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STABILIZED1, a Stress-Upregulated Nuclear Protein, Is Required for Pre-mRNA Splicing, mRNA Turnover, and Stress Tolerance in *Arabidopsis* [W]

Byeong-ha Lee,[a,1] Avnish Kapoor,[b] Jianhua Zhu,[b] and Jian-Kang Zhu[a,b,2]

[a] Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721
[b] Institute for Integrative Genome Biology and Department of Botany and Plant Sciences, University of California, Riverside, California 92521

In plants, many gene transcripts are very unstable, which is important for the tight control of their temporal and spatial expression patterns. To identify cellular factors controlling the stability of unstable mRNAs in plants, we used luciferase imaging in *Arabidopsis thaliana* to isolate a recessive mutant, *stabilized1-1* (*sta1-1*), with enhanced stability of the normally unstable luciferase transcript. The *sta1-1* mutation also causes the stabilization of some endogenous gene transcripts and has a range of developmental and stress response phenotypes. *STA1* encodes a nuclear protein similar to the human U5 small ribonucleoprotein–associated 102-kD protein and to the yeast pre-mRNA splicing factors Prp1p and Prp6p. *STA1* expression is upregulated by cold stress, and the *sta1-1* mutant is defective in the splicing of the cold-induced *COR15A* gene. Our results show that STA1 is a pre-mRNA splicing factor required not only for splicing but also for the turnover of unstable transcripts and that it has an important role in plant responses to abiotic stresses.

INTRODUCTION

Gene expression is controlled at the transcriptional and post-transcriptional levels. The instability of mRNAs facilitates the tight control of specific temporal and spatial expression patterns. In higher plants, the control of mRNA stability has been associated with growth, development, and response to hormones as well as biotic and abiotic stresses (Abler and Green, 1996; Carrington and Ambros, 2003; Kuhn and Schroeder, 2003; Shi et al., 2003). Much effort has been made to understand the RNA silencing pathway for the degradation of mRNAs containing sequences complementary to short regulatory RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Voinnet, 2002; Bartel and Bartel, 2003; Carrington and Ambros, 2003). miRNAs and siRNAs assemble in endonuclease-containing complexes termed RISC and miRNP, respectively, and can target homologous RNA sequences for endonucleolytic cleavage (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Hutvagner and Zamore, 2002). Factors involved in miRNA or siRNA biogenesis or actions are important determinants of the abundance of target mRNAs.

Many endogenous mRNAs with a high turnover rate are not targeted by miRNAs or siRNAs. Some of these unstable mRNAs in plants contain, as instability determinants, multiple overlapping AUUUA sequences or downstream element sequences that are not AU-rich (Ohme-Takagi et al., 1993; Johnson et al., 2000). However, the primary or secondary sequence features conferring instability to most of the unstable mRNAs are not known. The cellular machinery important for the degradation of the unstable mRNAs is expected to consist of RNases, RNase inhibitors, RNA binding proteins, and, potentially, other cellular factors. To identify the cellular factors regulating RNA stability, two *Arabidopsis thaliana* mutants defective in downstream element–mediated mRNA decay were isolated (Johnson et al., 2000). However, the genes responsible for the mutant phenotypes have not been identified (Johnson et al., 2000). In contrast with the paucity of genetic studies of mRNA stability control in multicellular organisms, including plants, extensive genetic analysis has been conducted in yeast and has elucidated general mRNA decay mechanisms. The main pathway for the turnover of both unstable and stable transcripts in yeast is the deadenylation-dependent decapping pathway (Caponigro and Parker, 1996). In addition, yeast has mRNA surveillance systems that detect and degrade aberrant mRNAs (Hilleren and Parker, 1999), which include malprocessed transcripts and transcripts with premature nonsense codons. Nonsense-mediated decay also occurs in plants, but whether the mechanisms are the same as in yeast is unclear.

Because of their sessile nature, plants have evolved sophisticated mechanisms to cope with environmental challenges (Zhu, 2002). Recently, RNA metabolism was shown to be important in plant responses to abiotic stresses (Forment et al., 2002; Gong et al., 2002b; Kuhn and Schroeder, 2003). The expression of the

[1] Current address: Cold Spring Harbor Laboratory, 1 Bungtown Road, Delbruck Building, Cold Spring Harbor, NY 11724.
[2] To whom correspondence should be addressed. E-mail jian-kang.zhu@ucr.edu; fax 951-827-7115.
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RS domain of an SR-like splicing protein, *SRL1*, conferred salt tolerance to *Arabidopsis*, suggesting an important role of pre-mRNA splicing in salt tolerance (Forment et al., 2002). *los4*, an *Arabidopsis* mutant defective in a DEAD box–RNA helicase similar to the yeast RNA export factor Dbp5p, showed impaired chilling and freezing tolerance (Gong et al., 2002b). At least five genes involved in RNA metabolism have been implicated in plant responses to drought and the stress hormone abscisic acid (ABA) (Kuhn and Schroeder, 2003; Razem et al., 2006). The ABA-hypersensitive *hyl1 Arabidopsis* mutant is defective in a double-stranded RNA binding protein (Lu and Fedoroff, 2000). HYL1 appears to affect the production of some miRNAs that in turn regulate the expression levels of their target genes (Han et al., 2004). *ABH1* and *SAD1* from *Arabidopsis* and *AKIP1* from *Vicia faba* appear to be directly involved in RNA processing, which somehow affects ABA responses. *ABH1* encodes a large subunit of a dimeric mRNA cap binding complex (Hugouvieux et al., 2001), whereas *SAD1* encodes an Lsm small ribonucleoprotein (snRNP) similar to the yeast Lsm5p (Xiong et al., 2001a). AKIP1 is a single-stranded RNA binding protein homologous with hnRNP A/B (Li et al., 2002). A very recent study revealed that FCA, an RNA binding protein that controls flowering time, is an ABA receptor important for ABA regulation of flowering (Razem et al., 2006).

We have used the firefly luciferase reporter gene driven by the stress-responsive *RD29A* promoter to facilitate genetic dissection of plant responses to abiotic stresses (Chinnusamy et al., 2002; Lee et al., 2002). The reporter gene system has allowed for the identification of a number of signaling components important for the transcriptional regulation of stress-responsive genes (Lee et al., 2001; Xiong et al., 2001a, 2001b, 2001c). In addition, this reporter system has led to the isolation of a DNA glycosylase that is essential for preventing transcriptional gene silencing (Gong et al., 2002a). We noticed that the luciferase transcript used in our studies is very unstable in *Arabidopsis* (Ishitani et al., 1998). Although the instability sequence in the luciferase reporter gene is not known, this unstable reporter has permitted us to isolate several *Arabidopsis* mutants with altered regulation of mRNA stability. Here, we present the characterization of one such mutant, *stabilized1* (*sta1*), the cloning of the *STA1* gene, and the surprising finding that *STA1* is required for both pre-mRNA splicing and the degradation of some transcripts. In addition, *STA1* is upregulated by cold stress, and *sta1-1* mutant plants show altered responses to various abiotic stresses.

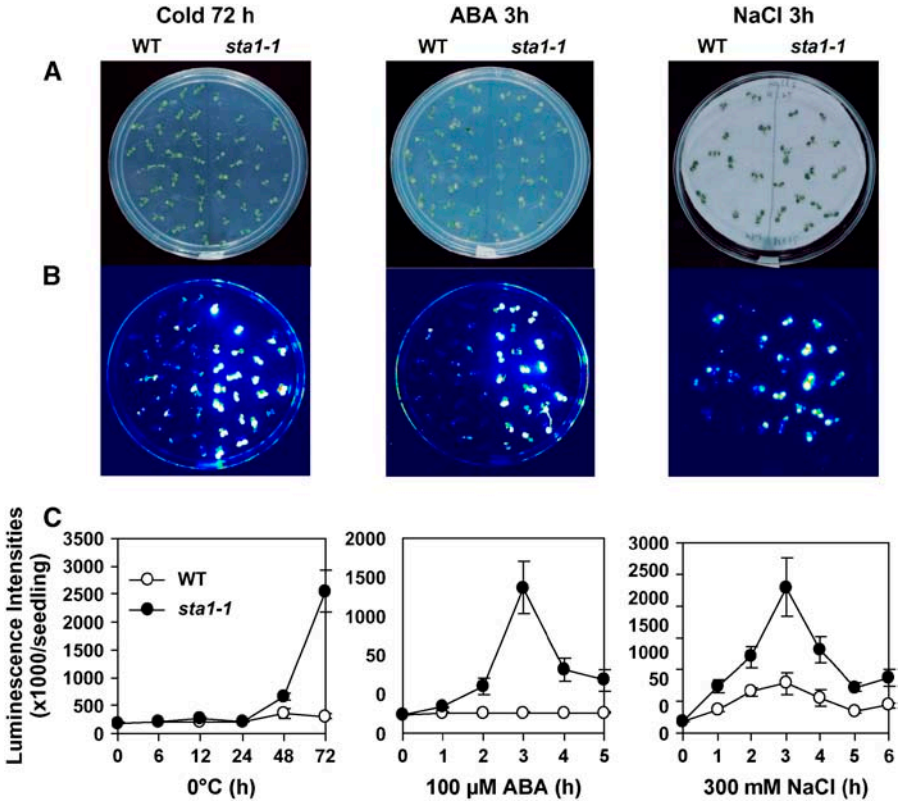

Figure 1. Comparison of Luminescence Images and Intensity between the Wild Type and *sta1-1* under Stress.

(A) Wild-type and *sta1-1* seedlings on MS agar plates.

(B) Luminescence images corresponding to plates in **(A)**.

(C) Quantification of luminescence intensities over the time periods indicated ($n = 20$ for cold stress and ABA, $n = 10$ for NaCl treatment; error bars indicate SD).

RESULTS

The *sta1-1* Mutation Enhances the Stability of the Luciferase Transcript

We previously described a mutant screening strategy that uses a low-light luminescence imaging system and transgenic *Arabidopsis* expressing the firefly luciferase reporter driven by the stress-inducible *RD29A* promoter (Chinnusamy et al., 2002; Lee et al., 2002). Using this system, we isolated many mutants that show altered luminescence responses under stress conditions. One such mutant, recovered from a population of ecotype Columbia plants expressing the *RD29A*-luciferase transgene (hereafter called the wild type) mutated with the use of ethyl methanesulfonate, showed higher luminescence than the wild type after cold, ABA, or NaCl treatment. The mutant, named *sta1-1*, was characterized in this study after several backcrosses were performed.

RD29A promoter–driven luciferase (*RD29A-LUC*) activity was tested with seedlings grown on Murashige and Skoog (MS) agar medium supplemented with 3% sucrose. Under the tested stress conditions, *sta1-1* mutant seedlings showed higher luminescence than did the wild type (Figure 1). Under cold conditions, 72 h of treatment enhanced the luminescence intensity in the *sta1-1* mutant much more than in the wild type (Figure 1C). ABA or NaCl treatment also induced higher luminescence in *sta1-1* than in the wild type, with a peak at 3 h of treatment.

The steady state levels of the luciferase transcript and the endogenous *RD29A* transcript were examined by RNA gel blot analysis in both the wild type and the *sta1-1* mutant. Because of its very unstable nature, the luciferase transcript was virtually undetectable in wild-type plants, even after cold, ABA, or NaCl treatment (Figure 2). However, a high level of luciferase mRNA was detected in *sta1-1* after 72 h of cold treatment (Figure 2). This result also revealed that the endogenous *RD29A* transcript level was not higher in the *sta1-1* mutant than in the wild type under any of the conditions tested (Figures 2A and 2B). Therefore, it is unlikely that the *sta1-1* mutation caused increased transcription from the *RD29A* promoter, because both the transgene and the endogenous *RD29A* gene have the same promoter.

To further examine whether the enhanced luciferase transcript level in *sta1-1* is attributable to increased transcription or posttranscriptional changes in stability, nuclear run-on assays were performed. We used samples collected after 72 h of cold treatment, when the dramatic difference between the wild type and *sta1-1* in luciferase transcript abundance was observed (Figures 2A and 2B). The nuclear run-on results showed that the *sta1-1* mutant did not have higher transcription rates for either the endogenous *RD29A* or the luciferase transgene than the wild type (Figure 2C). Together, these results suggest that the higher level of luciferase transcript in *sta1-1* is the result of enhanced luciferase transcript stability.

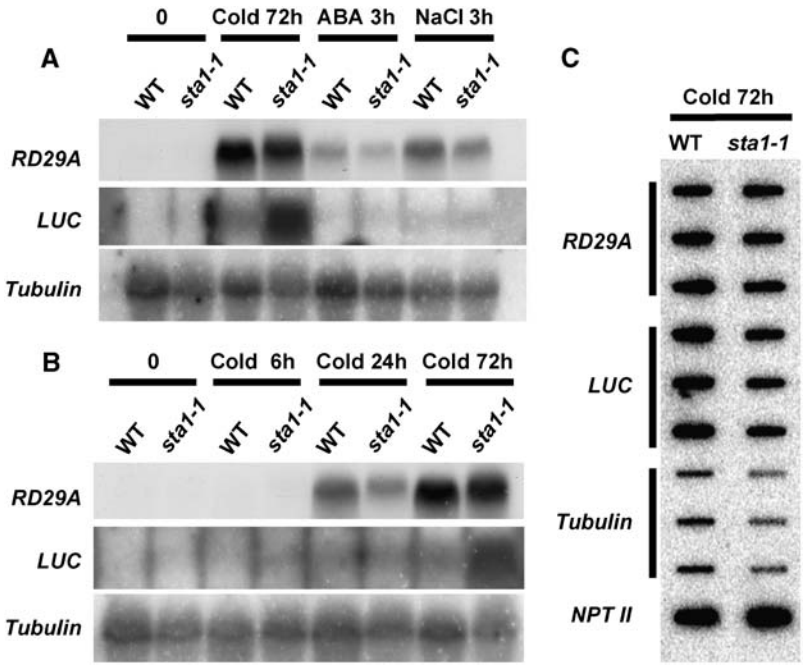

Figure 2. Comparison of Expression Levels between the Wild Type and *sta1-1* of Endogenous *RD29A* and the *RD29A-LUC* Transgene by RNA Hybridization and Nuclear Run-On Analysis.

(A) RNA hybridization with total RNA (20 μg) from samples treated with cold (72 h), ABA (3 h), or NaCl (3 h).
(B) RNA hybridization with total RNA (20 μg) from samples treated with cold (0, 6, 24, or 72 h).
(C) Nuclear run-on analysis with 72-h cold-treated samples.

Physiological and Developmental Phenotypes of the *sta1-1* Mutant

Because of the potential role of mRNA stability control in plant stress responses and development, the *sta1-1* mutant was examined for possible stress tolerance and developmental phenotypes.

ABA is a stress hormone with inhibitory functions in seed germination and root growth. For the germination test on ABA, we considered germination as the emergence of cotyledons rather than of radicles. In the absence of exogenous ABA, the wild type and *sta1-1* germinated completely on MS agar medium with 3% sucrose at 4 d after imbibition. However, when 0.1 μM ABA was added to the medium, the germination rate of *sta1-1* seeds at 4 d was reduced to virtually zero (i.e., no cotyledon emergence), whereas ~60% of the wild-type seeds could still germinate (Figure 3A). Three days later, the wild-type seeds overcame the inhibitory effect of 0.1 μM ABA to reach a germination rate of ~92%. At that time, the germination rate of *sta1-1* seeds was also improved to ~90% in the presence of 0.1 μM ABA (Figure 3B). *sta1-1* seeds, however, still showed a lower germination rate than did wild-type seeds at 0.5 and 1.0 μM ABA even at 7 d after imbibition (Figure 3B).

Low-temperature responses of the *sta1-1* mutant were tested at 4°C. Four-day-old seedlings grown under normal conditions on a vertical plate were transferred to 4°C under light. Under this cold condition, *sta1-1* was clearly damaged and all seedlings were eventually killed, whereas the wild-type seedlings were alive and growing (Figure 3C). These results showed that the *sta1-1* mutant is chilling-sensitive.

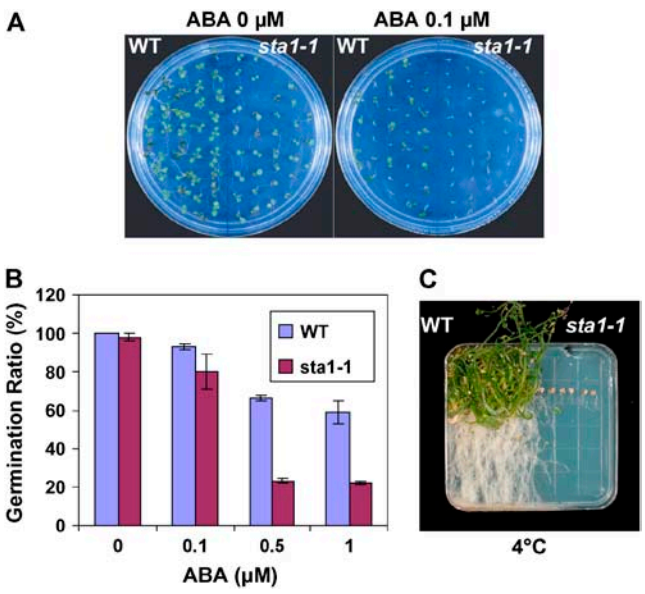

Figure 3. *sta1-1* Germination and Chilling Sensitivity.

(A) Germination test of the wild type and *sta1-1* on MS agar medium with ABA (0 or 0.1 μM). Photographs were taken at 4 d after imbibition.

(B) Germination rates of the wild type and *sta1-1* on MS medium with ABA (0, 0.1, 0.5, or 1 μM). Rates were scored at 7 d after imbibition. Data from three replicate experiments are shown. Error bars indicate SE.

(C) Chilling sensitivity test of the wild type and *sta1-1* at 4°C. Four-day-old seedlings were transferred to 4°C, and the photograph was taken ~6 months later.

Root growth is affected by various stress conditions and has often been used as an index for stress sensitivity. The root growth of the wild type and the *sta1-1* mutant was compared on MS agar medium supplemented with ABA, NaCl, mannitol, or LiCl. With ABA, *sta1-1* showed lower relative root growth than the wild type, although the difference became smaller at higher ABA concentrations (Figure 4A). ABA at low concentrations is known to have a stimulatory rather than an inhibitory effect on root growth (Davies and Zhang, 1991). In our analysis, this root growth–promoting effect of ABA was observed at 0.1 to 0.5 μM ABA (Figure 4A). Although both the wild type and *sta1-1* responded positively in root growth to 0.1 μM ABA, the positive ABA effect was much smaller in *sta1-1* than in the wild type. At 0.5 μM ABA, the root growth of the wild-type seedlings was still promoted, but *sta1-1* root growth was inhibited (Figure 4A). This result suggests that *sta1-1* mutant seedlings are hypersensitive to ABA inhibition of root growth.

The relative root growth rates of the wild type and *sta1-1* were not significantly different on NaCl-containing plates, although it appeared that wild-type root growth became more sensitive to high concentrations (120 and 150 mM) than *sta1-1* root growth (Figure 4B). Interestingly, the *sta1-1* mutant showed an apparently higher level of tolerance in relative root growth to osmotic stress caused by mannitol (Figure 4C). The osmotic stress tolerance of *sta1-1* can also be observed at the whole seedling level. The size of the wild-type seedlings on mannitol-containing plates was reduced greatly as the concentration of mannitol increased. By contrast, the size decrease in *sta1-1* seedlings by mannitol was relatively small (Figure 4E). Indeed, the appearances of the wild-type and *sta1-1* seedlings were very similar at 300 mM mannitol, whereas *sta1-1* seedlings were much smaller without mannitol.

Differences between the wild type and *sta1-1* were observed under LiCl treatment (Figures 4D and 4F). Approximately 100% of the relative root growth in the wild type was maintained with up to 10 mM LiCl, whereas *sta1-1* displayed only ~55% relative root growth at 10 mM LiCl (Figure 4D). The *sta1-1* seedlings were all killed at 20 mM LiCl, whereas the wild-type seedlings were all alive under this condition (Figure 4F).

The *sta1-1* plants were smaller in size and height than were wild-type plants (Figures 5A to 5C). *sta1-1* mutant leaves were also smaller than wild-type leaves. In addition, the mutant leaves were more serrated and had a pointed leaf tip (Figure 5D). Although *sta1-1* completed its life cycle at a similar rate as the wild type, the inflorescence of *sta1-1* started bolting earlier than that of the wild type (Figures 5E and 5F). *sta1-1* plants generally bolted at a leaf number of six, whereas the wild type started to bolt at a leaf number of approximately eight.

STA1 Encodes a Pre-mRNA Splicing Factor

None of the 31 F1 plants derived from a cross between the wild type and *sta1-1* showed a mutant phenotype in luminescence or development (see Supplemental Table 1 online). In the successive F2 generation, 23% of the progeny displayed mutant levels of luminescence (intensities of $>1 \times 10^6$ counts per seedling after

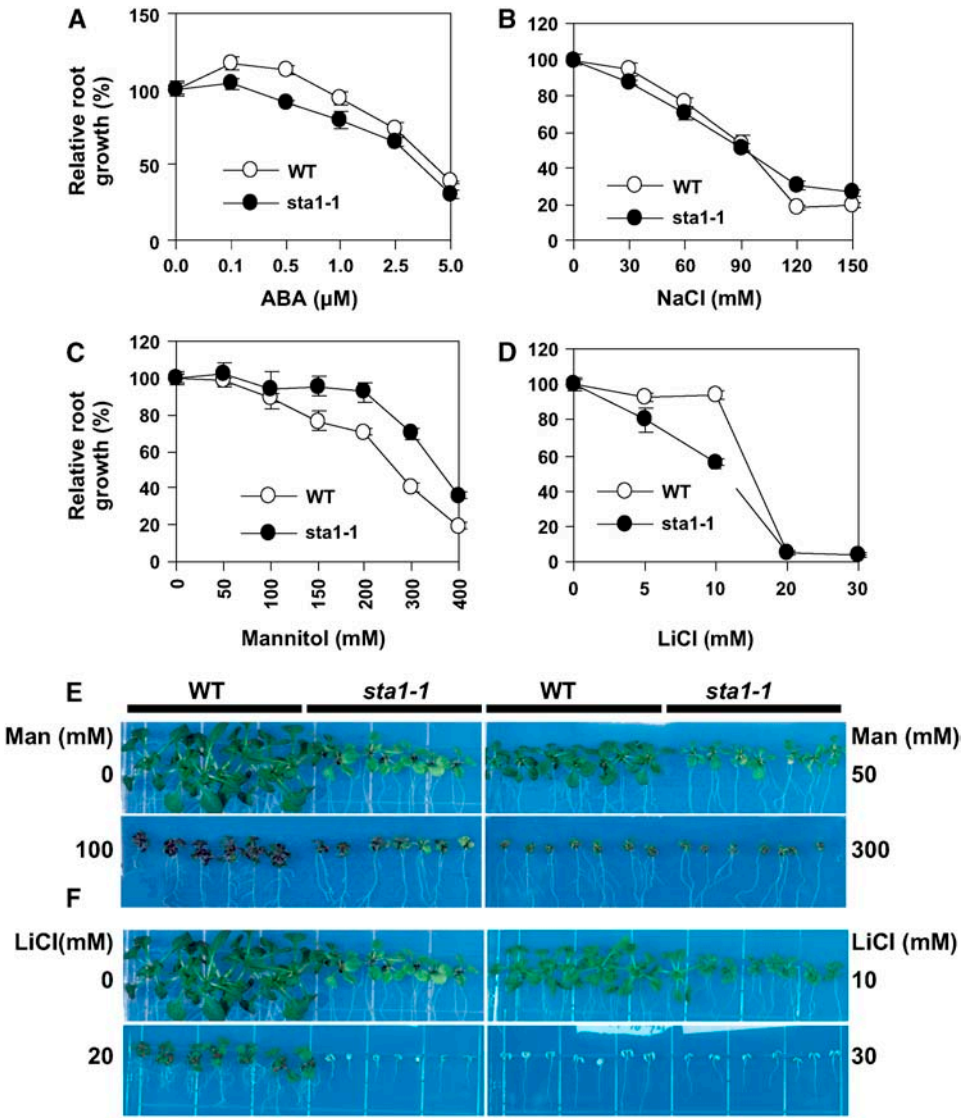

Figure 4. *sta1-1* Sensitivity to Various Salt and Osmotic Stress Conditions.

(A) to **(D)** Comparisons of the wild type and *sta1-1* in root growth on MS agar medium with ABA **(A)**, NaCl **(B)**, mannitol **(C)**, and LiCl **(D)**. Root growth was measured relative to controls. At least eight seedling roots were measured for each data point. Error bars indicate SE.

(E) and **(F)** Comparisons between the wild type and *sta1-1* in seedling growth on MS agar medium with mannitol (Man) **(E)** or LiCl **(F)**. Photographs were taken at 13 d after seedling transfer onto the treatment medium.

All experiments were performed three times except for LiCl treatment (two times) with different seeds lots, and each time nearly identical results were obtained.

72 h of cold treatment). These results suggest that a single recessive nuclear mutation is responsible for the mutant phenotypes conferred by *sta1-1*. To clone the mutation, F2 seeds from a cross between wild-type plants in the Landsberg *erecta* ecotype and *sta1-1* were used as a mapping population. Seedlings with high luminescence after 72 h of cold treatment were mapped with known simple sequence length polymorphism (SSLP) markers as well as SSLP markers that were developed in this study. The mutation was limited to a 50-kb region between positions 30 and 80 kb on BAC clone F9H3. Through sequencing of genomic DNA in this region in the *sta1-1* mutant, a mutation was found in the F9H3.5 gene (At4g03430) that had a computer annotation of ''putative pre-mRNA splicing factor.'' In the *sta1-1* mutant, 6 bp (1249 to 1254 bp from the translation initiation site) were deleted from At4g03430, which resulted in two amino acid deletions in-frame in the open reading frame (ORF) (Figure 6A).

To confirm that the correct gene was cloned, a genomic fragment containing 1513 bp upstream of the start codon, the 3090-bp ORF, and 253 bp downstream of the stop codon was introduced into the *sta1-1* mutant through *Agrobacterium tumefaciens*–mediated transformation. Transgenic plants (T1) expressing this genomic fragment did not display *sta1-1*

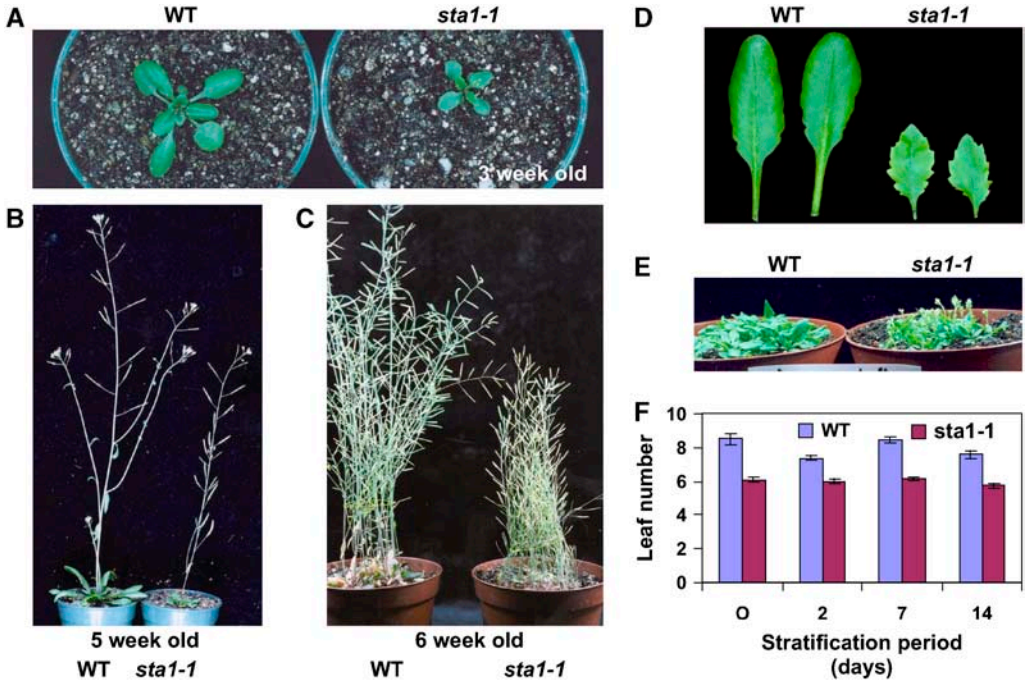

Figure 5. Developmental Phenotypes of *sta1-1*.

(A) to **(C)** Morphology of wild-type and *sta1-1* plants: 3 weeks old **(A)**, 5 weeks old **(B)**, and 6 weeks old **(C)**.
(D) Comparison of leaf morphology between the wild type and *sta1-1*.
(E) Early-bolting phenotype of *sta1-1*.
(F) Leaf number comparison between the wild type and *sta1-1* upon bolting after different stratification periods.

developmental phenotypes (Figure 6B), and the subsequent T2 seedlings showed a 3:1 segregation ratio between seedlings with normal luminescence intensity and high luminescence intensity after cold treatment (Figure 6C). These results confirm that At4g03430 is the correct gene responsible for the phenotypes conferred by *sta1-1*.

The At4g03430 gene does not contain any intron, and its ORF has been confirmed by Yamada et al. (2003). The *STA1* ORF is predicted to encode a polypeptide of 1029 amino acids with a molecular mass of ~115.6 kD. The deduced amino acid sequence of STA1 exhibits a significant degree of overall similarity with human U5 snRNP-associated 102-kD protein (accession number O94906; 53% identity and 69% similarity). STA1 protein is also similar to the fission yeast pre-mRNA splicing factor PRP1p (accession number Q12381) and the budding yeast pre-mRNA splicing factor Prp6p (accession number P19735), with identities of 42 and 31% and similarities of 61 and 48%, respectively. *STA1* exists as a single-copy gene in the *Arabidopsis* genome. We attempted to isolate a homozygous T-DNA knockout allele of *sta1-1*. The SALK line SALK_009304 contains a T-DNA insertion at 286 bp downstream from the translation initiation site, which likely represents a null allele. However, genotyping of 36 plants identified only wild-type and heterozygous alleles but not homozygous T-DNA mutants. This suggests that the homozygous T-DNA mutant is lethal.

Domain analysis predicted that the STA1 protein has 15 HAT (for half a tetratricopeptide repeat [TPR]) helix domains and 5 TPR domains as well as a PRP1 splicing factor N-terminal domain and a bipartite nuclear localization signal (Figure 7A; see Supplemental Table 2 online). The HAT domain has a sequence and structure similar to the TPR domain and is found in many RNA processing proteins (Preker and Keller, 1998). HAT domains are present in multiple repeats, and it is believed that intramolecular HAT–HAT interaction provides a protein–protein interaction surface. The *sta1-1* mutation took place in one of the HAT domains (Figure 7A). Therefore, it is likely that the protein interaction surface provided by the HAT domain is affected by the mutation in STA1. The TPR domain is a degenerate 34–amino acid repeat forming two α-helices and is often arranged in tandem. Proteins with TPR repeats are involved in many cellular events, such as cell cycle control, splicing, transcription, protein folding, and protein transport (Blatch and Lassle, 1999). TPR participates in these activities by mediating protein–protein interactions. Interestingly, the N-terminal 85 amino acid residues in STA1 are predicted to contain a ubiquitin domain by the PROSITE Database of Protein Families and Domains (http://www.expasy.org/prosite) (Figure 7A). This ubiquitin domain was also found in the STA1 homolog in rice (*Oryza sativa*), but not in humans or yeast, which suggests an evolutionary divergence of the plant protein.

To investigate the subcellular localization of STA1, we generated transgenic *Arabidopsis* expressing a green fluorescent protein (GFP)–STA1 fusion protein. The green fluorescence was detected in nuclei, which suggests a nuclear localization of the STA1 protein (Figures 7B to 7E). This observation is consistent with the presence

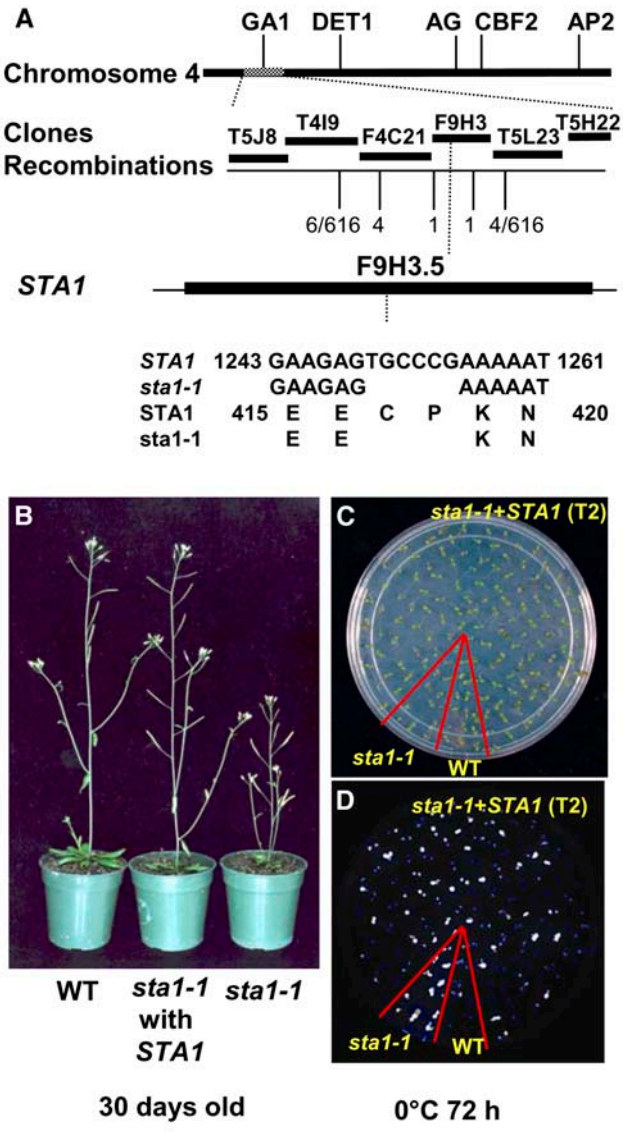

Figure 6. Molecular Cloning of *STA1* and Functional Complementation.

(A) Positional cloning of *STA1*. Numbers of recombinations are from 308 F2 progeny seedlings with the phenotype conferred by *sta1-1*. Markers used at the recombination positions were, from left, T4I9-29K, F4C21-27K, F9H3-80K, F9H3-32K, and F9H3-3K.

(B) Molecular complementation of the *sta1-1* developmental defect with the wild-type *STA1* gene.

(C) and **(D)** Molecular complementation of the *RD29A-LUC* expression defect with the wild type *STA1* gene. Shown are seedlings on an MS agar plate **(C)** and the corresponding luminescence image **(D)**.

of a nuclear localization signal in the STA1 amino acid sequence (Figure 7A; see Supplemental Table 2 online).

To determine the tissue distribution of *STA1* expression, an ~1.5-kb sequence upstream of the *STA1* initiation codon was amplified by PCR and used to drive the expression of the β-D-glucuronidase (*GUS*) reporter gene. *STA1* promoter–*GUS* transgenic *Arabidopsis* plants were assayed to detect *GUS* expression. The *GUS* reporter gene was expressed in all tissues tested, although leaf epidermal cells did not seem to have strong expression. In leaf epidermis, GUS staining was detected preferentially in guard cells and trichomes. The *STA1* promoter–*GUS* expression results suggest a largely ubiquitous expression pattern of *STA1* (Figures 7F to 7M).

STA1 Is Stress-Inducible and Required for Both Pre-mRNA Splicing and mRNA Turnover

The notion that STA1 is a pre-mRNA splicing factor and that the *sta1-1* mutant may be defective in pre-mRNA splicing is supported by experimental evidence. Results of RNA gel blot analysis revealed an additional, slightly higher band when the *COR15A* gene was used as a probe (Figures 8A and 8B). This higher band was present only in cold stress–treated *sta1-1* plants. The size of the higher band appeared to be the same as that of the unspliced *COR15A* transcript. To test this notion, we PCR-amplified the intron present in the *COR15A* ORF and labeled this fragment as a probe for RNA gel blot analysis. As expected, the intron probe detected a signal only in cold stress–treated *sta1-1* plants, and the size of the signal was the same as that of the upper band detected by the *COR15A* cDNA (Figures 8A and 8B). These results demonstrate that the *sta1-1* mutant is indeed defective in pre-mRNA splicing.

It is interesting that the *COR15A* splicing defect occurred only under cold stress conditions, even though *COR15A* was also induced by ABA or NaCl (Figure 8A). The preferential splicing defect under cold stress and the increased cold sensitivity of the *sta1-1* mutant prompted us to test whether *STA1* might be preferentially needed under cold stress and thus that its expression might be upregulated by cold. Indeed, we found that the *STA1* transcript level is upregulated by cold stress but not by ABA or NaCl (Figures 9A and 9B). Surprisingly, we found that the cold-induced *STA1* transcript level was substantially higher in *sta1-1* than in the wild type (Figures 9A and 9B). Results of nuclear run-on assays revealed no substantial difference in *STA1* transcription rates between wild-type and *sta1-1* plants (Figure 9C). Thus, the *STA1* transcript is highly unstable, because it was not detectable without cold stress by RNA gel blot analysis, even though the *STA1* promoter has strong constitutive activities (Figure 7). Therefore, the *sta1-1* mutation causes the stabilization of the normally unstable *STA1* transcript. It should be noted that our *STA1* promoter–driven *GUS* expression construct does not contain a 21-bp sequence immediately upstream of the start codon or the 3′ untranslated region of *STA1*, which might be important in posttranscriptional regulation of *STA1* expression. Thus, it is possible that *STA1* gene expression may not be constitutive or ubiquitous.

To identify other endogenous genes with enhanced transcript stability in the *sta1-1* mutant, full genome microarray analysis was performed with the use of Affymetrix 24K GeneChips. Total RNA extracted from 14-d-old seedlings of the wild type and the *sta1-1* mutant grown under normal conditions was used for the transcript profiling. After statistical analysis, we found that the transcript levels of 71 genes were significantly ($P \leq 0.05$) higher by at least twofold in *sta1-1* than in the wild type (see Supplemental Table 3 online). The *STA1* gene

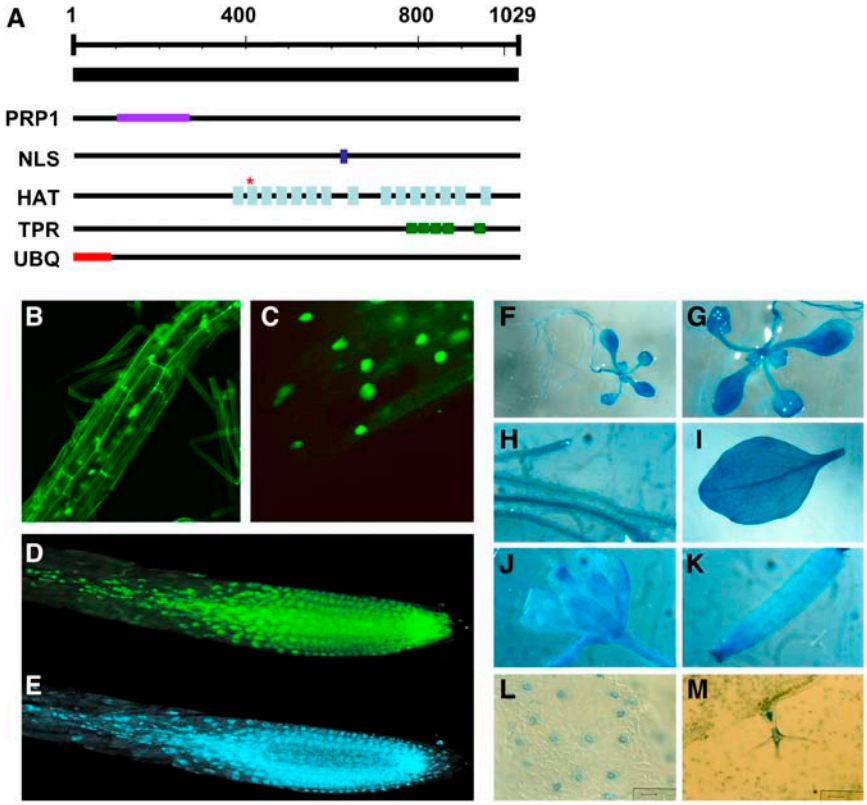

Figure 7. Characterization of *STA1*.

(A) Predicted domain in the STA1 protein. The asterisk represents the mutation site in *sta1-1*. PRP1, PRP1 splicing factor N-terminal domain; NLS, nuclear localization signal; HAT, half a TPR; TPR, tetratricopeptide repeat; UBQ, ubiquitin.
(B) to **(D)** Confocal microscopic images of an *Arabidopsis* root expressing the GFP-STA1 fusion protein.
(E) A 4′,6-diamidino-2-phenylindole–stained root corresponding to the root in **(D)**.
(F) to **(M)** Expression of *STA1* promoter–*GUS* in *Arabidopsis*. Expression in whole seedlings (**[F]** and **[G]**), root **(H)**, leaf **(I)**, flower **(J)**, silique **(K)**, guard cells **(L)**, and trichome **(M)**. For observation of guard cells and trichomes, the epidermal layer was peeled from leaves.

itself was not included in our list of 71 genes, probably because our microarray analysis was performed with seedlings not under cold treatment. However, the microarray result still indicated that the level of the *SAT1* transcript was ~1.97 times higher, with a P value of 0.016, in *sta1-1* than in the wild type grown under normal conditions. One of the 71 genes, steroid sulfotransferase (*STF*; At2g03760), which was determined to have a high transcript level in *sta1-1* by the microarray assay, was tested by RNA gel blot analysis. *STF* was found to be strongly upregulated by cold and NaCl stress and slightly upregulated by ABA (Figures 9A and 9B). Consistent with the microarray result, RNA gel blot analysis showed that the *STF* transcript level was higher in *sta1-1* than in the wild type, particularly after 72 h of cold stress (Figures 9A and 9B). It is noteworthy that under NaCl stress, the *STF* transcript level was only slightly higher in *sta1-1* than in the wild type (Figure 9A). This finding is consistent with the enhanced requirement for STA1 in facilitating transcript turnover under cold stress. To investigate whether the higher *STF* transcript level under cold stress is also attributable to transcript stabilization, we performed nuclear run-on assays, which revealed no substantial difference in the transcription rate for *STF* between the wild type and the *sta1-1* mutant (Figure 9D). Therefore, the higher level of *STF* transcript in the mutant appears to be also caused by enhanced transcript stability.

DISCUSSION

In this study, we used a genetic approach to identify a novel factor important in mRNA turnover. The recessive *sta1-1* mutation causes the stabilization of not only the firefly luciferase reporter gene transcript but also transcripts from at least two endogenous genes (*STA1* itself and *STF*). Interestingly, *STA1* encodes a pre-mRNA splicing factor. Indeed, *sta1-1* mutant plants are defective in the splicing of the *COR15A* gene. Our work thus identifies a cellular factor required for both transcript turnover and RNA splicing. Furthermore, we found that *STA1* expression is upregulated by cold stress, and the gene appears to be essential under cold stress conditions.

Pre-mRNA splicing is an indispensable process for removing introns from pre-mRNA for proper gene expression in eukaryotic cells and is performed by the spliceosome, a multicomponent

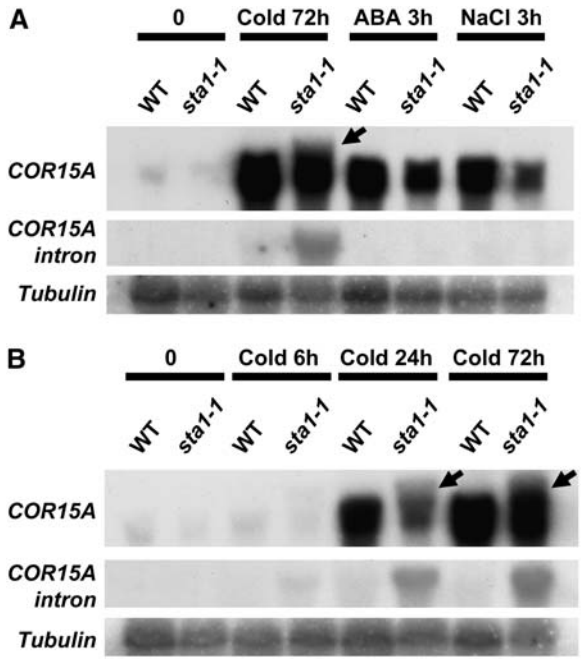

Figure 8. *COR15A* Expression in *sta1-1*.

Total RNA (20 μg) from 14-d-old seedlings after cold, ABA, or NaCl treatment **(A)** or different cold stress durations **(B)** was subjected to RNA hybridization with the probes shown. Arrows indicate *COR15A* nonspliced transcript.

complex of small nuclear RNAs and many protein factors (Jurica and Moore, 2003). Small nuclear RNAs include U1, U2, U4, U5, and U6, and each constitutes a snRNP with several protein subunits. Although the spliceosome is made up of ~300 polypeptides that include many other proteins (Rappsilber et al., 2002; Zhou et al., 2002), these snRNPs form the core. The remaining non-snRNP protein factors are known to participate in recruiting the core splicing machinery and/or connecting splicing to other processes, such as transcription, 5′ end capping, and 3′ end cleavage/polyadenylation (Proudfoot et al., 2002). During the splicing processes, the structures and components of the snRNP complexes change dynamically. For example, during the spliceosome activation process, U5 snRNP undergoes a dramatic remodeling by tightly associating with SKIP (for Ski Oncogene–Interacting Protein), the Prp19 complex, and other factors in exchange for other U5 snRNP–associated proteins, such as 15- and 100-kD proteins (Makarov et al., 2002). The newly remodeled 35S U5 snRNPs persist throughout the splicing catalytic processes until dissociation from mRNA. After the dissociation, 35S U5 snRNP is converted into 20S U5 snRNP, an abundant form of U5 snRNP.

The sequence of STA1 suggests that it is a U5 snRNP–associated protein. It has high similarities to human U5 snRNP–associated 102-kD protein (accession number O94906), fission yeast pre-mRNA splicing factor Prp1p (accession number Q12381), and budding yeast pre-mRNA splicing factor Prp6p (accession number P19735). In budding yeast, Prp6p mediates the interactions between U4/U6 snRNP and U5 snRNP. U4/U6 snRNP and U5 snRNP form a tri-snRNP before being integrated into the spliceosome. Galisson and Legrain (1993) showed that the U4/U6-U5 tri-snRNP did not accumulate in the *prp6* mutant. Instead, the U4/U6 snRNP and U5 snRNP were present separately in the mutant, which suggests a Prp6p requirement in U4/U6-U5 tri-snRNP formation. The U5 snRNP–associated 102-kD protein, human Prp6p homolog, is also thought to bridge U4/U6 snRNP and U5 snRNP through specific interaction with the U4/U6 snRNP–associated 61-kD protein (Makarov et al., 2000; Makarova et al., 2002). Thus, it is likely that the *STA1* gene product has a similar function in plant U4/U6-U5 tri-snRNP formation. However, we found that *STA1* was not capable of complementing the temperature-sensitive growth phenotype of the *prp6-1* yeast mutant Se1 (Urushiyama et al., 1997) (data not shown). Nevertheless, the in vivo splicing defect in *sta1-1*, together with the strong sequence similarity between STA1 and the human and yeast proteins, support STA1 as a bona fide splicing factor. The inability of *STA1* to complement the yeast mutant is likely attributable to sequence and possibly functional divergence of the plant protein. The N-terminal region of STA1 has a ubiquitin domain not observed in nonplant proteins. Interestingly, the Patch-Calling database, which provides data on global protein–protein interactions by use of industrialized, high-throughput yeast two-hybrid technology (Uetz et al., 2000; http://portal.curagen.com/pathcalling_portal/index.htm), contained an interaction between Prp6p and Ubc9p, a ubiquitin-conjugating enzyme in budding yeast. This suggests that ubiquitin conjugation may also be required for the proper function of STA1 homologs in nonplant systems. STA1 might have acquired the ubiquitin moiety as an intramolecular domain during evolution.

It is interesting to speculate on how the *sta1-1* mutation enhances transcript stability or how STA1 normally promotes transcript turnover. Evidence suggests that posttranscriptional processes such as RNA processing, export, translational regulation, and degradation are interconnected. In addition, RNA processing events, including splicing, can be coupled to transcription in higher eukaryotes (Proudfoot et al., 2002; Jensen et al., 2003). As RNA emerges from transcription, it is packaged into a messenger ribonucleoprotein complex. Failure to form proper messenger ribonucleoprotein may lead to retention by a nuclear surveillance system, resulting in mRNA degradation. We suggest that STA1 and possibly the entire spliceosome may be part of the nuclear surveillance system that recognizes and degrades certain transcripts before they exit the nucleus. It is not known what primary or secondary sequence features are recognized by this surveillance system. It is interesting that all three transcripts (luciferase, *STA1*, and *STF*) confirmed to be stabilized in *sta1-1* do not have introns. However, many of the 71 genes showing enhanced transcript levels in the mutant do have introns. We do not know whether the intronless genes are directly or indirectly affected by the *sta1-1* mutation.

Among the 71 genes with high expression levels in the *sta1-1* mutant are two spliceosomal proteins. They include proteins that are homologous with U5 snRNP–associated 200-kD protein (At2g42270) and U4/U6 snRNP–associated 90-kD Prp3 protein (At1g28060). Results of many studies showed these proteins to be present in the spliceosome, along with U5 snRNP–associated 102-kD protein, the human counterpart of STA1 (Anthony et al.,

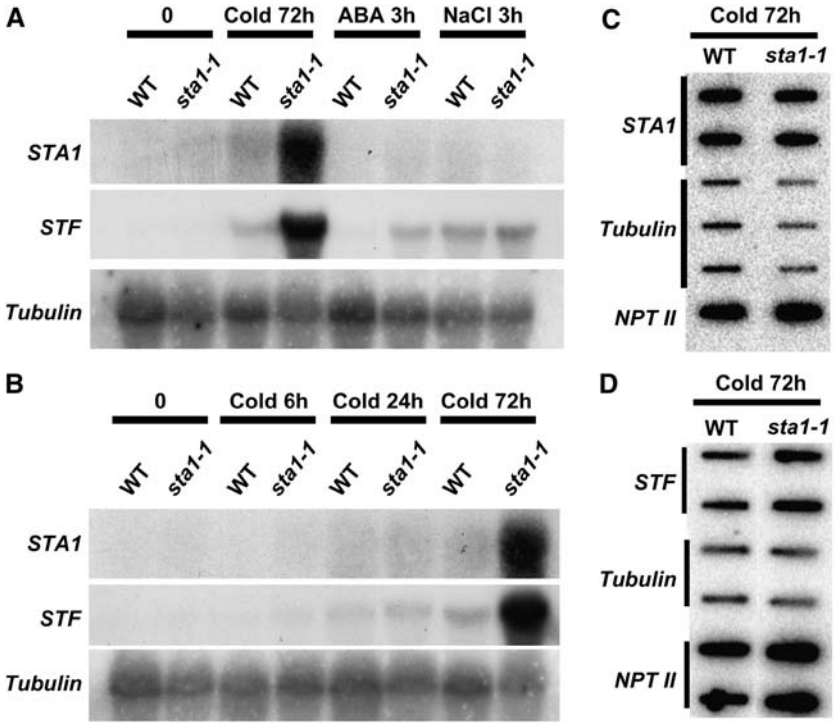

Figure 9. Expression of *STF* and *STA1* in the Wild Type and *sta1-1*.

(A) and **(B)** RNA gel blot analysis of wild-type and *sta1-1* total RNA (20 μg) with *STA1* and *STF* probes after different treatments **(A)** or different cold durations **(B)**. **(C)** and **(D)** Nuclear run-on analysis with samples after 72 h of cold treatment. *STA1* **(C)** and *STF* **(D)** were analyzed.

1997; Jurica et al., 2002; Makarov et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). Therefore, the higher expression of one U5 snRNP–associated protein gene and one U4/U6 snRNP–associated protein gene in the *sta1-1* mutant might imply a cellular mechanism to compensate for the defect in *STA1*. Complementation of defects in the splicing machinery by overexpressing other components is not surprising. The addition of a high amount of SR proteins, auxiliary splicing factors rich in Arg-Ser (RS) dipeptides, compensated for the loss of U1 snRNP function or U2AF depletion in an in vitro splicing system (Crispino et al., 1994; Tarn and Steitz, 1994; MacMillan et al., 1997). A cellular compensation system for *sta1-1* defects could possibly affect RNA processing, which in turn may have resulted in the enhanced transcript stability of certain genes in the *sta1-1* mutant. Recently, UBP1, a U-rich intron and 3′ untranslated region binding hnRNP, was cloned and characterized in tobacco (*Nicotiana tabacum*) (Lambermon et al., 2000). UBP1 overexpression led to the enhanced stability of certain transcripts. UBP1 can bind the 3′ untranslated region of mRNAs and thus may protect the mRNAs from exonucleolytic degradation (Lambermon et al., 2000). Interestingly, UBP1-enhanced transcript stability was observed only for intronless gene transcripts or less efficiently spliced intron-containing transcripts (Lambermon et al., 2000).

The function of STA1 appears to be essential in *Arabidopsis*, because *sta1* null mutants are lethal. Yeast *prp6* null mutants also appear to be lethal, as indicated in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) (Giaever et al., 2002). It is likely that the in-frame deletion of two amino acids in *sta1-1* represents a weak mutant allele. Promoter-GUS analyses suggest that *STA1* expression may be constitutive. Indeed, the *sta1-1* mutant exhibits a phenotypic defect in the absence of cold or other stress, which also suggests that *sta1-1* is not strictly a temperature-sensitive allele. However, the mutant defect is most severe under cold stress, as indicated by the malsplicing of *COR15A* in the cold and the dramatic chilling sensitivity of the mutant plants. The cold stress phenotypes of *sta1-1* plants suggest an important requirement of *STA1* under cold stress. This requirement is reflected in the cold stress upregulation of *STA1* expression. Nevertheless, it is still possible that the *sta1-1* allele may be sensitive to cold and thus that cold temperature may exacerbate the mutant defect by causing a more severe defect in the mutant protein.

sta1-1 plants are also altered in their responses to ABA and salt stress. The mutant is hypersensitive to ABA in germination and root growth, as are the phenotypes of *abh1* and *sad1*, both of which affect RNA metabolism (Hugouvieux et al., 2001; Xiong et al., 2001a). In addition, *sta1-1* plants show a serrated leaf phenotype that is also observed in the *abh1*, although *sta1-1* but not *abh1* is smaller and bolts early (Hugouvieux et al., 2002). However, the ABA hypersensitivity in *sta1-1* is not as strong as that in *abh1* or *sad1*. Unlike the *abh1* or *sad1* mutant, *sta1-1* does not have a detectable phenotype in stomatal opening or closing (data not shown), even though the *STA1* promoter–GUS is expressed in guard cells. The weak existence or absence of certain ABA phenotypes in *sta1-1* plants may be because the mutation (an in-frame deletion of two amino acid residues)

causes only a partial loss of function under these conditions. *sta1-1* plants are also hypersensitive to LiCl (Figures 4D and 4F). This phenotype is consistent with the defect of *sta1-1* in pre-mRNA splicing, as LiCl is well known for its inhibitory effect on RNA-processing enzymes (Dichtl et al., 1997). A recently identified ABA receptor, FCA, is a nuclear RNA binding protein that regulates flowering in response to ABA (Razem et al., 2006). Associated with FY, FCA autoregulates its own mRNA by promoting premature cleavage and polyadenylation (Macknight et al., 2002; Quesada et al., 2003; Simpson et al., 2003). Thus, future investigations of the potential involvement of *STA1* in FCA-mediated ABA signaling would be interesting.

METHODS

Plant Materials and Growth Conditions

RD29A-LUC–expressing *Arabidopsis thaliana* ecotype Columbia *gl1* (referred to here as the wild type) plants were mutated by ethyl methanesulfonate to generate M2 seeds. Surface-sterilized M2 seeds were plated on MS (Murashige and Skoog salt base; JRH Biosciences) agar (0.6%) plates supplemented with 3% sucrose and placed at room temperature (22 ± 1°C) under continuous light after 2 to 3 d of cold stratification. Seven-day-old seedlings were used to screen for altered LUC expression in response to low temperature, ABA, or NaCl treatment with the use of a video-imaging system consisting of a charge-coupled device camera (CCD-512SB; Princeton Instruments), a controller (Princeton Instruments), and a computer with WinView image-processing software, as described previously (Chinnusamy et al., 2002; Lee et al., 2002). When necessary, seedlings were transferred to soil pots and allowed to grow in a growth chamber with cycles of 16 h of light at 22°C and 8 h of dark at 18°C.

Physiological Characterization

Stresses were applied to 1-week-old wild-type and mutant seedlings grown on the same MS agar plate. For cold treatment, the plates were placed at 0°C in the dark for the designated times. For ABA treatment, 100 μM ABA [(±)-*cis*,*trans*-ABA; Sigma-Aldrich] dissolved in sterile water was sprayed uniformly on the leaves of the seedlings. ABA-treated plates were kept at room temperature (22 ± 1°C) under cool-white light for the designated times. For NaCl treatment, seedlings were transferred to a filter paper saturated with 300 mM NaCl in MS solution. The seedlings were then incubated under light at room temperature for the designated times.

For germination tests, surface-sterilized seeds were placed on MS agar (0.6%) plates supplemented with ABA at the designated concentrations. The plates were cold-treated for 2 d at 4°C to promote uniform germination. Seven days later, germination was scored. Cotyledon appearance was considered to be germination.

For growth analysis, 4-d-old seedlings grown vertically on MS agar (1.2%) square plates were transferred onto MS agar (1.2%) supplemented with various salts. Root length was measured 4 d later, and seedling growth phenotypes were examined 13 d later. For chilling tests, 4-d-old seedlings grown at room temperature on MS agar (1.2%) square plates were transferred to 4°C under continuous light conditions. Phenotypes were then monitored.

Gene Expression Analysis

Nine-day-old seedlings grown on MS agar plates were used for RNA gel blot analysis. After stress treatments as described in the text, total RNA was extracted and analyzed as described previously (Liu and Zhu, 1997).

For nuclear run-on analysis, nuclei were isolated from 2-week-old seedlings treated with cold (0°C) for 72 h. The nuclei isolation and in vitro transcription reactions were performed as described (Dorweiler et al., 2000). Comparable amounts of labeled RNA for treated and untreated samples were used for filter hybridization. Slot blots on nitrocellulose filter membranes were prepared with 100 ng of denatured purified gene fragments or an equivalent amount of denatured linearized plasmid per slot. For comparison, two to three slots were used for each probe. Prehybridization and hybridization were performed as described by Dorweiler et al. (2000). After hybridization, the strips were washed for 15 min with 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 42°C and then with 2× SSC and 0.1% SDS for 15 min at room temperature. The strips were visualized with the use of a STORM 860 PhosphorImager (Molecular Dynamics), and signals were quantified with ImageQuant software (Molecular Dynamics). Background was subtracted from each signal before normalizing the probe to the signals for control.

Probes used for both RNA hybridization analysis and nuclear run-on analysis were as follows: the *RD29A* gene–specific probe from the 3′ noncoding region (Liu and Zhu, 1997); the *COR15A* cDNA (Gilmour et al., 1992; Lin and Thomashow, 1992), kindly provided by M.F. Thomashow; and the *COR15A* intron fragment amplified from *Arabidopsis* genomic DNA by use of the primer pair COR15AI-F (5′-AAGGATCTTAGCAGGCAATGTT-3′) and COR15AI-R (5′-CAAAGGTTTCAAAACACATATCCA-3′). Full-length coding regions of both luciferase and *STA1* were used to detect each transcript signal. STF (At2g03760), NPT II, and tubulin probe fragments were amplified from plasmid construct, cDNA clone, or genomic DNA by PCR with the following primer pairs: STF-F (5′-TGAAGCTAAAGATTCCGACATTATC-3′) and STF-R (5′-AGTATCTCTCCATCCTCCAATCTCT-3′); NPT II-F (5′-ATGACTGGGCACAACAGACA-3′) and NPT II-R (5′-AATATCACGGGTAGCCAACG-3′); and Tubulin-F (5′-CGTGGATCACAGCAATACAGAGCC-3′) and Tubulin-R (5′-CCTCCTGCACTTCCACTTCGTCTTC-3′).

For Affymetrix GeneChip array analysis, 20 μg of total RNA from wild-type and *sta1-1* seedlings grown for 14 d at 22°C with a cycle of 16 h of light and 8 h of darkness was extracted by use of the RNeasy plant mini kit (Qiagen) and used to make biotin-labeled complementary RNA targets. Affymetrix *Arabidopsis* ATH1 genome array GeneChips, which contain >22,500 probe sets representing ~24,000 genes, were used, and hybridization, washing, and staining were performed at the Genetic Analysis and Technology Core Facility at the University of Arizona. The microarray assay included data sets from five biological replicates of wild-type plants and two biological replicates of the *sta1-1* mutant, and these seven data sets were used for statistical analysis to determine genes with higher transcript levels in *sta1-1*. Expression measures from Affymetrix cell intensity files were background-corrected, normalized, and summarized using the robust multiarray average algorithm from the affy package of Bioconductor (Irizarry et al., 2003; Gentleman et al., 2004). Differentially expressed genes were identified by statistical analysis implemented in the LIMMA package of Bioconductor (Gentleman et al., 2004; Smyth, 2004). Through this analysis, the P values were obtained from the distribution of the moderated *t* statistic, the ratio of the $\log_2$(fold change between the wild type and *sta1-1*) to its SE, and were corrected for multiple testing according to Benjamini and Hochberg (1995). Genes with at least a twofold higher transcript level than the wild type and a false discovery rate–adjusted P value of <0.05 were considered to be genes with significantly higher transcript levels in *sta1-1*.

Positional Cloning

For genetic mapping of the *sta1-1* mutation, *sta1-1* in the Columbia ecotype was crossed with the wild type in ecotype Landsberg *erecta*. The resulting F1 plants were allowed to self, and the F2 seeds were collected. Homozygous *sta1-1* mutants in the segregating F2 population were selected on the basis of their high luminescence under stress conditions.

Mapping of the mutation was performed with SSLP markers (Bell and Ecker, 1994). For fine mapping, new SSLP markers were developed with the use of the Cereon *Arabidopsis* polymorphism collection at http://www.Arabidopsis.org/Cereon/index.html. Primers for SSLP markers were as follows: T4I9-29K (5′-TTGATCGATCGTCTCGTATTTC-3′ and 5′-TTGGCCATTACTTTGGATCA-3′), F4C21-27K (5′-GCTCGTGACGTGGCTATCTT-3′ and 5′-TGGGGGTCAAAACTCAAAAC-3′), F9H3-80K (5′-GATCGGAAAACCAGAAACGA-3′ and 5′-TTTCCGGCAAAATTGTAACAG-3′), F9H3-32K (5′-CCGTTACACATAATAAAGGGTTTTC-3′ and 5′-CGTTACTAATGGATTTAGAGTGAGTGA-3′), and F9H3-3K (5′-GTAGGTCCCCAGCCTTGATT-3′ and 5′-TTGAAAACTGCTGACGGAGA-3′).

Plasmid Construction and Plant Transformation

The F9H3 BAC clone was obtained from the ABRC and used as a PCR template. For *sta1-1* complementation, the 4954-bp genomic DNA fragment of *STA1* covering from 1513 bp upstream of the start codon to 253 bp downstream of the stop codon was amplified with LA Taq polymerase (Takara Shuzo), with F9H3 BAC DNA used as a template with the following primers: F9H3.5gKpnI-F (5′-TGTTGGTACCCTTATTGTAGCAATACTTGTTCTTA-3′) and F9H3.5gSalI-R (5′-TACAGTCGACAAAAGAAGTTTAATAGCTGAACA-3′). The resulting fragment was cloned into pCAMBIA1200 between the *Kpn*I and *Sal*I sites, generating pCAM1200-HC15.

For the *STA1* promoter–driven GUS construct, a 1493-bp fragment spanning from −1513 to ~−21 bp upstream of the *STA1* ORF was amplified by PCR, with F9H3 BAC DNA used as a template and the primer pair F9H3.5pSalI-F (5′-GTTGGTCGACTTATTGTAGCAATACTTGTTCTTA-3′) and F9H3.5pNcoI-R (5′-CCGGTCCATGGAACCAAACTATAAAAATCTCT-3′). The *STA1* promoter fragment was then cloned into pCAMBIA1381 between the *Sal*I and *Nco*I sites, resulting in pCAM1381-HC15-GUS.

For the construct for the GFP-STA1 fusion protein, the *STA1* ORF was amplified by PCR with F9H3 BAC DNA used as a template and the primer pair F9H3.5gSalI-F (5′-GATTAGGTCGACATGGTGTTTCTCTCGATTCCAAAC-3′) and F9H3.5gXmaI-R (5′-ATTGATCCCGGGCAGCAGAATTCTCTTCCTTGCTCAA-3′). The amplified *STA1* ORF was subcloned into pEZTNL between the *Xho*I and *Xma*I sites, resulting in pEZTNL-HC15-GFP.

pCAM1200-HC15 and pCAM1381-HC15-GUS were transferred to *Agrobacterium tumefaciens* GV3101 (pMP90), and pEZTNL-HC15-GFP was transferred to *A. tumefaciens* LBA4404, by electroporation at 1250 V with capacitance of 25 μF and resistance of 400 Ω. After appropriate antibiotic selection and PCR confirmation, selected agrobacteria were grown at 28°C in Luria-Bertani broth (1% [w/v] bacto-tryptone, 0.5% [w/v] bacto-yeast extract, and 1% [w/v] NaCl, pH 7.0) overnight and then used for in planta floral infiltration.

GUS Staining

Hygromycin-resistant, *STA1* promoter–*GUS* transgenic *Arabidopsis* seedlings and plant parts (T1 generation) were stained in GUS assay buffer (3 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.1 M Na-phosphate, pH 7, 0.1% Triton X-100, and 8 mM β-mercaptoethanol) for 12 h at 37°C, followed by incubation in 70% ethanol to remove chlorophyll.

Microscopy

Glufosinate-ammonium–resistant *GFP-STA1* transgenic seedlings selected in soil by spraying 30 mg/L Finale (AgrEvo Environmental Health) were mounted on glass slides, and green fluorescence images were taken with use of a Bio-Rad MRC1024 confocal laser-scanning microscope with a 488-nm excitation laser and a 522/DF35 emission filter.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library under accession numbers At2g03760 (*STF*), At2G42540 (*COR15A*), At4g03430 (*STA1*), and At5G52310 (*RD29A*) and in the GenBank/EMBL data libraries under accession numbers O94906 (human U5 snRNP–associated 102-kD protein), Q12381 (fission yeast pre-mRNA splicing factor Prp1p), and P19735 (budding yeast pre-mRNA splicing factor Prp6p). Microarray data are available from the Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE4662.

Supplemental Data

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

Supplemental Table 1. Genetic Analysis of the *sta1-1* Mutant.

Supplemental Table 2. Predicted Domains in the STA1 Protein.

Supplemental Table 3. List of Genes with Expression Levels Significantly Higher by at Least Twofold in *sta1-1* Than in the Wild Type.

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