTTCAAGCT-3′; *FRS8*, 5′-GTCGACGGATCCATGGAAGAGCAGCTGGTTGTT-3′ and 5′-TCTAGACTATGAAGGCTTCTCTGTCAC-3′; *FRS9*, 5′-GTCGACTCATGAGCAGGGTCGAGCATGT-3′ and 5′-TCTAGATCACTCTTTCAAGCTTAGTC-3′; *FRS10*, 5′-CTCGAGTCTATGGCGTTGAAGCCATTGAAC-3′ and 5′-TCTAGATCATGGCTGATACAAGCAATT-3′; and *FRS12*, 5′-GGTACCAGATCTATGGAGAGTGTAGATACTGAG-3′ and 5′-GCTAGCTCATCTCTGCCAACAAAGTTTC-3′. The RT-PCR products were cloned into the pCR-TOPO2.1 vector (Invitrogen) to generate pTOPO-FRS clones. A separate RT-PCR was performed to obtain the C-terminal fragment of *FRS1* missed in our original pTOPO-FRS1 cDNA clone. The primers used were: 5′-GGAATGTGTACAGCTCAGAGA-3′ and 5′-AGTGGATCCGTCTAGTTCAAGTCTTTTACC-3′. A full-length cDNA clone of *FRS1* was created by ligating the original FRS cDNA with the second RT-PCR product at the common *Bsr*GI site.

For semiquantitative RT-PCR analysis of *FRS* gene expression, total RNAs were extracted from different organs of adult plants. RT-PCR was performed with the one-step RT-PCR system (Promega, Madison, WI) using the following program: 45 min at 48°C for RT, then 2 min at 94°C followed by 32 cycles of 30 s at 94°C; 1 min at 60°C; 2 min at 68°C, and a final extension at 68°C for 7 min. The actin-1 gene of Arabidopsis was used as a control. The primer pairs used were: *FHY3*, 5′-GCTGTGAGTGAACAGACCAG-3′ and 5′-CATCAGTCATGTAGGTTGGTG-3′; *FAR1*, 5′-GGATTCAGAGGAATGTCAAG-3′ and 5′-GTCTCCATAGACTCATCAGC-3′; *FRS1*, 5′-GATCAGACAGTGTGAACTCTG-3′ and 5′-GCTGTAACCGATTCTGACTC-3′; *FRS2*, 5′-TGGAGATGCAGGATAAGCAG-3′ and 5′-ACTGAACCCACTCATTCTCG-3′; *FRS3*, 5′-GAAGCCATCAAGTATGCTGAG-3′ and 5′-GACATCCATGTCTGCTGAGG-3′; *FRS4*, 5′-GTTGTCCTCCAAATGTCGGG-3′ and 5′-ACTCCTGGCATTGTGTTGTG-3′; *FRS5*, 5′-CGGAGATGTTGATGACGACG-3′ and 5′-GGTTGTCAGCATTCATCTGC-3′; *FRS6*, 5′-GCTGGAGAGACAATGGAGTC-3′ and 5′-CACGAAAGGTCCATCGACATG-3′; *FRS7*, 5′-GTCATATAGAGGAGGCTCAG-3′ and 5′-GGTACTACTGATGTGATTGC-3′; *FRS8*, 5′-CTATGTCTGGCTCTTCAGAG-3′ and 5′-GACATTGCTGAGACCTCGTC-3′; *FRS9*, 5′-TCTTGGGACTCTATCGTCAG-3′ and 5′-AGATACCGAGATGGAAGAGC-3′; *FRS10*, 5′-GAGTGATACGTTGGAGCTTC-3′ and 5′-GAGTGACAAGAAGATCCCAC-3′; *FRS11*, 5′-GGAGTTGGCAAAGGACTTAC-3′ and 5′-CCAACCCATATTCCAAGTGG-3′; *FRS12*, 5′-GAGGTGAAGGTAGTGTTGAG-3′ and 5′-TTGACATCCAATTCGACAGC-3′; and actin, 5′-CATCAGGAAGGACTTGTACGG-3′ and 5′-GATGGACCTGACTCGTCATAC-3′.

Plasmid Construction

For *FRS*::*GUS* reporter gene constructs, up to 2-kb upstream sequences of the predicted ATG start codons (including 5′-untranslated regions) were PCR amplified with gene-specific oligonucleotides containing proper restriction sites. PCRs were carried out on total genomic DNA extracted with the DNeasy Plant Mini kit (Qiagen). These generated promoter fragments were cloned into the binary vector pCAMBIA3301 (CAMBIA, http://www.cambia.org) to replace the 35S promoter and drive the GUS gene expression. The primer pairs used were: *FHY3*, 5′-GACAAGCTTCGATTTTACCTGAAGAGTGTGAG-3′ and 5′-GAGCCATGGCCATGACAAACCTATAGTCTCAGGC-3′; *FAR1*, 5′-GACAAGCTTGTGACTCAGAGCACAACTCTCGTAC-3′ and 5′-GATCCATGGGTATCAAAGTCTATACCATTTCGTG-3′; *FRS1*, 5′-GACCAATTGATCCTCGAAGCAGAGAGCTGCTATG-3′ and 5′-GATCCATGGACGACATTTCGAAATCAATACCTG-3′; *FRS2*, 5′-GACAAGCTTGAGAAGTGAGAAGATCCAATGTTTG-3′ and 5′-GATCCATGGCACTAGTTCCAACTCTGTTAACCG-3′; *FRS3*, 5′-GACAAGCTTCCAGACAAGATGGATTCGTAAGC-3′ and 5′-GATCCATGGCCTTCACTTAGTAGAGAAGTTC-3′; *FRS4*, 5′-GACCAATTGGAATGGTCTCTTGACAGCACAAGG-3′ and 5′-GATCCATGGGAGAAGCTTGATGAATCCACACC-3′; *FRS5*, 5′-GACAAGCTTCTTGAGAAGAAGGAATC-3′ and 5′-GATCCATGGCCTCCTTGTCTTCAGGGATGAATT G-3′; *FRS6*, 5′-GACGAATTCGCAAAAGAGTTGAACCTCAGCTGC-3′ and 5′-GATCCATGGTCTCCATTGGAGGTTTGGTTCCTC-3′; *FRS7*, 5′-GACCAATTGCCTTGTATCGTTGATCATCTATG-3′ and 5′-GATCCATGGTGTTCCCACAATTTAAACTGGTAGG-3′; *FRS8*, 5′-GACAAGCTTGTCCACCTTATACTGTCCTTGATG-3′ and 5′-GTCAGATCTACCATCTGAAGACCATCAAATTC-3′; *FRS9*, 5′-GATCAATTGCCAATACTCTCTCATAGCGTGGAG-3′ and 5′-GTCAGATCTACCATGCTTACTTCTTCCACTAATTCTG-3′; *FRS10*, 5′-GATTCTAGAAGGTCTTTACAATACGGTCCAAC-3′ and 5′-GATCCATGGATGGCTTCAACGCCATAAGACAAGG-3′; *FRS11*, 5′-GATTCTAGAAACACGTCTAGGTTCATGGATGCAC-3′ and 5′-GATCCATGGAGCTCCTAAGGTATATTCAGCAC-3′; and *FRS12*, 5′-GATGAATTCGCGTTATACGTGATTGCATTCGGTG-3′ and 5′-GATCCATGGTAGT-AAGCTCAGTATCTACACTGC-3′.

For the GFP-FRS fusion protein constructs, the full-length cDNA fragment for each *FRS* gene was cloned into the pRTL2-S65TGFP vector that contains the strong CaMV 35S promoter to drive transient gene expression (Restrepo et al., 1990; von Arnim et al., 1998). Briefly, for *FRS1*, *FRS2*, *FRS3*, *FRS4*, *FRS5*, *FRS8*, and *FRS12*, their pTOPO-FRSs clones were digested with *Bgl*II/*Bam*HI and *Xba*I/*Spe*I/*Nhe*I to release their cDNA fragments, and then the cDNA fragments were gel purified and cloned into the pRTL2-S65TGFP vector digested with *Bgl*II and *Xba*I. For *FRS9*, pTOPO-FRS9 was digested by *Sal*I (blunted by Klenow fragment) and *Xba*I, and then the released cDNA fragment was cloned into the pRTL2-S65TGFP vector digested with *Bgl*II (blunted with Klenow fragment) and *Xba*I. For *FRS6*, *FRS7*, and *FRS11*, first their full-length cDNAs with modified restriction sites at both ends were PCR amplified from cDNA clones obtained from ABRC and cloned into the pGEM-T Easy vector (Promega) to generate corresponding pGEM-FRS clones. The primers used for PCRs were: *FRS6*, 5′-GATATCTCGGATCCTATGGAGAGAAGTGAGTCCGTTG-3′ and 5′-TCTAGAGTCGACCCCGGGACACTAGTCTTGTTTTTCTTCTAC-3′; *FRS7*, 5′-GGATCCATGGTTGTCAAAACTTATCC-3′and 5′-GTCTCTAGATCATCTCTGCCAACACAG-3′; and *FRS11*, 5′-CTCGAGTCGGTACCAGATCTATGTCGGATGATCCTGGAC-3′ and 5′-GTCGACTCTAGATCAAAAATTCCTCTGCACAG-3′. The full-length cDNA fragments of *FRS7* and *FRS11* were released by digestion with *Bam*HI/*Xba*I (for pGEM-FRS7) or *Bgl*II/*Spe*I (for pGEM-FRS11) and then cloned into *Bgl*II/*Xba*I digested pRTL2-S65TGFP vector. For *FRS6*, pGEM-FRS6 was digested with *Bam*HI and *Xba*I, and the released cDNA fragment was cloned into the *Bam*HI/*Xba*I sites of pRTL2-GFP-hCOP1 (Wang et al., 1999). The junction sites of all subcloning steps were confirmed by sequencing.

For RNAi silencing of *FRS9*, the first PCR fragment (348 bp) was amplified from *FRS9* cDNA clone with primers S9-B (5′-CGCGGATCCGTGGATGCATCAACTACAATG-3′) containing a *Bam*HI site, and S9-C (5′-TCCATCGATCTCAAACATTTGACAGCTGC-3) with a *Cla*I site. The PCR product was digested with *Bam*HI and *Cla*I and then cloned into the *Bam*HI/*Cla*I sites of the pHANNIBAL vector (Commonwealth Scientific and Industrial Research Organization [CSIRO]; http://www.csiro.au), generating pHANNIBAL-9BC. The second PCR fragment was amplified by primers S9-X (5′-GACCTCGAGGTGGATGCATCAACTACAATG-3′) containing an *Xho*I site, and S9-K (5′-CGGGGTACCCTCAAACATTTGACAGCTGC-3) containing a *Khn*I site. The PCR fragment was digested with *Xho*I and *Kpn*I, and then inserted into *Xho*I/*Kpn*I digested pHANNIBAL-9BC to generate pHANNIBAL-FRS9. A *Not*I fragment containing the inverted repeat sequences of *FRS9* was released from pHANNIBAL-FRS9 and cloned into *Not*I-digested pART27 binary vector (Gleave, 1992), giving rise to pART-FRS9.

Plant Transformation and Selection of Transgenic Plants

Each of the *FRS*::*GUS* reporter gene constructs and the pART-FRS9 RNAi gene silencing constructs were electroporated into the Agrobacterium strain GV3101 and then introduced into Arabidopsis wild-type plants (Col ecotype) via a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on germination plates containing 20 μg/mL glufosinate-ammonium (for *FRS*::*GUS* reporter gene lines) or 50 μg/mL Kanamycin (for *FRS9* RNAi lines). We selected about 20 T1 transgenic lines with a single T-DNA insertion and allowed them to self to produce T2 seeds. Phenotypic analyses were conducted with T2 plants and then confirmed in T3 generation. Histochemical GUS staining of *FRS*::*GUS* reporter gene was conducted according to a described procedure (Jefferson et al., 1987).

Protein Localization Studies

Onion (*Allium cepa*) epidermal cells were transfected with pRTL2-GFP-FRSs constructs using helium biolistic gun transformation system (Bio-Rad,

Hercules, CA) as described (Ang et al., 1998) and incubated in light or darkness for 24 to 48 h at 22°C. The subcellular localization of GFP fusion proteins was visualized with a fluorescence microscope.

RNA Gel-Blotting

Arabidopsis seedlings were grown either in darkness for 5 d, or in darkness for 4 d and then were transferred to red light for 12 h. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen). Five micrograms total RNA per lane was size-fractionated on a formaldehyde agarose gel and subsequently transferred to a nylon membrane. After hybridization in 0.25 M sodium phosphate, 1 mM EDTA, 1% (w/v) casein, 7% (w/v) SDS at 65°C with random prime-labeled DNA probes (Roche, Indianapolis), membranes were washed two times each of 2 × SSC, 0.1% SDS; 0.2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS. *CAB* and *RBCS* probes were described previously (Wang and Deng, 2002).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Sequence data from this article (the new full-length cDNA sequence of *FRS1*) have been deposited with the EMBL/GenBank data libraries under accession number AY763412.

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