Posttranscriptional Control of Photosynthetic mRNA Decay under Stress Conditions Requires 3’ and 5’ Untranslated Regions and Correlates with Differential Polysome Association in Rice

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Abiotic stress, including drought, salinity, and temperature extremes, regulates gene expression at the transcriptional and posttranscriptional levels. Expression profiling of total messenger RNAs (mRNAs) from rice (Oryza sativa) leaves grown under stress conditions revealed that the transcript levels of photosynthetic genes are reduced more rapidly than others, a phenomenon referred to as stress-induced mRNA decay (SMD). By comparing RNA polymerase II engagement with the steady-state mRNA level, we show here that SMD is a posttranscriptional event. The SMD of photosynthetic genes was further verified by measuring the half-lives of the small subunit of Rubisco (RbcS1) and Chlorophyll a/b-Binding Protein1 (Cab1) mRNAs during stress conditions in the presence of the transcription inhibitor cordycepin. To discern any correlation between SMD and the process of translation, changes in total and polysome-associated mRNA levels under stress were measured. Total and polysome-associated mRNA levels of two photosynthetic (RbcS1 and Cab1) and two stress-inducible (Dehydration Stress-Inducible Protein1 and Salt-Induced Protein) genes were found to be markedly similar. This demonstrated the importance of polysome association for transcript stability under stress conditions. Microarray experiments performed on total and polysomal mRNAs indicate that approximately half of all mRNAs that undergo SMD remain polysome associated during stress treatments. To delineate the functional determinant(s) of mRNAs responsible for SMD, the RbcS1 and Cab1 transcripts were dissected into several components. The expressions of different combinations of the mRNA components were analyzed under stress conditions, revealing that both 3’ and 5’ untranslated regions are necessary for SMD. Our results, therefore, suggest that the posttranscriptional control of photosynthetic mRNA decay under stress conditions requires both 3’ and 5’ untranslated regions and correlates with differential polysome association.

In response to certain environmental stimuli, such as dehydration, high salinity, and low temperature, genes undergo various changes in expression as part of the stress tolerance response of plants. Plants, at once sessile and developmentally indeterminate, are unique in that each gene expression change must account for both long-range genetically determined programs and short-term environmental responses. As such, plants use a number of regulatory mechanisms to achieve appropriate gene expression, including the control of RNA stability (Bailey-Serres et al., 2009; Belostotsky and Sieburth, 2009).

The RNA regulatory elements that control transcript stability can reside anywhere along its sequence. Stability elements have been reported to occur within the 5’ untranslated region (UTR) of several transcripts of nucleus-encoded chloroplast proteins. The pea (Pisum sativum) and Arabidopsis (Arabidopsis thaliana) Ferredoxin-1 (Fed-1) mRNAs, which stabilize upon the excitation of phytochrome, are the best characterized examples of mRNAs with 5’ UTR stability elements (Chiba and Green, 2009). Phytochrome-mediated stabilization of Fed-1 mRNA requires active translation, the 5’ UTR, and active photosynthetic electron transport (Chiba and Green, 2009). Light-mediated increases in transcript stability have also been reported for small subunit of Rubisco (RbcS)
transcripts in petunia (Petunia hybrida; Sathish et al., 2007) and the light-harvesting chlorophyll-binding (Lhc) transcripts in pea (Warpeha et al., 2007).

Unlike stabilization elements, many of the recognized destabilization sequences reside within the 3′ UTR, such as multiple overlapping AUUUA sequences or other AU-rich elements, which have been reported in mammals. Several proto-oncogene, cytokine, and transcription factor mRNAs involved in growth and differentiation are subjected to rapid decay via AU-rich elements (Vasudevan and Peltz, 2001; Bevilacqua et al., 2003; Reznik and Lykke-Andersen, 2010). The best characterized instability sequence in plants is the so-called downstream (DST) element, which has a complex structure and recognition requirements that are unique to plants (Sullivan and Green, 1996; Feldbrügge et al., 2002). The DST instability sequence was originally found within the 3′ UTR of the small auxin up RNA (SAUr) genes and has also been shown to function in tobacco (Nicotiana tabacum; Feldbrügge et al., 2002; Streathfield, 2007). Additional investigations of DST elements in Arabidopsis have revealed that unstable transcripts encoding proteins associated with circadian control possess DST elements in their 3′ UTRs (Gutierrez et al., 2002; Lidder et al., 2005; Streathfield, 2007).

Genome-wide expression profiling is an excellent experimental tool for the analysis of mRNA decay. Using complementary DNA (cDNA) microarray analysis, approximately 325 Arabidopsis transcripts have been found to be unstable, with half-lives of less than 2 h. This rapid transcript decay has been found to be associated with a group of touch- and circadian clock-controlled genes (Gutierrez et al., 2002). When the half-lives of mRNAs from Arabidopsis suspension culture cells were assessed using expression microarrays, the measurements varied from minutes to more than 24 h and revealed two mechanisms that appear to affect differential decay (Narsai et al., 2007). Conserved sequence elements were identified within both the 5′ and 3′ UTRs that correlated with stable and unstable mRNAs, with genes containing introns giving rise to more stable mRNAs than intronless genes.

Accumulating evidence indicates that mRNA decay mechanisms are often coupled to translation in plants (Chiba and Green, 2009) and other organisms (Balagopal and Parker, 2009). With respect to general determinants, the presence of a 5′-m′Gppp cap and a 3′-poly(A) tail, which contribute to mRNA stability, is even more important to ensure efficient translation (Gallie, 1996). Hence, mechanisms that remove these elements would be expected to decrease gene expression at the levels of both mRNA stability and translation. Another observation that links these functions is the stabilizing effect that the protein synthesis inhibitor cycloheximide has on a number of unstable mRNAs (Chiba and Green, 2009). This stabilization could occur because translation of a labile transacting factor, or translation of the mRNA itself, is required for rapid degradation. The role of polyribosomes (polysomes) as sites of mRNA decay is supported by reports that the decay intermediates of SR54 (small subunit of ribulose-1,5-bisphosphate carboxylase in soybean [Glycine max]) and PHYA (phytochrome A in oat [Avena sativa]) mRNAs are polysome associated (Thompson et al., 1992; Higgs and Colbert, 1994) and that most in vitro decay systems are polysome based (Tanzler and Meagher, 1994; Ross, 1995). In another model system, several mutations of the pea Fed-1 gene that block translational initiation or elongation also abolish the light response of Fed-1, consistent with the model of translation being a requirement for the stabilization of Fed-1 in a light environment (Chiba and Green, 2009).

In addition to the genes that are up-regulated under stress conditions, we have here identified a comparable number of down-regulated genes in rice (Oryza sativa) plants. The latter group includes most of the photosynthetic genes involved in light and dark reactions. The mRNAs of photosynthetic genes degrade rapidly upon exposure to stress conditions, a phenomenon referred to as stress-induced mRNA decay (SMD). Experiments incorporating polysome fractionation followed by microarray analysis revealed that photosynthetic mRNAs remain polysome associated during SMD. Dissection of the photosynthetic mRNAs Rubisco Small Subunit1 (RbcS1) and Chlorophyll a/b-Binding Protein1 (Cab1) in transgenic plants has identified the 3′ UTR as the site of the regulatory mRNA elements that mediate SMD.

RESULTS

SMD of Photosynthetic Genes during Stress Conditions

We have previously identified stress-regulated genes in rice plants through expression profiling using the Rice 3′-Tiling microarray (GreenGene Biotech) and RNAs from the 14-d-old leaves of rice seedlings subjected to drought, high salt, and low temperature (Oh et al., 2009; Jeong et al., 2010). In addition to up-regulated genes, we also identified many genes that were down-regulated under stress conditions, including most of the photosynthetic genes involved in light and dark reactions. For example, mRNA levels of the light reaction genes Cab1, Plastocyanin (PCy), Cab26, OEE1, PSI-D and PSI-K and the dark reaction genes RbcS1, Rubisco Activase (RA), SEDP2ase, GAPDH, TK, and FBPase-P are rapidly reduced in response to both drought and salt stress conditions. In contrast, the mRNA levels of these genes are not reduced by cold stress. These findings were further confirmed by RNA gel-blot and real-time (RT)-PCR analyses (Fig. 1A; Supplemental Fig. S1). Thus, photosynthetic gene mRNAs appear to decay in response to different stressors (i.e. to undergo SMD).

To investigate whether SMD occurs at the transcriptional or posttranscriptional level, we measured RNA polymerase II (Pol II) engagement, a proxy for active transcription, and steady-state mRNA levels for three different types of representative genes. These genes included the down-regulated transcripts RbcS1 and Cab1, up-regulated Dehydration Stress-Inducible Protein1 (Dip1) and Salt-Induced Protein (SatT), and also OsCc1 and Ubil, which remain unperturbed under stress conditions. The transcription and steady-state
Figure 1. Changes in steady-state mRNA and transcription activity levels under stress conditions. Total RNA was isolated from the leaf tissue of 2-week-old wild-type seedlings that were subjected to drought, high salinity, and low temperature stress for 0 to 6 h. RNA gel-blot hybridizations were then performed using the probes described in “Materials and Methods.” Dip1 (AY587109) and SalT (AK062520; Claes et al., 1990) served as stress marker genes. A, Total cellular RNA gel-blot analysis. Cab1 (AK060851) and PCY (AK070447) are involved in the light reaction of photosynthesis. RbcS1 (AK121444) and RA (AK119513) genes are involved in the dark reaction. Ethidium bromide (EtBr)-stained rRNAs served as a loading control. B, Transcription (RNA Pol II engagement) and steady-state mRNA levels were measured in leaf tissues exposed to drought and salt stress for the indicated times. Transcription of RbcS1, Cab1, Dip1, SalT, OsCc1 (Jang et al., 2002), and Ubi1 (AK121590; Kim et al., 1994) were measured using a Pol II-ChIP assay (Supplemental Fig. S2). Steady-state mRNA levels were measured by qRT-PCR analysis using cDNA synthesized using total RNAs from stress-treated leaves. Values are means ± SD of three independent q-PCR experiments and are presented relative to the results from unstressed controls with values set at 1. C, Quantification of the decrease in mRNA abundance and transcript half-life estimation. The transcript (RbcS1, Cab1, Dip1, and SalT) levels in nontransgenic plants over a time course after exposure to drought or salt stress in the presence or absence of cordycepin were measured. Those levels under normal growth conditions in the presence or absence of cordycepin were also measured. The signal for EIF-4A (AB046414) did not change significantly during the time course and was used as an internal control to normalize the mRNA levels. The half-life values were calculated as shown in Table I. Steady-state mRNA levels were measured by qRT-PCR analysis as described in B.
mRNA levels of the six genes were analyzed using the RNA Pol II-chromatin immunoprecipitation (ChIP) assay and the quantitative (q)RT-PCR method, respectively (Fig. 1B). Rice leaves treated with 2 and 6 h of drought or salt stress, and untreated control leaves, were used for the Pol II-ChIP and qRT-PCR experiments. The steady-state RbcS1 and Cab1 mRNA levels dropped by 12- to 15-fold, whereas their transcription remained unaltered. In contrast, the steady-state mRNA levels of Dip1 and SalT, both stress-inducible genes, were induced by 80- to 100-fold, whereas the transcription levels in each case were only modestly increased. Neither the steady-state mRNA levels nor the transcription of OsCc1 and Ubi1 was significantly altered under stress conditions, further validating their constitutive expression in seedling leaves.

To confirm the posttranscriptional controls of the down-regulated (RbcS1 and Cab1) and up-regulated (Dip1 and SalT) transcripts during drought and salt stress, their half-lives were measured in the presence of the transcription inhibitor cordycepin (Fig. 1C). Cordycepin treatments were effective in rice in blocking transcription, as evidenced by the reduced half-lives of EXPL2 (AK068088) and SEN1 (AK120910), rice homologs of Arabidopsis genes that produce very unstable mRNAs (Gutierrez et al., 2002; Lidder et al., 2005; Xu and Chua, 2009), under normal growth conditions (Supplemental Fig. S5). The half-lives of the RbcS1 and Cab1 transcripts were 123 and 239 min under normal growth conditions, respectively, whereas they decreased to 44 to 53 min under drought and salt stress conditions (Fig. 1C; Table I). Drought and salt stress caused a stabilization of the Dip1 and SalT transcripts at the posttranscriptional level (Fig. 1C). Similar posttranscriptional stabilization has been observed previously in salt stress-regulated genes such as PEPCase (Cushman et al., 1990), AtP5S (Hua et al., 2001), and SOS1 (Shi et al., 2003; Chung et al., 2008) and in abscisic acid- and water stress-regulated genes such as α-amylase/subtilisin inhibitor (BASI; Liu and Hill, 1995), le25, and his1-s (Cohen et al., 1999). Taken together, our results suggest that the SMD of RbcS1 and Cab1 as well as the control of the stress-inducible Dip1 and SalT genes are posttranscriptional events.

The mRNAs of Photosynthetic Genes Remain Polysome Associated during Stress Conditions

To investigate a possible correlation between SMD and translation, the levels of polysome-associated mRNAs were assessed in 14-d-old leaves after exposure to drought, salt, or cold stress conditions (Supplemental Fig. S3). To obtain polysomes, crude leaf tissue was homogenized in the presence of cycloheximide to attenuate translation elongation. The extracts were then centrifuged through Suc gradients, an absorbance (254 nm) profile was obtained, polysomal fractions (includes two or more ribosomes; fractions 8-13) were collected, and polysome-associated mRNA was extracted (Fig. 2A). Approximately 70% of the total polyadenylated mRNAs (referred to as “total mRNA”) was polysome associated under untreated conditions, but 17% to 19% of the mRNA associated with polysome (referred to as “polysomal mRNA”) became dissociated after exposure to drought, salt, or cold stress conditions (Fig. 2B). To test whether the stress-induced decline in translation affected the photosynthetic mRNA levels, RbcS1 and Cab1 transcripts, together with stress-inducible Dip1 and SalT mRNAs in polysomes, were quantified by qRT-PCR (Fig. 2C). The total mRNA levels were generally consistent with the results for polysomal mRNAs after stress treatment, suggesting that the mRNA polysomal association is important for maintaining stability (Fig. 2).

To more broadly evaluate the effects of stress on the mRNA association with polysomes (translation state), we conducted a genome-level analysis of total and polysomal mRNAs using the Rice 3’-Tiling microarray (GreenGene Biotech) that contains probes for 29,389 genes (Fig. 3). A total of 8,129 mRNAs were identified to be significantly up- or down-regulated by stress treatments (2-fold or greater; P < 0.05). The overall patterns of SMD were similar under drought and salt stress but distinct under cold stress (Fig. 3A). More specifically, 42%, 38%, and 9% of mRNAs were both subjected to SMD and polysome associated under drought, salt, and cold stress, respectively (Table II). In contrast, the levels of 8%, 15%, and 15% of mRNAs (Polysomal mRNA in Table II) dropped by more than 2-fold only in polysome fractions, with no change in their total mRNA levels upon treatment with drought, salt, and cold stress, respectively. This indicated the escape of these transcripts from the polysome under stress treatments but no loss of stability. Overall, we found from our analyses that approximately 50% of all of the mRNAs that undergo SMD remain polysome associated (Table II). More importantly, the photosynthetic genes responsible for light and dark reactions were found to be regulated via SMD in a polysome-associated manner under drought and salt but not

### Table 1. Half-lives of RbcS1 and Cab1 mRNAs under normal and stress conditions

| Gene  | Half-Life |  |  | Half-Life, Decay and Transcription Combined |
|-------|-----------|----------------|----------------|-------------------------------------------|
|       | Normal + Cordycepin | Drought + Cordycepin | Salt + Cordycepin | Drought | Salt |
| RbcS1 | 123       | 44             | 51              | 50     | 55   |
| Cab1  | 239       | 50             | 53              | 73     | 77   |
under cold stress conditions (Table III; Supplemental Fig. S6).

The 3' UTR of a Transcript Is a Major Determinant of SMD during Stress Conditions

It has been reported previously that specific cis-acting mRNA elements in mammalian cells, typically found in their 5' and 3' UTRs, are involved in the induction of mRNA instability in response to extracellular stimuli (Shim and Karin, 2002). To define the photosynthetic mRNA regulatory elements that act in SMD, we dissected RbcS1 and Cab1 genes into three components: the upstream promoter region encompassing the 5’ UTR, the transit peptide (Tp), and the 3’ UTR (Fig. 4). For each gene, four different constructs were created containing various combinations of these three components and the GFP (gfp) coding region (Fig. 4A). Two respective Tps were linked to their own promoter, as shown by the small schemes above the graphs in Figure 4, B and C, and in Supplemental Figure S4, A and B. Transgenic rice plants expressing these constructs were generated via the Agrobacterium tumefaciens-mediated method, and the T3 homozygous plants were analyzed.

We next evaluated gfp transcription and the steady-state mRNA levels in transgenic plants treated with 2 and 6 h of drought or salt stress using the Pol II-ChIP and qRT-PCR methods, respectively (Fig. 4; Supplemental Fig. S4). Over the time course of stress treatments, the steady-state gfp mRNA levels varied depending upon the transgenic construct, whereas transcription from the RbcS1 and Cab1 promoters did not change (Fig. 4, B and C). The steady-state gfp mRNA levels in the P-RbcS1/Tp/
gfp/3’ RbcS1 (RTG/R) and P-Cab1/Tp/gfp/3’ Cab1 (CTG/C) transgenic plants dropped by 18- to 20-fold, respectively, in response to drought stress. In contrast, the steady-state gfp mRNA levels in P-RbcS1/Tp/gfp/3’ PinII (RTG/P) and P-Cab1/Tp/gfp/3’ PinII (CTG/P) transgenic plants in which the 3’ UTR of RbcS1 and the 3’ Cab1 region was replaced with the 3’ UTR of PinII, a potato (Solanum tuberosum) proteinase inhibitor II gene, were only marginally reduced upon exposure to drought. This indicated the importance of the 3’ UTR for SMD. Removal of the transit peptide sequences RbcS1-Tp from RTG/R and RTG/P and Cab1-Tp from CTG/C and CTG/P did not impact on the relative steady-state levels of gfp mRNA over the drought time course. These patterns of gfp transcription and steady-state mRNA accumulation in the transgenic plants were very similar under salt stress conditions (Supplemental Fig. S4). Changes in the steady-state mRNA levels of gfp and endogenous RbcS1 and Cab1 during the stress treatments were verified by RNA gel-blot analysis (Fig. 4, B and C; Supplemental Fig. S4). The Dip1 and/or SalT genes were used as stress-inducible controls for drought and/or salt stress treatments, respectively. Interestingly, replacement of the RbcS1 and Cab1 promoters and respective 5’ UTRs with those of the constitutively expressed OsCc1 gene yielded transgenes that no longer responded to drought or salt stress conditions (Fig. 4, A and D; Supplemental Fig. S4), suggesting a coupling between the 3’ UTRs and 5’ UTRs.

Table II. Changes in total and polysomal mRNA populations after drought, salt, and cold stress

| Change                  | Drought | Salt | Cold | Drought and Salt |
|-------------------------|---------|------|------|------------------|
|                         | Total mRNA | Overlap | Polysomal mRNA | Total mRNA | Overlap | Polysomal mRNA | Total mRNA | Overlap | Polysomal mRNA | Total mRNA | Overlap | Polysomal mRNA |
| Significant increase    | 10 (132)  | 73 (2,929) | 17 (5,38)      | 10 (319)  | 55 (1,853) | 15 (1,173)  | 15 (223)  | 11 (156) | 74 (1,065) | 6 (128)  | 75 (1,606) | 19 (399)   |
| Significant decrease    | 50 (1,301) | 42 (1,085) | 8 (195)       | 47 (1,380) | 38 (1,114) | 15 (451)   | 76 (230) | 9 (26)  | 15 (45)   | 42 (678) | 52 (841)  | 6 (111)    |

Percentage and number (in parentheses) of mRNAs were scored from microarray data sets in triplicate determinations to identify significantly increased or decreased transcripts in total or polysomal mRNA populations (2-fold or greater; P < 0.05). Overlap indicates mRNAs with a significant change in both total and polysomal mRNA populations. P values were analyzed using one-way ANOVA.
Table III. Microarray data for photosynthetic genes among both total and polysomal mRNAs under drought, salt, and cold stress conditions

Groups L1 to L7 and D1 to D5 represent genes involved in the light and dark reactions of photosynthesis, respectively (Supplemental Fig S6): L1 (PSII), L2 (plastoquinone), L3 (cytochrome b₆f), L4 (plastocyanin), L5 (ferredoxin, ferredoxin-NADP⁺ reductase), L6 (PSII), L7 (ATP synthase), D1 (carboxylation of the Calvin cycle), D2 (reduction of the Calvin cycle), D3 (regeneration of the Calvin cycle), D4 (carbon output), and D5 (photorespiratory cycle).

| Group/Gene                        | Accession No. | Light reaction | Drought | Salt | Cold | Polyosomal mRNA |
|-----------------------------------|---------------|----------------|---------|------|------|-----------------|
|                                   |               |                | Mean    | P    | Mean | Mean            | Mean | P    |
|                                   |               | Drought        | Salt    | Cold |      |                 |      |      |
|                                   |               | Mean | P | Mean | P | Mean | P |
|                                   |               | Mean | P | Mean | P | Mean | P |
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|                                   |               | Mean | P | Mean | P | Mean | P |
|                                   |               | Mean | P | Mean | P | Mean | P |
Table III. (Continued from previous page.)

| Group/Gene Accession No. | Total mRNA | Polysomal mRNA |
|-------------------------|------------|----------------|
|                         | Drought P  | Salt P | Cold P | Drought P | Salt P | Cold P |
| Pyruvate kinase isoyme G Os10g0571200 -1.2 | -2.3 | 0.00 | -2.0 | 0.00 | -1.1 | 0.82 |
| D3 Triose phosphate isomerase Os09g0535000 -1.5 | -2.8 | 0.00 | -2.1 | 0.00 | -1.4 | 0.16 |
| Fru-1,6-bisphosphatase Os06g0608700 -1.9 | -3.7 | 0.00 | -3.2 | 0.00 | 1.0 | 0.94 |
| Fru-1,6-bisphosphatase Os06g0654200 -1.3 | -2.5 | 0.00 | -2.3 | 0.00 | -1.3 | 0.29 |
| Pyrophosphate-Fru-6-P 1-phosphotransferase Os06g0247500 -1.1 | -2.2 | 0.00 | -2.0 | 0.00 | -1.1 | 0.49 |
| Fru-6-P 2-kinase/ Fru-bisphosphatase Os03g0294200 -1.0 | -2.0 | 0.00 | -2.0 | 0.00 | 1.2 | 0.48 |
| Transketolase Os06g0133800 -1.0 | -2.0 | 0.00 | -2.2 | 0.00 | -1.2 | 0.40 |
| Rib-5-P isomerase Os03g0781400 -1.7 | -3.2 | 0.00 | -2.1 | 0.00 | 1.1 | 0.43 |
| Rib-5-P isomerase Os02g0158300 -1.3 | -2.5 | 0.00 | -2.1 | 0.00 | 1.1 | 0.91 |
| Phospho 2-dehydro 3-deoxyxyanote aldolase 2 Os10g0564400 -1.1 | -2.2 | 0.00 | -2.0 | 0.00 | -1.3 | 0.28 |
| NAD-dependent epimerase/ dehydratase Os12g0420200 -1.4 | -2.6 | 0.00 | -2.8 | 0.00 | -1.4 | 0.07 |
| HpcH/Hpal aldolase family protein Os09g0529900 -1.4 | -2.6 | 0.00 | -2.3 | 0.00 | 1.0 | 0.92 |
| D4 Triose phosphate/phosphate translocator Os08g0344600 -1.4 | -2.6 | 0.00 | -2.6 | 0.00 | -1.4 | 0.13 |
| Phosphoglucomutase Os10g0189100 -1.0 | -2.0 | 0.00 | -2.1 | 0.00 | -1.3 | 0.11 |
| Fructokinase Os08g0113100 -1.1 | -2.1 | 0.00 | -2.3 | 0.00 | -1.1 | 0.55 |
| Phosphofructokinase Os06g00151900 -1.6 | -3.0 | 0.00 | -3.7 | 0.00 | 1.0 | 0.99 |
| β-Fructofuranosidase Os01g0966700 -1.3 | -2.5 | 0.00 | -2.8 | 0.00 | -1.4 | 0.10 |
| Plasticid α-1,4-glucan phosphorylase 2 Os03g0758100 -1.2 | -2.3 | 0.00 | -2.0 | 0.00 | -1.4 | 0.19 |
| Granule-bound starch synthase 1B Os07g0412100 -1.4 | -2.6 | 0.00 | -2.6 | 0.00 | -1.1 | 0.78 |
| Starch synthase isoform zSTSII-2 Os02g0744700 -1.9 | -3.7 | 0.00 | -3.5 | 0.00 | 1.1 | 0.71 |
| D5 Gln synthetase Os06g0699700 -1.7 | -3.2 | 0.00 | -2.8 | 0.00 | 1.0 | 0.9 |
| Gln synthetase Os05g0430800 -1.1 | -2.1 | 0.00 | -2.0 | 0.00 | -1.3 | 0.2 |
| Ferredoxin-dependent Glu synthase Os07g0676200 -1.4 | -2.6 | 0.00 | -2.3 | 0.00 | -1.1 | 0.30 |
| Ferredoxin-dependent Glu synthase Os07g0658400 -1.3 | -2.5 | 0.00 | -2.3 | 0.00 | -1.3 | 0.38 |

aAccession numbers for full-length cDNA sequences of the corresponding genes. bNumbers represent the mean fold values of three independent biological replicates. cP values were analyzed by one-way ANOVA.

5’ UTRs/promoter. The half-lives of gfp transcripts measured in the RTG/R, CTG/C, CcG/R, and CcG/C transgenic plants after exposure to drought stress conditions were in agreement with the results from qRT-PCR shown in Figure 4, suggesting again the importance of the 3’ UTR for SMD (Supplemental Fig. S7). In conclusion, the segmentation and evaluation of two photo-synthetic genes in this study has revealed that both 3’ and 5’ UTRs are the major mediators of SMD.

**DISCUSSION**

The regulation of mRNA stability is an important process in the control of gene expression. In plant cells, as in mammalian cells, the range of mRNA half-lives spans several orders of magnitude. Unstable mRNAs might have half-lives of less than 60 min, whereas those of stable mRNAs are in the order of days, with the average being several hours (Pérez-Ortín et al., 2007; Chiba and Green, 2009). Unstable mRNAs have attracted attention because they have regulatory functions that are important for growth and development. For example, mRNAs of the transcription factors c-myc and c-fos in mammalian cells (Guhainiyogi and Brewer, 2001) and mating-type genes in yeast (Patel and Jacobson, 1992) are known to be highly unstable. In plants, transcripts for the photo-labile phytochrome in oat (Seeley et al., 1992) and those of several auxin-inducible genes in pea (Koshiba et al., 1995) are included in this category.
Global expression profiling has been used to monitor mRNA stability under different conditions. For example, Gutierrez et al. (2002) previously examined changes in mRNA degradation in Arabidopsis using cDNA arrays in response to different environmental conditions and/or developmental stages. These investigators identified a total of 325 Arabidopsis transcripts with estimated half-lives of 2 h or less. Similar experiments performed with Arabidopsis suspension cell cultures exposed to diverse abiotic stress events further showed that mRNA half-lives can vary widely (Narsai et al., 2007). In this study, we analyzed global mRNAs that become unstable under drought, high salt, or cold stress conditions using a 135K 3'-Tiling microarray that includes all 29,389 rice genes. The results of this analysis indicate that within 2 h of drought and salt stress conditions, the levels of 2,386 and 2,494 mRNAs, respectively, dropped by more than 2-fold (Fig. 3B; Table II). An RNA Pol II-ChIP (Fig. 1B) assay was employed to measure the transcriptional activity of selected rice genes. Because in yeast to human Pol II is known to be often preloaded onto the promoter prior to activation (Yearling et al., 2011), we measured the levels of RNA Pol II binding to the coding region of six representative genes that undergo stress-induced changes in transcription. To evaluate the specific effects of different stress treatments on mRNA decay, we calibrated the general decrease in transcription under stress conditions by normalizing the ChIP-q-PCR signals.

**Figure 4.** Changes in transcription and steady-state mRNA levels of gfp in transgenic rice plants under drought stress conditions. A, Gene construct diagrams illustrating the various combinations of promoter, transit peptide, and 3’ UTR sequences from RbcS1 and Cab1 and the heterologous constitutive promoter of OsCc1. RbcS1 and Cab1, which are present as single copies in the rice genome (top panel), were dissected into three regions: promoter (P RbcS and P Cab), transit peptide (Tp), and 3’ UTR sequence (3’S’Rbc and 3’S’Cab). The respective DNA fragments were amplified by genomic PCR and fused to the gfp coding sequence to generate the expression vectors shown. The 3’ PinII terminator sequence (An et al., 1989) was used as a negative control. B to D, Transcription and steady-state mRNA levels were measured using leaf tissues from transgenic rice plants transformed with the constructs shown in A after exposure to drought stress for the indicated times (top panels). Small schemes showing the configuration of the GFP constructs are shown above the graphs. Transcription of the gfp transgene was measured by Pol II-ChIP assays. Steady-state levels of gfp mRNA were measured by q-PCR analysis with cDNA synthesized from total RNAs of stress-treated transgenic leaves. Values are means ± SD of three independent q-PCR runs and are presented relative to results from unstressed controls with values set at 1. All mRNA levels were normalized to an internal control gene, Ubi1 (AK121590). Steady-state mRNA levels were also measured by RNA gel-blot analyses using gene-specific probes for gfp, Cab1, and Dip1 (bottom panels). For RbcS1, the probe used contained the transit peptide sequence. Thus, the slower migrating transcripts are from the RTG/P and RTG/R transgenes (Tp:gfp; indicated with arrowheads in B), whereas the faster migrating transcripts are from the endogenous RbcS1 gene (Tp:RbcS1). Total RNA was isolated from transgenic leaf tissues after exposure to drought stress for the indicated times. Nontransgenic (NT) control plants are indicated. Ethidium bromide (EtBr) staining of rRNAs served as a loading control. B, RbcS1 constructs under drought stress conditions. C, Cab1 constructs under drought stress conditions. D, OsCc1 constructs under drought stress conditions.
to the signals of the input DNA controls. As a result, the transcriptional activity of the six representative genes including \( \text{RbcS1}, \text{Cab1}, \text{Dip1}, \) and \( \text{SalT} \) remained relatively unaltered by stress treatments. Thus, our results suggest the presence of SMD, an active mechanism that drives mRNA turnover upon exposure to stress.

SMD was particularly evident for genes involved in photosynthesis, as the mRNA levels for light and dark reaction genes were reduced more rapidly than for other genes under stress conditions (Table III; Supplemental Fig. S6). The mRNA turnover during the SMD of photosynthetic genes was verified by measuring the half-lives of \( \text{RbcS1} \) and \( \text{Cab1} \) during drought and salt stress conditions in the presence of the transcription inhibitor cordycepin (Fig. 1C). These half-lives dropped sharply from 123 and 239 min under normal growth conditions to 44 to 53 min, respectively, under drought and salt stress conditions (Fig. 1C; Table I), suggesting the importance of SMD in the stress-responsive regulation of photosynthesis. Photosynthesis is among the primary processes that are down-regulated under drought or salinity stress (Chaves, 1991; Munns et al., 2006; Chaves et al., 2009). Down-regulation has been attributed to consequences of decreased CO₂ availability caused by diffusion limitations through the stomata and mesophyll (Flexas et al., 2004, 2007), alterations in photosynthetic metabolism (Lawlor and Cowan, 2002), or they can arise as secondary consequences of oxidative stress (Ort, 2001). When the supply of CO₂ to Rubisco is impaired, the photosynthetic apparatus is predisposed to increased energy dissipation and down-regulates photosynthesis (Chaves et al., 2009). Additionally, increased levels of soluble sugars (Suc, Glc, and Fru) after moderate drought and salt stress contribute to the down-regulation of photosynthesis (Chaves and Oliveira, 2004). These changes in turn interact with hormones as part of the sugar signaling network (Rolland et al., 2006). Photosynthetic gene transcripts have been reported to decrease when the cellular sugar content is high (Stitt et al., 2007). The reaction centers of PSI and PSII in thylakoids are the major sites of reactive oxygen species (ROS) generation during photosynthesis. The photoproduction and subsequent scavenging of ROS not only protects chloroplasts from their direct effects but also relaxes the stress induced by excess photons (electrons; Asada, 2006). On the other hand, the continuation of photosynthesis during stress conditions may result in an elevation of ROS to levels sufficient to cause cell death. Retrograde (chloroplast-to-nucleus) signals, including ROS and carbohydrates from chloroplasts, regulate the expression of nuclear genes encoding photosynthetic proteins in accordance with the metabolic and developmental state of the organelle (Gray et al., 2003; Kleine et al., 2009). Thus, under stress conditions, nucleus-encoded photosynthetic mRNAs may need to turn over to enable plants to effectively cope with limited CO₂ availability, unbalanced levels of soluble sugars, and increased chloroplast concentrations of ROS.

Universally, mRNA translation involves three distinct steps: initiation, elongation, and termination (Bailey-Serres, 1998). The polysomal level of an mRNA molecule reflects the efficiency of initiation and reinitiation as well as the rates of elongation and termination. The results of this study revealed that the polysome content is reduced by 17% to 19% depending upon the exposure levels to drought, high salinity, or cold stress (Fig. 2B). Similar global declines in protein synthesis were observed previously by monitoring the polysome content in response to water deficiency in soybean (Bensen et al., 1988; Mason et al., 1988), oat (Dhindasa and Cleland, 1975), maize (Zea mays; Hsiao, 1970), and tobacco (Kawaguchi et al., 2003) plants and in the seedlings of barley (Hordeum vulgare), pea, pumpkin (Cucurbita maxima), sunflower (Helianthus annuus), and safflower (Carthamus tinctorius; Rhodes and Matsuda, 1976). A partial reduction in polysome content in response to stress appears to be common among plants (Kawaguchi et al., 2004; Kawaguchi and Bailey-Serres, 2005). The analysis of polysomal mRNAs in tobacco has revealed that a subset of cellular mRNAs escape translational repression under drought conditions (Kawaguchi et al., 2003). In another previous study, two mRNA species encoding a putative lipid transfer protein and osmotin remained associated with large polysomes under drought stress conditions, whereas polysome-associated mRNAs encoding \( \text{RbcS} \) and eukaryotic initiation factor 4A decreased (Kawaguchi et al., 2003). Likewise, we found in this analysis that the mRNA levels of \( \text{Dip1} \) and \( \text{SalT} \), both stress-inducible genes, remained associated with polysomes even under stress conditions, whereas the polysome association of \( \text{RbcS1} \) and \( \text{Cab1} \) mRNAs decreased. More specifically, 42%, 38%, and 9% of mRNAs that are subjected to SMD under drought, salt, and cold stress conditions, respectively, appear to remain associated with polysomes (Table II). In contrast, 50%, 47%, and 76% of mRNAs that were found to be repressed in the total mRNA pool, but not in the polysomal pool, could either remain associated with polysomes or be degraded in a place other than polysomes. The former was revealed by our analysis of the spot intensities on microarray data sets on total and polysomal mRNA pools. Spot intensities of many of those genes, in fact, remained comparably high in both stress-treated and untreated polysomal mRNA pools, 21 genes of which are shown in Supplemental Table S2 as a representative example. Transcripts that are not associated with polysomes under stress conditions, however, could be stored in either P-bodies (Parker and Sheth, 2007) or stress granules (Anderson and Kedersha, 2008) such as translationally repressed messenger ribonucleoproteins found in eukaryotes, and subsequently degraded.

As shown previously in clusters of orthologous groups (Tatusov et al., 1997, 2003), our analysis here (Supplemental Fig. S8) revealed that transcripts in the functional categories T (signal transduction mechanisms) and O (posttranslational modification, protein turnover, chaperones) are controlled by both polysome-associated and unassociated SMD. In contrast, C (energy production and conversion) and G (carbohydrate transport and
metabolism) category mRNAs are controlled mainly by polysome-independent SMD. Most of the mRNAs (8%, 15%, and 15% of mRNAs denoted as Polysomal mRNA in Table II) for which the polysomal but not the total mRNA levels were repressed upon treatment with drought, salt, and cold stress, respectively, were found to be in categories J (translation, ribosomal structure, and biogenesis), Q (secondary metabolite biosynthesis, transport, and metabolism), and T (Supplemental Fig. S8).

Translational regulation is largely determined by the characteristics of the 5′ and 3′ UTRs, including the m′Gppp cap and the poly(A) tail, the context of the AUG start codon. To prove orthcom open reading frames, specific nucleotide content, as well as primary structure and structural elements (Wilkie et al., 2003; Kawaguchi and Bailey-Serres, 2005; Hughes, 2006; Sonenberg and Hinnebusch, 2009). SAUR transcripts, which are among the most unstable plant mRNAs, with half-lives of between 10 and 50 min, are induced within minutes of the application of auxin. The instability of SAUR mRNAs has been attributed mainly to the presence of a preserved DST element in their 3′ UTRs (Gil et al., 1994; Gil and Green, 1996). Consistent with data obtained for the SAURs, our analysis of two photosynthetic genes led to the observation that the 3′ UTR of RbcS1 and Cab1 mRNA harbors one of the important determinants of instability of these transcripts under stress conditions. In addition, the 3′ UTRs of RbcS1 and Cab1 mRNA must be coupled to their respective promoters and 5′ UTRs in order to manifest SMD. Replacement of the RbcS1 and Cab1 promoters with the 5′ UTRs that with constitutively expressed OsCc1 abolished the SMD of the 3′ UTRs of RbcS1 and Cab1 mRNA (Fig. 4). Thus, our findings here demonstrate that in rice, the mRNAs of photosynthetic genes are destabilized upon exposure to stress conditions in a polysome-associated manner. This stress-induced mRNA decay appears to be mediated by determinants within the 3′ UTR that are augmented by the presence of the cognate promoter and 5′ UTR. These findings indicate that the posttranscriptional regulation of photosynthetic genes is a significant component of abiotic stress responses in rice and that the control of mRNA stability is an alternative strategy for improving stress tolerance in plants.

**MATERIALS AND METHODS**

**Plant Materials and Treatments**

Transgenic and nontransgenic rice (Oryza sativa ‘Nakdong’) plants were grown as follows. Sterilized seeds were germinated in Murashige and Skoog solid medium in a growth chamber in the dark at 28°C for 3 d and then in the light at 28°C for 1 d, transplanted into soil, and grown in a greenhouse (16-h light/8-h-dark cycle) at 28°C to 30°C. Each plant was grown in a pot [4.5 \times 4 \times 5 cm; six plants per pot] filled with rice nursery soil (Bio-Media) for 14 d after germination. Stress treatments were performed as described previously (Oh et al., 2009; Jeong et al., 2010; Redillas et al., 2011). Briefly, for drought stress, 14-d seedlings were air-dried in the greenhouse under continuous light of approximately 900 to 1,000 μmol m⁻² s⁻¹. For salt stress treatments, 14-d seedlings were transferred to a 400 mM NaCl solution in the greenhouse under identical light conditions. For cold stress treatments, 14-d seedlings were exposed to 4°C in a cold chamber under continuous light of 150 μmol m⁻² s⁻¹. Before stress treatments were applied, plants had been grown in the greenhouse under continuous light of approximately 900 to 1,000 μmol m⁻² s⁻¹ in a pot for the drought stress group and in tap water for 3 d for the salt stress group for environmental adaptation. Nontreated control seedlings were grown in parallel in the greenhouse and a growth chamber under identical light conditions and harvested at time zero. After each experimental procedure, leaf tissue was rapidly harvested using liquid nitrogen and stored at −80°C until use.

**Plasmid Construction and Rice Transformation**

RbcS1 and Cab1 were dissected into three cis-acting elements: promoter (P RbcS and P Cab), transit peptide sequence (Tp), and 3′ UTRs (3′ RbcS and 3′ Cab). Respective DNA fragments were then isolated using genomic PCR with the primer pairs listed in Supplemental Table S1 and then fused with gfp (Chiu et al., 1996) to generate expression constructs. Plasmids were introduced into Agrobacterium tumefaciens LBA4404 by triparental mating, and embryogenic (cv Nakdong) calli from mature seeds were transformed as described previously (Jang et al., 1999). Three T3 homozygous lines were initially analyzed for each GFP construct, and one representative line was chosen for more detailed analysis.

**RNA Gel-Blot Analysis**

Total RNA was extracted from the leaves of transgenic and nontransgenic rice plants using Tri Reagent (Molecular Research Center). Ten micrograms of total RNA was electrophoresed on a 1.2% (w/v) agarose gel containing iodoacetamide and blotted onto a Hybond N+ nylon membrane (Amersham). Prepared membranes were hybridized with the gene-specific probes for RbcS1, Cab1, RA, PCY, Dip1, Salt, and gfp genes. Probe DNAs were labeled with [α-32P]dCTP using a random primer labeling kit (Takara) in accordance with the manufacturer’s instructions. After hybridization, the membranes were washed in sequence with 2× SSC (0.3 M NaCl, 50 mM sodium citrate, pH 7.0) with 0.1% (w/v) SDS solution, 1× SSC with 0.1% (w/v) SDS solution, and then 0.5× SSC with 0.1% (w/v) SDS solution at 65°C for 15 min each. Membranes were then exposed to film on an intensifying plate and analyzed using a phosphoimage analyzer (FLA 3000; Fuji).

**RNA Pol II-ChIP**

Pol II-ChIP is a method for estimating transcriptional activity in vivo (Supplemental Fig. S2; Bowler et al., 2004; Sandovol et al., 2004; Tsuji et al., 2006; Chung et al., 2009). The antibody used in the ChIP experiments herein was anti-Pol II CTD (sc-900; Santa Cruz Biotechnology). ChIP assays were performed according to Chung et al. (2009). Rice plants (14 d old) were harvested and then immediately immersed in cross-linking buffer (0.4 M Suc, 10 mM Tris-HCl, pH 8.0, 5 mM mercaptoethanol, and 1% formaldehyde) under a vacuum for 15 min. Cross-linking was stopped by the addition of Gly to a final concentration of 125 mM under a vacuum. After washing the plants in cold water, the leaves were removed, frozen in liquid nitrogen, finely ground in buffer 1 (0.4 M Suc, 10 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol), filtered through two layers of Miracloth (Calbiochem, EMD, Merck; http://splash.emdbiosciences.com), and then centrifuged at 2,000g for 20 min. The resulting pellet was dissolved in buffer 2 (0.25 M Suc, 10 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol), and the RNA was precipitated with 2 volumes of 95% ethanol. After ethanol precipitation, RNA was dissolved in water, and the RNA concentration was determined by spectrophotometry. The RNA was then precipitated with 2 volumes of 95% ethanol and washed with 2 volumes of 75% ethanol.

**RESULTS**

**Posttranscriptional Control of Photosynthetic mRNA Decay**

Posttranscriptional regulation of photosynthetic gene expression is largely determined by the characteristics of the UTRs (Fig. 4). Thus, our findings here demonstrate that in rice, the mRNAs of photosynthetic genes are destabilized upon exposure to stress conditions in a polysome-associated manner. This stress-induced mRNA decay appears to be mediated by determinants within the 3′ UTR that are augmented by the presence of the cognate promoter and 5′ UTR. These findings indicate that the posttranscriptional regulation of photosynthetic genes is a significant component of abiotic stress responses in rice and that the control of mRNA stability is an alternative strategy for improving stress tolerance in plants.
typically at a 1:50 dilution. Immunoprecipitates were collected after incubation with a 1/50th volume of salmon sperm DNA/protein A-agarose at 4°C for 2 h. The protein A-agarose beads bearing immunoprecipitates were then subjected to sequential washes with low-salt wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0), high-salt wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0), LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the immunoprecipitates were eluted twice with 250 μl of elution buffer (1% SDS and 0.1 mM NaF) at 65°C. To reverse the cross-linking of the chromatin fractions, the eluted solutions were mixed with NaCl to a final concentration of 0.3 M and incubated at 65°C for 6 h. Finally, RNA and protein were removed by treatment with RNase A at 65°C for 1 h and with proteinase K at 45°C for 1 h, respectively. Immunoprecipitated DNA samples were purified using a phenol/chloroform extraction and ethanol precipitation. Purified DNA was used as a template for real-time PCR using gene-specific primers (Supplemental Table S1). The primer positions are as follows: RvCl1, 128 bp from +372 to +499; Cafl, 142 bp from +674 to +815; DiiP, 124 bp from +299 to +382; Safl, 121 bp from +264 to +384; OsCcl, 115 bp from +195 to +309; and Uhl1, 187 bp from +1,149 to +1,267. Input DNA controls were diluted 1:100 and quantified by RT-PCR. The values obtained were used to normalize the levels of DNA after immunoprecipitation. The signal intensities shown in Figures 1B and 4 and Supplemental Figure S4 are presented as ChIP-PCR signals normalized using input DNA controls.

Measurements of mRNA Half-Lives

mRNA half-lives were determined as described previously by Lidder et al. (2005). Briefly, 2-week-old rice seedlings were transferred to tap water and grown for 2 d. Cordycepin (3′-deoxyadenosine) was added to a final concentration of 1 μM. Tissue samples were then harvested twice a day over a time course and frozen in liquid nitrogen. Total RNA was isolated using TRI Reagent (Molecular Research Center) and analyzed by q-RT-PCR. Half-lives were performed to RT-PCR was performed in triplicate for each cDNA sample. The microarray data sets used in this study can be found at the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE32065.

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure S1. Changes in the steady-state mRNA levels of photosynthesis-related genes under drought, salt stress, and cold treatment conditions. Supplemental Figure S2. RNA Pol II-ChIP assay. Supplemental Figure S3. Outline of the evaluation of total and polysomal mRNA abundance under nontreated, drought, salt, and cold conditions. Supplemental Figure S4. Changes in the gfp transcription and steady-state mRNA levels in transgenic rice plants in response to salt stress. Supplemental Figure S5. Cordycepin is effective in blocking transcription in rice plants. Supplemental Figure S6. Groups (L1–L7 and D1–D5 from Table III) of photosynthetic genes involved in light and dark reactions. Supplemental Figure S7. Quantification of the decrease in mRNA abundance and half-life estimations in transgenic plants. Supplemental Figure S8. Numbers of total and polysomal mRNAs with altered expression under drought, salt, and cold stress conditions. Supplemental Table S1. Primers used in this study for qRT-PCR, qPCR, and semiquantitative-RT-PCR analyses and for plasmid construction. Supplemental Table S2. Microarray data for 21 genes that were repressed in total mRNA pools but not in polysomal mRNA pools under drought, salt, and cold stress conditions.
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