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Development and Environmental Stress Employ Different Mechanisms in the Expression of a Plant Gene Family

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Ribulose bisphosphate carboxylase small subunit (*RbcS*) genes in the common ice plant, as in all higher plants, constitute a multigene family. We have measured transcription activity and steady state mRNA levels of individual members of the family, six *RbcS* genes, in the ice plant with emphasis on the transition from C3 photosynthesis to Crassulacean acid metabolism (CAM), which this plant undergoes during development and under environmental stress. Four *RbcS* genes are differentially expressed in leaves but are regulated in a coordinate fashion. A developmentally engrained, sharp decline in the steady state mRNA levels, which is observed during the juvenile-to-adult growth phase transition, coincides with the time interval when the C3-to-CAM switch occurs. Developmental down regulation of *RbcS* is due to down regulation of transcription. In contrast, NaCl stress specifically affected *RbcS* transcript accumulation post-transcriptionally, resulting in decreased *RbcS* mRNA levels. Antagonistic regulatory programs are apparent in stress/stress relief experiments. The results indicate complex controls, affecting both transcriptional and post-transcriptional processes, that act differentially during plant development, stress, and recovery from stress.

INTRODUCTION

As in all higher plants, the common ice plant's small subunit proteins (SSU) of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are encoded by a nuclear multigene family, *RbcS* (Dean et al., 1989b). This gene family consists of six genes in the ice plant (DeRocher et al., 1993), four of which are represented in cDNA libraries from leaves (DeRocher et al., 1991). The SSU is a subunit of the Rubisco enzyme essential for carbon dioxide assimilation by pathways known as C3, C4, or Crassulacean acid metabolism (CAM). The halophyte, ice plant, switches from C3 to CAM during development, and this transition is accelerated by environmental stresses that affect water availability. Under stress conditions, the switch to CAM provides two advantages to the plant. First, water loss via transpiration is reduced by limiting gas exchange to night time when ambient temperatures are lower. Second, the activities of CAM-specific enzymes induced by the switch result in increased CO_2 concentrations in the leaves. CAM buildup involves changes in expression of a number of photosynthesis-related genes and results in major physiological changes in the leaves and in long-term morphological changes (Bohnert et al., 1992; Cushman and Bohnert, 1993). Due to the central role of Rubisco in plant metabolism, subunit genes and proteins are possibly regulated in the process of CAM induction. Encoding SSU by multiple genes could allow a variety of developmental and environmental signals to be integrated into overall small subunit gene expression by distributing control mechanisms responsive to such signals among the members of the gene family.

Differential regulation of the *RbcS* gene family has been shown to occur in tomato in which three of five *RbcS* genes expressed in leaves are turned off during fruit ripening (Sugita and Gruissem, 1987) as a result of changes in transcription of specific members of the gene family (Wanner and Gruissem, 1991). Tomato and Arabidopsis *RbcS* genes (to include only species in which all *RbcS* family members have been studied) have also been demonstrated to be differentially regulated by light (Manzara et al., 1991; Dedonder et al., 1993). Only three of the five tomato *RbcS* genes are expressed in etiolated seedlings, and the remaining two genes are rapidly induced once the dark-grown seedlings are exposed to light (Wanner and Gruissem, 1991). Differential regulation has been shown between leaves and roots for six of the 12 *RbcS* genes of *Lemna gibba*. One of the most highly expressed *L. gibba RbcS* genes in leaves has the lowest expression level in roots, and this difference appears to result from post-transcriptional regulation (Silverthorne and Tobin, 1990; Silverthorne et al., 1990). Most other previous studies on differential regulation of *RbcS* genes have focused on tissue-specific differential regulation. Only subtle differences in expression of members of the *RbcS* gene family were seen in different tissues of, for example, petunia (Dean et al., 1985, 1987a, 1987b, 1989a) and pea (Coruzzi et al., 1985; Fluhr et al., 1986).

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There have been no previous studies on the regulation of *RbcS* gene family members in response to environmental stresses. By studying molecular processes involved in the transition from C3 photosynthesis to CAM mode in the ice plant during NaCl stress, we surmised that we could find alterations in the CO_2 assimilation pathway that would affect expression of the *RbcS* gene family and expected regulatory changes in response to NaCl stress. It was shown previously that such stress caused a decline in the steady state mRNA of the *Mesembryanthemum crystallinum RbcS* gene family as a whole (Michalowski et al. 1989a). In contrast, a study that investigated the transcriptional induction of the *Ppc1* gene, encoding a CAM-specific phosphoenolpyruvate carboxylase isoform (Cushman et al., 1989, 1990), indicated slight increases in transcription of the *RbcS* gene family as a whole.

Previous studies had also shown that genes induced during the appearance of CAM were not only stress responsive, but were also under developmental regulation. For example, genes for CAM enzymes are not inducible in young plants (Cushman et al., 1990; Vernon and Bohnert, 1992). This indicated that our studies had to monitor not only stress-related changes, but that these changes also had to be studied during the entire plant life. To determine the contributions of the individual *RbcS* gene family members to changes in *RbcS* gene expression during CAM buildup in the ice plant, we have now determined expression characteristics of the genes at the levels of transcription and mRNA accumulation. Because of the role of Rubisco in both C3 photosynthesis and CAM, the expression properties of the *RbcS* gene family were also studied during development. The work presented here addresses differential regulation of the individual members of the *RbcS* gene family and the roles of transcriptional and post-transcriptional regulation in the expression of each of the *RbcS* genes during environmental stress (and subsequent stress relief) and development. We demonstrate transcription control during development and post-transcriptional regulation under environmental stress as two equally important mechanisms in *RbcS* gene expression in *M. crystallinum*.

RESULTS

Gene-Specific Probes and Signal Normalization

The sequences of the ice plant *RbcS* gene family have been reported previously (DeRocher et al., 1993). With the availability of *RbcS* gene–specific probes, it was possible to assess contributions of individual ice plant *RbcS* genes to total *RbcS* expression, to determine if the genes were differentially regulated, and to establish the roles of transcriptional and post-transcriptional regulation in expression of each of the *RbcS* genes. Probes specific for the expressed genes *RbcS-1*, *RbcS-2*, *RbcS-3*, and *RbcS-4* were made (see Methods) to determine their expression dynamics in response to abiotic stress and relief from stress and during development. The *RbcS-5* gene is almost entirely sequence identical with *RbcS-4* and, thus, no probe that would distinguish between these two genes can be generated. No cDNA clone corresponding to *RbcS-5* was found in the two cDNA libraries, indicating that *RbcS-5* may be not expressed or is expressed at a very low level, less than 0.1% of all *RbcS* transcripts. Because the *RbcS-4* probe would hybridize to any *RbcS-5* mRNA that might exist, hybridization signals obtained with this probe are referred to as *RbcS-4/5*. A probe for *RbcS-6* was also made, but no data are included here because *RbcS-6* transcripts could not be detected in RNA from leaves at any time point or during stress treatments (we have not found a corresponding cDNA after an extensive search of transcript libraries generated from both stressed and unstressed plants; DeRocher et al., 1991). An *RbcS* coding region probe that hybridized to all *RbcS* transcripts was included in all RNA analyses to verify that changes in total *RbcS* mRNA amounts reflected changes seen for individual genes. The results of RNA gel blot analysis and nuclear run-on transcription assays can be directly compared in all experiments, because RNA and nuclei were isolated from the same set of plants for every time point.

Normalization for the expression of control genes, tubulin, actin, and 18S rRNA genes, included measurements of run-on transcription and mRNA amount. The transcriptional activities of these three constitutively expressed genes were used as internal standards in run-on transcription assays to provide a basis for determining relative changes in *RbcS* transcription. Steady state mRNA amounts were determined for the three control genes (all multigene families in *M. crystallinum*) in unstressed and NaCl-stressed plants over the full range of development used (data not included). Expression of the three gene families as a whole is relatively constant with duplicate measurements for each of 23 time points giving variation in steady state mRNA amounts of ±21.5% for 18S rRNA, ±26.9% for actin, and ±27.4% for tubulin. Given this constancy over a wide range of conditions and developmental states, we assumed constant transcriptional activity. In the following analysis, transcription activities of the *RbcS* genes are given with respect to 18S rRNA transcription. Calculation of *RbcS* transcription activities with respect to actin or tubulin gave similar results (data not shown; see also Cushman, 1993). In addition, *Cab* (an ice plant probe for a light-harvesting chlorophyll *a/b* binding protein, LHCP-II gene; E.J. DeRocher, unpublished data) transcriptional activity is included. *Cab* exemplifies a gene that is regulated by stress and during development, but in a manner different from that of the *RbcS* genes. The difference in behavior of these two photosynthesis-related genes indicates that the observed changes in *RbcS* transcriptional activity are *RbcS* specific and do not reflect a global down regulation of chloroplast functions.

RbcS Expression during Development

Experiments over several years have indicated an optimal response period to salt stress during development (Ostrem et

al., 1987) that, under our growth conditions, was reached 5 weeks after germination. This period coincided with the appearance of axillary side shoots in the plants that initiates flower development. To test the possibility that *RbcS* expression undergoes a developmentally regulated change during this same period, RNA and nuclei were isolated from unstressed plants at 3, 4, 5, 6, and 7 weeks of age. RNA gel blot analysis of *RbcS* mRNA amounts is shown in Figure 1A. There was a sharp transition in the amounts of mRNA for the four expressed *RbcS* genes that occurred between 5 and 6 weeks from high amounts in young plants to very low amounts in plants older than ~7 weeks. The mRNA amounts differed between 5 and 6 weeks by a factor of 9.6 for *RbcS-1*, 5.5 for *RbcS-2*, 19.8 for *RbcS-3*, and 19.1 for *RbcS-4/5*. In addition, Figures 1B and 1C show that the kinetics with which the mRNA amounts declined fall into two groups. The amounts of *RbcS-1* and *RbcS-2* mRNAs remained essentially constant at 3, 4, and 5 weeks and then dropped sharply. The mRNA amounts of *RbcS-3* and *RbcS-4/5*, however, showed a gradual decline from 3 to 5 weeks followed by a sharp drop to the amounts present at 6 weeks.

The results of run-on transcription assays are shown in Figure 2, again showing a sharp transition in transcription between young and old plants. Transcriptional activities for the *RbcS-1*, *RbcS-2*, and *RbcS-3* genes changed from high levels in young plants to low levels in old plants, preceding the decline in mRNA amounts by ~1 week. Transcriptional activity of *RbcS-4/5*, however, was low throughout development and may actually have increased slightly with plant age.

RbcS Expression during NaCl Stress

Five-week-old ice plants were NaCl stressed, and RNA and nuclei were isolated over a 5-day time course consisting of a 0-day unstressed control, plants stressed for 1, 3, and 5 days, and a 5-day unstressed control. RNA blot analysis was done for all time points, as shown in Figure 3. A representative RNA blot series is shown in Figure 3A. All four genes for which transcript had been detected exhibited a decline in mRNA amounts within 1 day after the start of the NaCl stress. The mRNA amounts showed no recovery during the 5 days of NaCl stress. To quantitate the changes seen in the RNA blots, the relative amounts of mRNA for each *RbcS* gene at each time point were determined by direct measurement of hybridization signals using a Betascope. The data are graphed (after normalization of probe intensities) in Figures 3B and 3C. Although all four genes responded to NaCl stress in a qualitatively similar manner, they were differentially regulated in the degree of response to NaCl stress. The amount of mRNA differed between the 0-day unstressed control and the 1-day stressed plants by a factor of 4.7 for *RbcS-1*, 2.8 for *RbcS-2*, 13.5 for *RbcS-3*, and 12.3 for *RbcS-4*. This is the same rank order of change as is seen during development, as documented in the previous section. The mRNA amounts for all four genes declined between the 0- and 5-day unstressed control plants by ~50%. This decline is the result of developmental regulation of *RbcS*

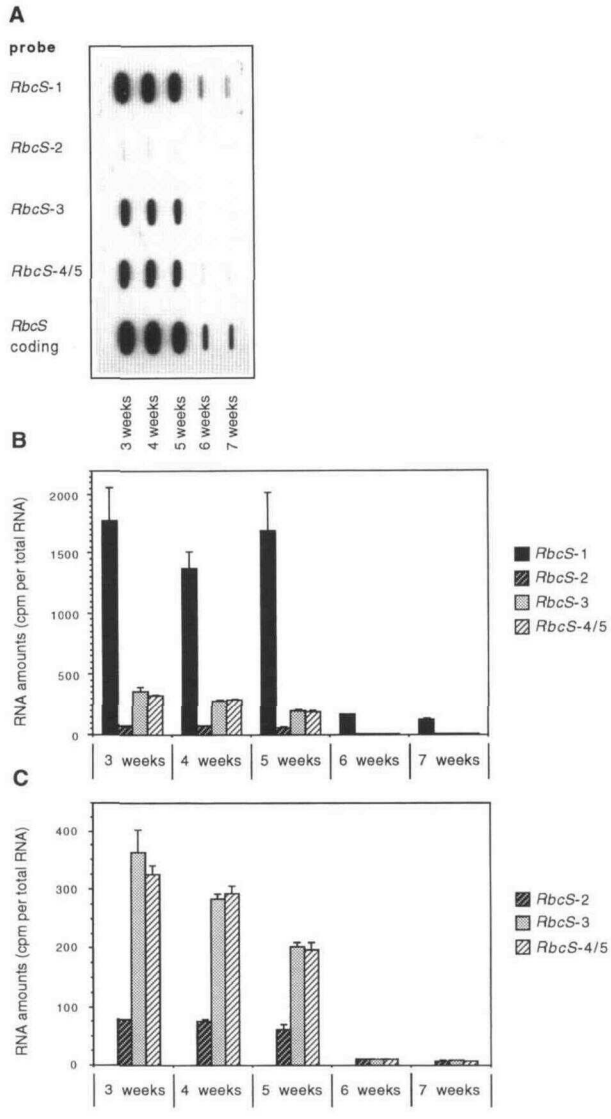

Figure 1. Changes in Amounts of *RbcS* mRNA during Development.

Total RNA was isolated from unstressed plants at 3, 4, 5, 6, and 7 weeks after germination. Steady state mRNA amounts were determined for individual *RbcS* genes and the gene family by RNA blot analysis.

(A) A representative autoradiogram of hybridization signals obtained after probing total RNA with gene-specific radiolabeled *RbcS* probes. In addition, a probe specific for the coding region, hybridizing to all genes, is included.

(B) Quantitation of hybridization signals for the *RbcS* gene–specific probes as determined by Betascope measurements. Columns represent the average of two hybridizations, and error bars indicate the range of values.

(C) Vertical expansion of the data for *RbcS-2*, *RbcS-3*, and *RbcS-4/5* shown in **(B)**.

expression over the 5-day time course documented in Figures 1 and 2. Just as was seen for the changes in *RbcS* mRNA amounts in response to development, the changes in *RbcS* mRNAs between 5 and 6 weeks were qualitatively the same but quantitatively different.

The rates of transcription of the *RbcS* genes were determined from nuclear run-on assays with nuclei isolated from the plants at each time point in the NaCl stress time course, as shown in Figure 4. A representative set of autoradiograms for the run-on assays in which exposure times have been normalized to 18S rRNA signals is shown in Figure 4A. Transcription for all four genes declined during the first day of NaCl stress. During the third and fifth days, transcription rates returned to prestress levels. This decline and recovery in transcription was specific for the *RbcS* genes, as can be seen by comparison to *Cab* transcriptional activity, which also declined in response to NaCl stress but did not recover during the 5-day time course. The transcriptional activities of the *RbcS* genes were

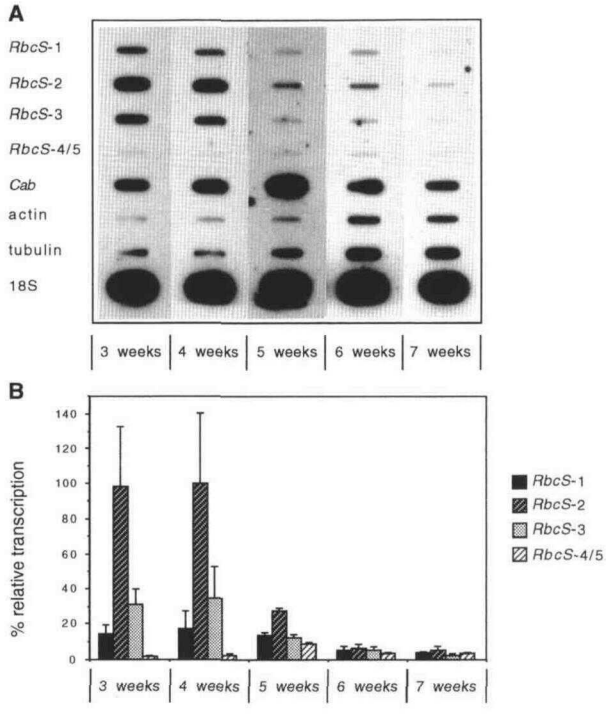

Figure 2. Changes in *RbcS* Transcription during Development.

Transcriptional activities were determined by in vitro nuclear run-on transcription assays using nuclei isolated from unstressed plants at 3, 4, 5, 6, and 7 weeks after germination.

(A) Representative autoradiogram of hybridization signals obtained after radiolabeled transcripts were synthesized in vitro and hybridized to DNA attached to nitrocellulose filters. Exposures for each time point were normalized to the 18S rRNA signals from the same experiment quantitated with a Betascope.

(B) Quantitation of hybridization signals after nuclear run-on transcription assays as determined by Betascope measurements. Columns represent the average of two assays, and error bars indicate the range of values.

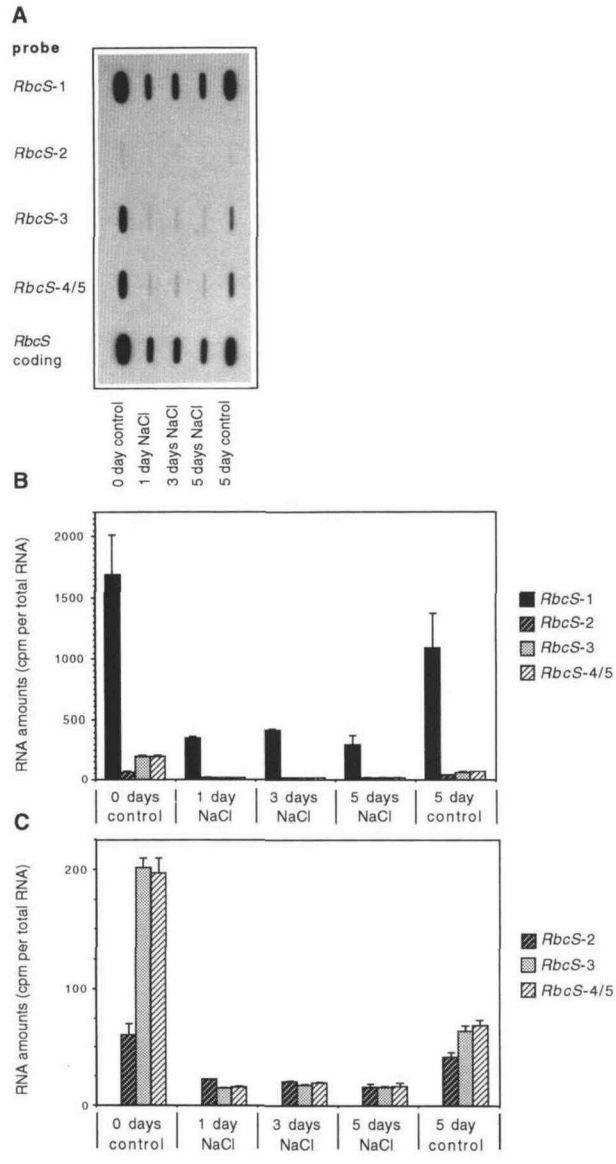

Figure 3. Effect of NaCl Stress on mRNA Levels of *RbcS* Gene Family Members.

Total RNA was isolated from control plants and plants stressed with NaCl for 1, 3, and 5 days starting when the plants were 5 weeks old. Steady state mRNA amounts were determined for individual *RbcS* genes and the gene family by RNA blot analysis.

(A) A representative autoradiogram of hybridization signals obtained after probing slot-blotted total RNA with gene-specific *RbcS* probes and *RbcS* probes detecting the coding region of all genes.

(B) Quantitation of hybridization signals for the *RbcS* gene–specific probes as determined by Betascope measurements. Columns represent the average of two hybridizations, and error bars indicate the range of values.

(C) Vertical expansion of the data for *RbcS-2*, *RbcS-3*, and *RbcS-4/5* shown in **(B)**.

quantitated from duplicate run-on assays using a Betascope, and the values were normalized to 18S rRNA transcription (Figure 4B). Transcription after 5 days of stress not only recovered to prestress levels but was higher than the transcriptional activities seen in the 5-day unstressed control plants. Although *RbcS* transcriptional activities recovered over 5 days of stress, mRNA amounts remained at a constant low level after the first day of stress. The lack of recovery of *RbcS* mRNA in the face of increased transcription indicated that accumulation of *RbcS* mRNAs was controlled post-transcriptionally during NaCl stress. Similar to the decline in *RbcS* mRNA amounts seen between the 0- and 5-day unstressed controls, the decline in transcriptional activities seen between these time points resulted from developmental regulation.

Stress and Stress Relief ("Destress")

Plants that were NaCl stressed for 5 days were "destressed" (see Methods) to determine if the down regulation of *RbcS*

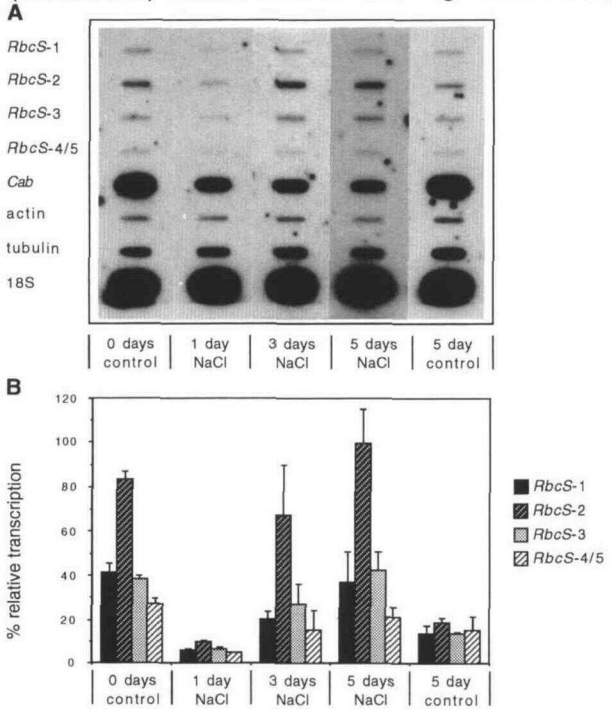

Figure 4. Effect of NaCl Stress on Transcription of *RbcS* Genes.

Transcriptional activities were determined by in vitro nuclear run-on transcription assays using nuclei isolated from control plants and plants NaCl stressed for 1, 3, and 5 days.

(A) Representative autoradiogram of hybridization signals obtained after radiolabeled transcripts were synthesized in vitro and hybridized to DNA attached to nitrocellulose filters. Exposures for each time point were normalized to 18S rRNA signals quantitated with a Betascope.

(B) Quantitation of hybridization signals after nuclear run-on transcription assays as determined by Betascope measurements. Columns represent the average of two assays, and error bars indicate the range of values.

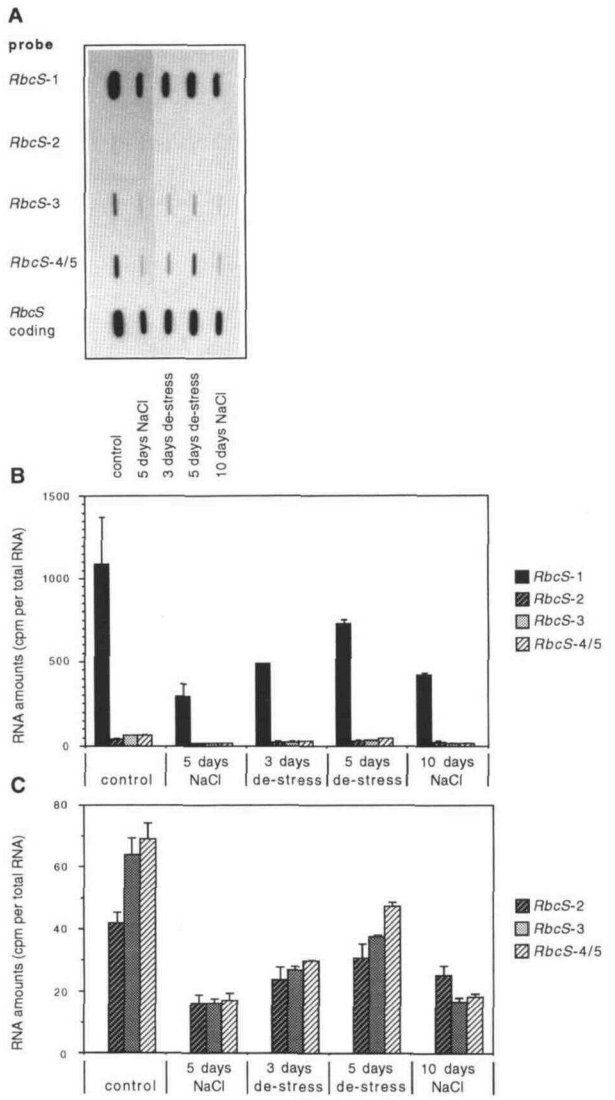

Figure 5. *RbcS* mRNA Levels after Destress.

Total RNA was isolated from control plants, plants stressed with NaCl for 5 days starting when the plants were 5 weeks old, and plants destressed 3 and 5 days. Steady state mRNA levels were determined for individual *RbcS* genes and the gene family by RNA blot analysis.

(A) Representative autoradiogram of hybridization signals obtained after probing total RNA with gene-specific *RbcS* probes and *RbcS* probes hybridizing to the coding regions.

(B) Quantitation of hybridization signals for the *RbcS* gene–specific probes as determined by Betascope measurements. Columns represent the average of two hybridizations, and error bars indicate the range of values.

(C) Vertical expansion of the data for *RbcS-2*, *RbcS-3*, and *RbcS-4/5* shown in **(B)**.

expression by NaCl stress was reversible. Changes in mRNA amounts and transcription rates during destress are shown in Figures 5 and 6, respectively. The mRNA amounts of all four genes increased proportionally during 5 days of destress. During the same time, transcription of all four genes declined,

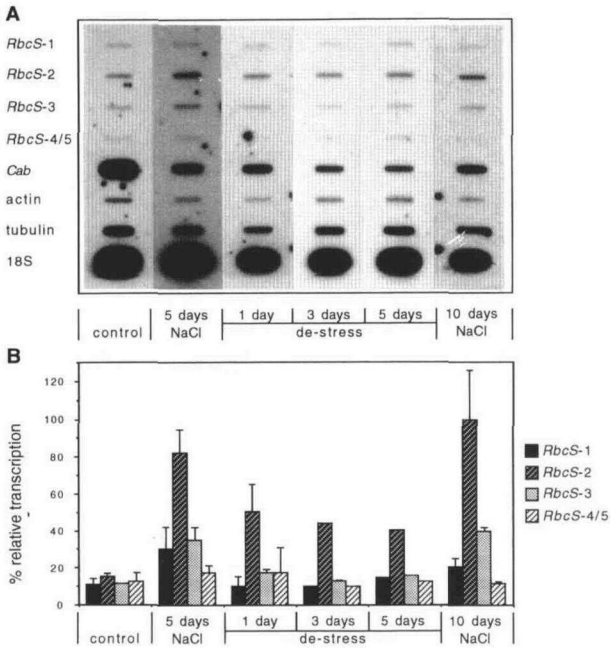

Figure 6. *RbcS* Transcriptional Activities after Destress.

Transcriptional activities were determined by in vitro nuclear run-on transcription assays using nuclei isolated from control plants, plants stressed with NaCl for 5 days starting when the plants were 5 weeks old, and plants destressed 1, 3, and 5 days.
(A) Representative autoradiogram of hybridization signals obtained after radiolabeled transcripts were synthesized in vitro and hybridized to DNA attached to nitrocellulose filters. Exposures for each time point were normalized to the 18S rRNA signals quantitated with a Betascope.
(B) Quantitation of hybridization signals after nuclear run-on transcription assays as determined by Betascope measurements. Columns represent the average of two assays, and error bars indicate the range of values.

indicating that post-transcriptional down regulation of *RbcS* transcripts by NaCl stress can be reversed upon removal of the stress. The mRNA amounts and transcriptional activities for the 10-day stress time point show that there was still no recovery to unstressed mRNA amounts after 10 days of NaCl stress.

RbcS Expression in Response to NaCl Stress during Development

Previously, we studied how development and salt stress influence transcription and mRNA amounts of the transcribed *RbcS* genes. To test whether the responsiveness of *RbcS* expression to NaCl stress is developmentally regulated, like that of CAM genes (Cushman et al., 1990), plants were NaCl stressed for 5 days starting at 3, 4, 5, and 6 weeks after germination. RNA and nuclei were isolated from the stressed plants and control plants.

The results of RNA blot analysis for the effect of NaCl stress at different ages are shown in Figure 7. A representative

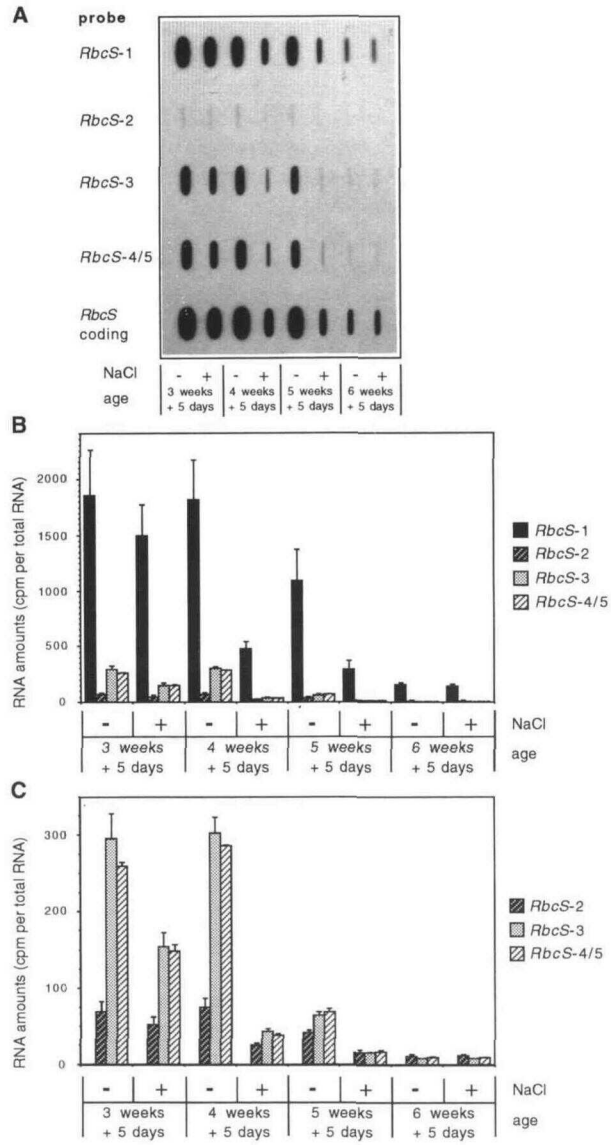

Figure 7. Effect of NaCl Stress on *RbcS* mRNA Levels at Different Points during Development.

Total RNA was isolated from control plants and plants stressed with NaCl starting at 3, 4, 5, and 6 weeks after germination. Steady state mRNA amounts were determined for individual *RbcS* genes and the gene family by RNA blot analysis.
(A) A representative autoradiogram of hybridization signals obtained after probing slot-blotted total RNA with gene-specific *RbcS* probes and *RbcS* probes detecting the coding regions of all genes.
(B) Quantitation of hybridization signals for the *RbcS* gene–specific probes as determined by Betascope measurements. Columns represent the average of two hybridizations, and error bars indicate the range of values.
(C) Vertical expansion of the data for *RbcS-2*, *RbcS-3*, and *RbcS-4/5* shown in **(B)**.

autoradiogram is shown in Figure 7A, and quantitated RNA blot hybridization data are shown in Figures 7B and 7C. Overall, the qualitative changes in mRNA amounts of the four *RbcS* genes in response to NaCl stress during development were similar to each other. That is, the effect of NaCl stress on the mRNA amounts of each *RbcS* gene varied with development, and all four genes showed a window of responsiveness to NaCl stress between 4 and 5 weeks after germination. When plants were stressed for 5 days starting at 6 weeks, the mRNA amounts for all four genes were unchanged. Three-week-old plants stressed for 5 days were less responsive to NaCl stress than plants between 4 and 5 weeks of age, although the behavior of the four genes fell into two groups. The mRNA amounts for *RbcS-1* and *RbcS-2* showed little if any decrease in response to NaCl stress. The mRNA amounts for *RbcS-3* and *RbcS-4/5* decreased by ~50%. At 4 weeks (plus 5 days of stress), all four genes showed major declines in mRNA amounts in response to NaCl stress. During the 5-day stress period started at 5 weeks, declines in mRNA amounts due to development could be seen in control plants. NaCl stress superimposes a further reduction in mRNA amounts upon the developmental down regulation of the *RbcS* genes at this plant age.

Nuclear run-on transcription assays were performed for the developmental time points, and the results are presented in Figure 8. The qualitative changes in transcriptional activities of each of the *RbcS* genes in response to NaCl stress during development were similar to those changes in mRNA amounts. However, the changes in transcriptional activity did not correspond to the changes in mRNA amounts. Transcriptional activities did not show a developmental window of responsiveness to NaCl stress. Instead, NaCl stress resulted in increased transcription of all four genes after 5 days of stress started at 4, 5, or 6 weeks after germination. Five days of NaCl stress

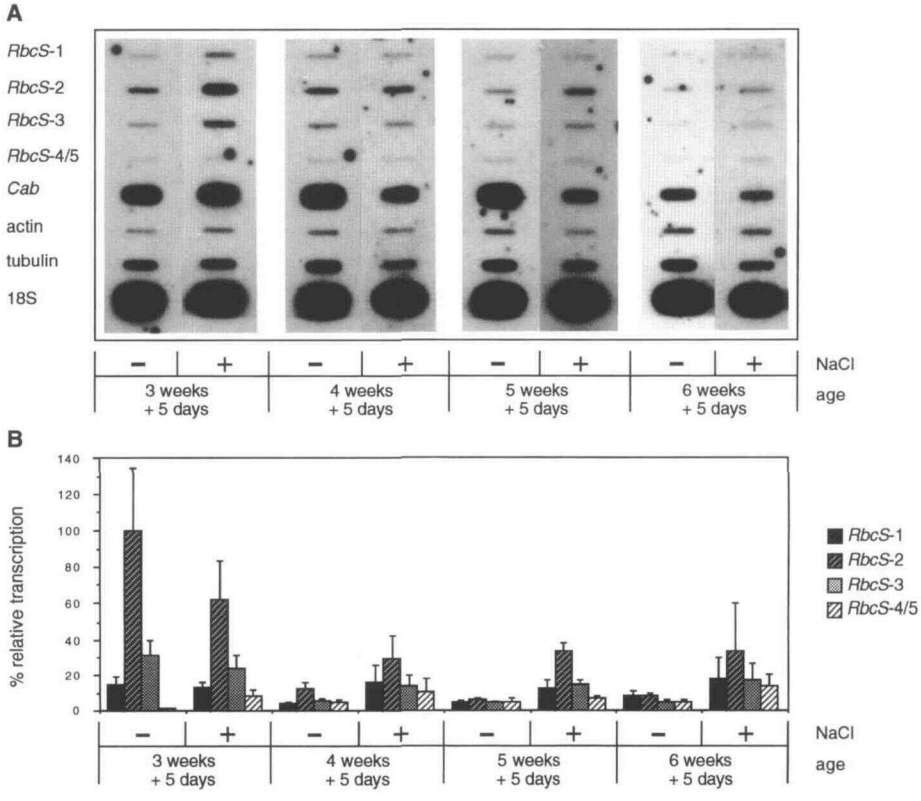

Figure 8. Effect of NaCl Stress on *RbcS* Transcriptional Activities at Different Points during Development.

Transcriptional activities were determined by in vitro nuclear run-on transcription assays using nuclei isolated from control plants and plants stressed with NaCl starting at 3, 4, 5, and 6 weeks after germination.

(A) Representative autoradiogram of hybridization signals obtained after radiolabeled transcripts were synthesized in vitro and hybridized to DNA attached to nitrocellulose filters. Exposures for each time point were normalized to the 18S rRNA signals quantitated with a Betascope.

(B) Quantitation of hybridization signals after nuclear run-on transcription assays as determined by Betascope measurements. Columns represent the average of two assays, and error bars indicate the range of values.

started at 3 weeks caused no significant change in the transcriptional activities of *RbcS-1*, *RbcS-2*, and *RbcS-3*, while that of *RbcS-4/5* increased.

The *RbcS* genes were differentially regulated in response to NaCl stress in 3-week-old plants. Expression of *RbcS-1* and *RbcS-2* was insensitive to NaCl stress, but both *RbcS-3* and *RbcS-4* were down regulated. As shown in Figure 7, there were no significant changes in the mRNA amounts of *RbcS-1* and *RbcS-2* due to NaCl stress started at 3 weeks. The ~50% decline in *RbcS-3* mRNA, however, occurred with no significant accompanying decline in transcriptional activity. The amount of *RbcS-4/5* mRNA also declined ~50%, but the transcriptional activity of *RbcS-4* showed an increase. The decreased accumulation of *RbcS-3* and *RbcS-4/5* in 3-week-old plants was apparently due to post-transcriptional regulation. Four weeks after germination, the *RbcS* genes were no longer differentially regulated in response to NaCl stress. Five days of NaCl stress started at 4 or 5 weeks resulted in decreased mRNA amounts and increased transcriptional activities for all four genes, indicating that the down regulation is post-transcriptionally controlled. After 5 days of stress started at week 6, the mRNA amounts for all four genes did not change from the already lowered levels due to developmental regulation. Transcriptional activities, however, were increased by NaCl stress, indicating post-transcriptional modification of *RbcS* transcripts in response to NaCl.

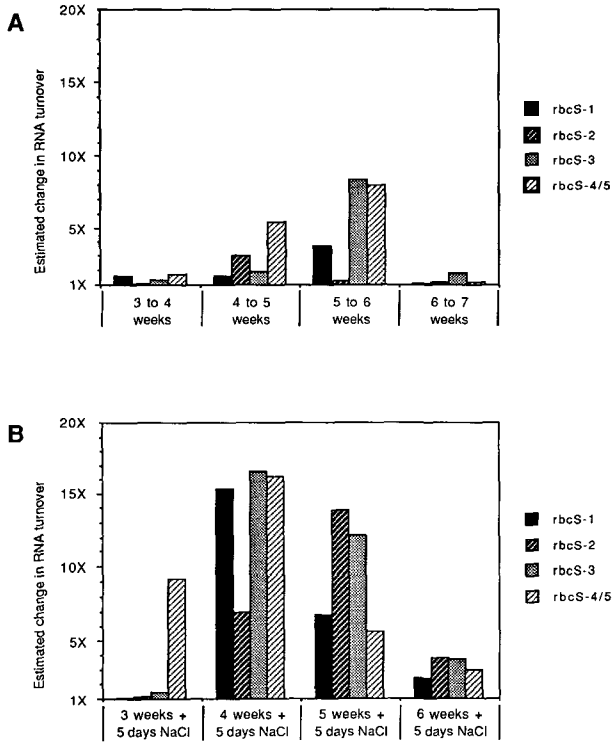

Figure 9. Changes in RNA Levels during Development and NaCl Stress.

Columns represent estimated changes in *RbcS* turnover.
(A) Changes calculated from discrepancies between changes in transcriptional activities and changes in steady state *RbcS* mRNA levels during development.
(B) Same as given in **(A)**, except changes calculated during NaCl stress.

Table 1. *Changes in RbcS Transcription and Amount during* Development

Plant Age	*RbcS* Gene	Δ Tr[a] Tr A2[b] / Tr A1[c]	Δ mRNA mRNA A2 / mRNA A1	Factor of Change[d]	Net Change[e]
3 weeks	1	0.87	0.81	1.1	+
vs	2	1.20	0.77	1.6	+
4 weeks	3	1.12	0.78	1.4	+
	4/5	1.56	0.90	1.7	+
4 weeks	1	0.79	1.23	1.6	−
vs	2	0.27	0.81	3.0	−
5 weeks	3	0.37	0.71	1.9	−
	4/5	3.64	0.67	5.4	+
5 weeks	1	0.37	0.10	3.7	+
vs	2	0.24	0.18	1.3	+
6 weeks	3	0.42	0.05	8.4	+
	4/5	0.40	0.05	8.0	+
6 weeks	1	0.82	0.77	1.1	+
vs	2	0.86	0.73	1.2	+
7 weeks	3	0.51	0.90	1.8	−
	4/5	0.97	0.78	1.2	+

[a] Tr, relative transcription activity.
[b] A2, older plants in a comparison.
[c] A1, younger plants in a comparison.
[d] X-fold difference between transcription and mRNA amount.
[e] +, increase; −, decrease.

Figure 9 summarizes the changes during development (Figure 9A) and during stress on a developmental time scale (Figure 9B). The decline in *RbcS* mRNA amounts seen during development was mediated primarily by decreased transcription when changes in steady state mRNA amounts and transcriptional activities (Figures 1 and 2; weeks 3 to 7) were compared. Changes in transcription and changes in mRNA amounts were calculated to determine how well mRNA accumulation paralleled transcription rate, as shown in Table 1 and Figure 9A. Discrepancies between changes in RNA accumulation and transcription indicate the possible involvement of regulated turnover of nuclear pre-mRNA, cytoplasmic mRNA, or both (indicated in Table 1 as "factor of change"). In most cases, discrepancies were small, indicating that the *RbcS* genes are primarily under transcriptional control during development. The influence of development on gene expression of the *RbcS* gene family members and on selected other genes, mostly CAM-related genes, is also shown in Figure 10. The approximately eightfold greater decrease in mRNA amounts than in transcriptional activities for the *RbcS-3* and *RbcS-4/5* genes between weeks 5 and 6 suggests a possible contribution of increased RNA turnover to decreased expression of these two genes.

Figure 9B presents a similar analysis during stress conditions. The observed changes in steady state mRNA amounts

and transcriptional activities (Figures 7 and 8) were compared between plants stressed with NaCl starting at 3, 4, 5, and 6 weeks and the corresponding unstressed control plants. Differences between changes in transcription and changes in mRNA amounts were calculated and used to estimate changes in RNA turnover that could account for discrepancies, as shown in Table 2. When stressed at 3 weeks, only *RbcS-4* showed a significant discrepancy in RNA turnover. NaCl stress started at 4 or 5 weeks resulted in substantial discrepancies between changes in mRNA amounts and changes in transcriptional activities for all four *RbcS* genes. In plants stressed starting at 6 weeks, discrepancies for all four *RbcS* transcripts by NaCl were still observed, but to a substantially lower degree than in 4- and 5-week-old plants. The apparently increased RNA turnover almost exactly counteracted the NaCl-caused increase in transcription such that no net change occurred in the mRNA amounts of the four *RbcS* genes. Figure 9B provides evidence for post-transcriptional regulation of *RbcS* transcript amounts in response to NaCl stress and transcriptional regulation during development.

Table 2. Changes in *RbcS* Transcription and Amount during NaCl Stress

Plant Age and Treatment with NaCl	*RbcS* Gene	Δ Tr[a] Tr NaCl[b] / Tr Control[c]	Δ mRNA mRNA NaCl / mRNA Control	Factor of Change[d]	Net Change[e]
3 weeks	1	0.87	0.81	1.1	+
+	2	0.62	0.76	1.2	–
5 days	3	0.8	0.5	1.6	+
	4/5	5.3	0.6	8.8	+
4 weeks	1	4.00	0.26	15.4	+
+	2	2.37	0.34	7.0	+
5 days	3	2.33	0.14	16.6	+
	4/5	2.11	0.13	16.2	+
5 weeks	1	1.87	0.27	6.8	+
+	2	5.27	0.38	13.9	+
5 days	3	3.04	0.25	12.2	+
	4/5	1.39	0.25	5.7	+
6 weeks	1	2.19	0.90	2.4	+
+	2	3.95	1.03	3.8	+
5 days	3	3.41	0.93	3.7	+
	4/5	2.84	0.95	3.0	+

[a] Tr, relative transcriptional activity.
[b] NaCl-stressed plants.
[c] Control, unstressed plants.
[d] X-fold difference between transcription and mRNA amount.
[e] +, increase; –, decrease.

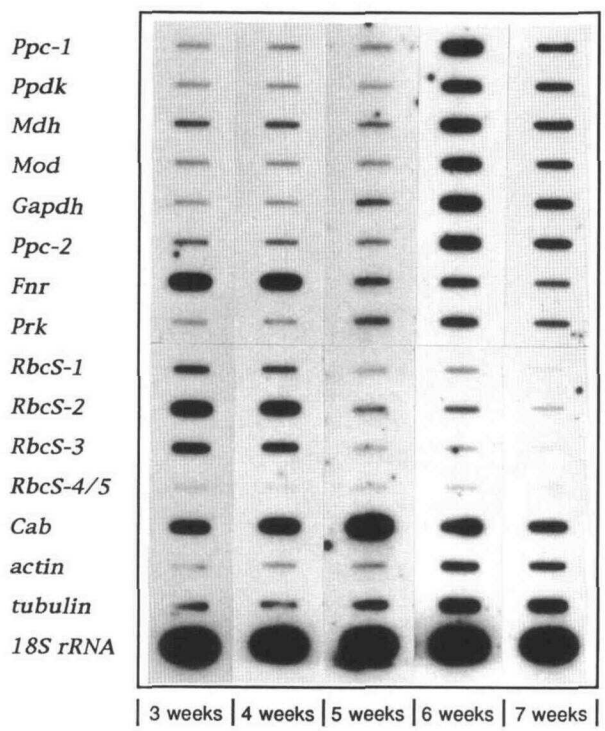

Figure 10. Transcriptional Activities of 16 Common Ice Plant Genes during Development.

Transcriptional activities were determined by in vitro nuclear run-on transcription assays using nuclei isolated from unstressed plants at 3, 4, 5, 6, and 7 weeks after germination. The autoradiogram of hybridization signals was obtained after radiolabeled transcripts were synthesized in vitro and hybridized to DNA attached to nitrocellulose filters. Exposures for each time point were normalized to the 18S rRNA signals quantitated with a Betascope. The following gene probes from ice plant were used: *Ppc1*, phosphoenolpyruvate carboxylase-1 (CAM form); *Ppdk*, pyruvate, orthophosphate dikinase; *Mdh*, malate dehydrogenase; *Mod*, malic enzyme; *Gapdh*, glyceraldehyde phosphate dehydrogenase; *Ppc2*, phosphoenolpyruvate carboxylase-2 (C3 form); *Fnr*, ferredoxin NADP$^+$ oxidoreductase; *Prk*, phosphoribulokinase; *Cab*, chlorophyll *a/b* binding protein.

A remarkable feature of expression of the *RbcS* gene family, irrespective of widely varying relative transcript stability and irrespective of changes in mRNA amounts during development and under stress, is that the relative contribution of each gene to the total amount of mRNA was maintained within a narrow range, as shown in Table 3. The largest portion of *RbcS* mRNA was consistently made up of *RbcS-1* transcripts ranging from 68 to 86% of total *RbcS* mRNA, and 14 to 32% of the transcripts were contributed by the remaining three genes. Table 3 indicates subtle trends in the relative expression of the *RbcS* transcripts. The amount of *RbcS-1* mRNA gradually increased from under 70% up to 84% as the plants aged from 3 to 7 weeks (Table 3). A gradual change occurred in the distribution of transcripts of the other three genes during this time period. In 3-week-old plants, *RbcS-2* contributed approximately fourfold less mRNA than *RbcS-3* or *RbcS-4/5*. By 7 weeks, this had gradually shifted such that all three genes contributed nearly equally to the total amount of *RbcS* mRNA. The effect of NaCl stress was to make relative expression levels at one developmental age more like the relative expression levels

Table 3. Relative Expression of Ice Plant *RbcS* Genes during Development and Stress

Plant Age and Treatment	*RbcS-1* (%)	*RbcS-2* (%)	*RbcS-3* (%)	*RbcS-4/5* (%)
3 weeks	69.8	3.1	14.3	12.8
4 weeks	67.7	3.8	14.0	14.5
5 weeks	78.6	2.8	9.4	9.2
6 weeks	84.8	5.3	4.9	5.0
7 weeks	84.2	5.0	5.8	5.0
3 weeks + 5 days NaCl	80.8	2.8	8.4	8.0
4 weeks + 5 days NaCl	81.9	4.3	7.3	6.5
5 weeks + 5 days NaCl	85.7	4.7	4.7	4.9
6 weeks + 5 days NaCl	83.5	6.6	4.6	5.3
5 weeks + 3 days cold[a]	85.1	4.8	5.5	4.7

[a] Data indicating similar changes in the gene-specific distribution of mRNAs under low-temperature stress are included for comparison.

characteristic for a later stage in development. After 3-week-old plants were NaCl stressed for 5 days, for example, the relative *RbcS* expression levels were most similar to those of unstressed 5-week-old plants, and a similar acceleration was observed when stress commenced at other stages in the plant's life.

DISCUSSION

We have determined expression characteristics of individual members of the *RbcS* gene family of the common ice plant during the plant's development and under environmental stress. These genes are expressed at different levels and show subtle differences in regulation. Comparing changes in the rate of transcription to changes in mRNA amounts, we demonstrated that both transcriptional and post-transcriptional processes play important roles. The relative contributions of transcriptional and post-transcriptional processes to establishing steady state *RbcS* mRNA levels depend on the developmental state of the plants and on environmental conditions, with each *RbcS* gene showing its own characteristic behavior.

Measurements of relative transcription rates and mRNA quantification let us gauge inherent transcript stability variations. In all instances, *RbcS-1* transcripts were the most abundant and *RbcS-2* transcripts were consistently extremely low, yet the transcription activity of *RbcS-2* was always higher than that of *RbcS-1*. For example, in the 0-day unstressed control plants of the NaCl stress shown in Figure 3, *RbcS-1* mRNA amounts exceeded those of *RbcS-2* by a factor of 28, but the *RbcS-1* transcription rate was approximately half that of *RbcS-2* (Figure 4). *RbcS-1* transcripts were the most stable and those of *RbcS-2* the least stable, with the stabilities of *RbcS-3* and *RbcS-4/5* transcripts being intermediate. The substantial differences in stability and the observed developmental stability regulation should be reflected in features that distinguish these transcripts and their genes. Considering the high sequence conservation of their coding regions, it seems likely that sequences involved in determining the different stabilities would be located in the 5′ leader sequences, introns, or in the 3′ noncoding regions. The 3′ noncoding regions of *RbcS-3* and *RbcS-4/5* are nearly identical but share practically no sequence identity to *RbcS-1* or *RbcS-2*, which in turn share very limited sequence identity with each other (DeRocher et al., 1993). It is interesting to note that all *RbcS-1*, *RbcS-3*, and *RbcS-4/5* cDNA clones that were sequenced (DeRocher et al., 1991) had poly(A) tails, whereas the 13 *RbcS-2* cDNA clones sequenced lacked poly(A) tails (or terminated in very short poly(A) stretches). Likewise, *RbcS-2* 3′ noncoding regions were significantly shorter than those of transcripts from the other genes. This might suggest an RNA degradation mechanism that operates from the 3′ end of the transcripts.

Another aspect of the differential expression of these genes is obvious when comparing the expression characteristics of the *RbcS* genes with the nucleotide sequences of their 5′ flanking regions. Based on sequence similarity, the presumptive *RbcS* promoter regions fall into two groups (DeRocher et al., 1993). The promoter of *RbcS-1* contains sequence motifs essential for regulation of *RbcS* genes in tomato and pea (Ueda et al., 1989; Gilmartin et al., 1990; Lam and Chua, 1990). All other *M. crystallinum RbcS* promoters, which share sequence similarity with each other but hardly any with *RbcS-1*, lack these elements. The *RbcS-1* sequence elements are very highly conserved in nucleotide sequence, in 5′ to 3′ order, and in spacing with elements found in the promoter of the highly transcribed tomato *RbcS-3A* gene (Wanner and Gruissem, 1991), implying considerable selective pressure to maintain promoter elements in these evolutionarily divergent species. It is interesting to note then that *RbcS-1* showed the lowest transcription activity of all ice plant *RbcS* genes, suggesting that the *RbcS-2*, *RbcS-3*, and *RbcS-4/5* genes may have evolved a different regulatory mechanism for controlling transcription initiation and that this mechanism is at least as effective as the one controlling *RbcS-1* transcription. At the same time, the transcribed genes show concerted changes in transcription during development, indicating that such different mechanisms may likely be governed by a common higher level hierarchy of gene expression control.

Quantitative data on differential expression of *RbcS* gene family members from other species have been obtained for tomato (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991) and petunia (Dean et al., 1985, 1987a, 1987b, 1989a) *RbcS* gene families, for three of the five pea *RbcS* genes (Coruzzi et al., 1985; Fluhr et al., 1986), and for six of the 12 *L. gibba RbcS* genes (Silverthorne et al., 1990). In all four species, the *RbcS* genes are differentially expressed but to varying degrees. The *RbcS* genes of petunia show the widest range of expression among individual genes (25-fold in leaves), and more than 70% of total *RbcS* mRNA is contributed by two of eight genes.

In tomato, pea, and *L. gibba*, the range of expression is much less variable (2.5-, 1.5-, and 5-fold, respectively). In the ice plant, up to 86% of *RbcS* transcripts were contributed by one gene, and the range of expression between genes is up to 18-fold. Just as there is considerable flexibility among plant species for the evolution of differently structured *RbcS* gene families, there appears to be comparable flexibility in how different species make use of the members of their respective *RbcS* gene families.

We show here a coordinated regulation of transcription of the four transcribed *RbcS* genes that leads to a subtle shift in the composition of the mRNA pool during development. Results from our studies on the responses under stress, which accelerates developmental progression of changes in expression, add a new level of complexity to the regulation of *RbcS* gene family expression. Under stress, it is not a dramatic shift in the differential expression of gene family members that leads to a continued supply of *RbcS* mRNA, but the shift from transcriptional control to post-transcriptional control. This appears to be different from programs of *RbcS* expression control reported in other systems. Differential *RbcS* regulation, a switch in expression from one subset of the gene family to another subset, has been reported for tomato. Different *RbcS* genes are expressed in leaves, and only some genes are expressed in fruit or in seedlings (Sugita and Gruissem, 1987; Manzara et al., 1991; Wanner and Gruissem, 1991). As in tomato, *RbcS* genes in Arabidopsis show differential regulation by light quality and quantity (Dedonder et al., 1993).

Ice plant *RbcS* mRNA amounts change dramatically both during development and stress. The developmentally induced change coincides with the period of maximum CAM inducibility (Cushman et al., 1990; Ostrem et al., 1990). CAM cannot be induced by NaCl stress in plants younger than ~4 weeks. Changes in mRNA amount are often interpreted as evidence of a change in transcription rate, disregarding the many steps essential before the establishment of a new equilibrium population of cytoplasmic mRNA. Any or all of the steps in between can be regulated independently of transcription. The data on *RbcS* expression in *M. crystallinum* exemplify this point. Comparisons of changes in *RbcS* steady state mRNA amounts and transcription activities measured over a 5-day time course of NaCl stress (Figures 3 and 4) demonstrate that the previously observed decrease in total *RbcS* mRNA concomitant with an increase in total *RbcS* transcriptional activity in response to NaCl stress (Cushman et al., 1989; Michalowski et al., 1989a) is representative of responses of individual *RbcS* genes to NaCl stress. All four *RbcS* genes show greatly reduced mRNA amounts at the same time that transcription rates increase over control levels. The net down regulation of all four *RbcS* genes is brought about post-transcriptionally.

The decline in *RbcS* mRNAs caused by NaCl stress is an active and reversible gene-specific process and not a pathological response to stress. Although *RbcS* transcripts are apparently turned over more rapidly under stress, the mRNA levels of a number of other genes are unaffected, such as the transcripts for the ferredoxin $NADP^+$ oxidoreductase-1 (*Fnr1*; Michalowski et al., 1989b) or the transcript levels of 18S rRNA, actin, and tubulin genes. Other ice plant genes have been previously shown to be induced by NaCl stress, including the genes *Ppc1* (Cushman et al., 1989), *Gpd1* (glyceraldehyde-*P*-dehydrogenase-1; Ostrem et al., 1990), *Imt1* (*myo*-inositol *O*-methyltransferase-1; Vernon and Bohnert, 1992), *Mod1* (malic enzyme-1; Cushman, 1992), and *Mdh1* (malate dehydrogenase-1; Cushman, 1993). The specific targeting of the *RbcS* genes for down regulation by NaCl stress means that the post-transcriptional regulation of the *RbcS* RNAs is an *RbcS*-specific regulatory mechanism. This is further supported by the finding that down regulation of the *RbcS* genes by NaCl stress is itself dependent on the age of the plant. The post-transcriptional mechanism controlling *RbcS* mRNA accumulation is inactive for the *RbcS-1*, *RbcS*-2, and *RbcS*-3 transcripts in plants less than 4 weeks old. This mechanism is active for all four genes in plants that are 4 to 6 weeks old and is still active for all four genes after plants are more than 6 weeks old, but at a much reduced level (Figures 7 and 8). A probably even more lucid demonstration for an *RbcS*-specific control is the observation that the post-transcriptional and transcriptional effects of NaCl stress are reversible (Figures 5 and 6).

The link between regulation of *RbcS* expression and overall plant development or age is particularly interesting due to the existence of similar linkage of the expression of properties of genes involved in CAM photosynthesis to overall development of the plant. Inducibility of CAM-related genes, like the down regulation of the *RbcS* genes, occurs only weakly if at all in young plants. When the plants reach 5 to 6 weeks of age under our growth conditions, induction of CAM genes in response to NaCl stress is enabled, reflecting, as has been suggested (Cushman et al., 1990), the plant's adaptation to its native habitat. We include evidence of the developmental program that appears to govern gene expression in the ice plant, which is shown in Figure 10, demonstrating the age-dependent transcriptional induction of CAM genes in unstressed plants. Declining *RbcS* transcription shows the opposite, a sharp decline, while transcription of other genes, such as *Cab*, actin, tubulin, and 18S rRNA genes, is not significantly affected during this period of development. There appears to be a coordinated but divergent shift in transcription of genes involved in CO_2 fixation during a particular period of ice plant development, with *Ppc1* increasing and *RbcS* decreasing.

The common ice plant is the first dicot species outside the Solanaceae for which the expression properties of the individual members of the *RbcS* gene family have been extensively characterized at the levels of transcriptional and post-transcriptional regulation, and these are the only *RbcS* expression studies on a CAM plant. Both transcriptional and post-transcriptional regulation play important roles in establishing steady state *RbcS* mRNA amounts in the ice plant. This is similar to what has been reported for tomato (Wanner and Gruissem, 1991), petunia (Dean et al., 1989a, 1989b), potato (Fritz et al., 1991), soybean (Thompson and Meagher, 1990; Thompson et al., 1992), and *L. gibba* (Silverthorne and Tobin, 1990). It is becoming increasingly clear that a better understanding of the regulation of gene expression in higher plants

will require more studies targeting the relative contributions of post-transcriptional events.

As one generalization from this work, we suggest that developmental control appears to be acting on transcription during ice plant development and the establishment of chloroplasts, similar to what has been observed in several other species. Under environmental stress conditions, at least in the ice plant but possibly in other plants as well, post-transcriptional regulation appears to play the major role in the control of *RbcS* gene expression. Under stress, *M. crystallinum* shows a number of protective adaptations among which probably the most obvious is that photosynthesis-related functions are protected. Several nuclear genes for chloroplast proteins are not affected either by stress on the level of transcription or on the level of mRNA accumulation (Michalowski et al., 1992). Although *RbcS* expression is modified under stress, we view the new equilibrium of mRNA amounts that is established during stress as sufficient to maintain carbon assimilation.

The down regulation of *RbcS* mRNA during development and in response to NaCl stress is interesting in light of the critical function of Rubisco in photosynthesis. Decreased *RbcS* mRNA amounts do not have a detrimental effect on the plant. This is likely to be due to the stability of the Rubisco holoenzyme. The developmental decrease in *RbcS* mRNA coincides with the switch from juvenile to adult, i.e., a switch from growth of primary leaves to axillary branches in which the flowers develop. Developmental down regulation may be an early step in the senescense of the primary leaves.

Down regulation by NaCl stress may be part of a process that accelerates the switch from vegetative to reproductive growth. Alternatively, the NaCl stress induction of CAM may lead to a reduced amount of Rubisco, which may be sufficient because of the CO_2-concentrating effect of the CAM enzyme phosphoenolpyruvate carboxylase. The coincident developmental enablement of both *RbcS* down regulation and CAM induction by NaCl stress indicates that the regulation of the *RbcS* and CAM genes is closely coordinated (Figure 10). This interpretation is further supported by the reversibility of both CAM induction (Vernon et al., 1988) and *RbcS* down regulation upon the removal of NaCl stress (Figures 5 and 6). Down regulation during development and down regulation during stress are distinct processes, as evidenced by transcriptional control during development and post-transcriptional regulation in response to NaCl stress. The post-transcriptional control is itself under developmental regulation, indicating a complex hierarchy of *RbcS* gene regulation by environmental stress and development.

METHODS

Plant Material

Mesembryanthemum crystallinum plants were grown in growth chambers (Conviron, Asheville, NC) under incandescent and fluorescent lights with an intensity of 500 to 550 $\mu E\ m^{-2}\ sec^{-1}$ with a day/night cycle of 12 hr and temperatures of 23°C during the light period and 18°C during the dark period. Seeds were germinated in trays filled with soil, and after 7 days, seedlings were transplanted to soil in 32-oz Styrofoam pots (one plant per pot) and watered with *M. crystallinum* nutrient solution (Ostrem et al., 1987). All plants were well watered throughout all experiments. Plants were NaCl stressed by watering with nutrient solution brought to 0.5 M NaCl. Salt-stress experiments were initiated by watering each plant with 1 L of 0.5 M NaCl/nutrient solution between 1 and 2 PM of the first day of the experiment. Stressed plants were watered with 500 mL of 0.5 M NaCl/nutrient solution between 1 and 2 PM every other day thereafter. "Destress" was accomplished by watering each NaCl-stressed plant with 2 L of distilled water between 1 and 2 PM of the first day of destress and with 500 mL of nutrient solution every other day thereafter. Plant ages were counted from the day in which cotyledons first appeared, which was usually the third day after placing seeds on soil.

RNA and Nuclei Preparation

To allow direct comparison of the results from RNA blot analysis and nuclear run-on assays, total RNA and nuclei were isolated from the same set of plants for each time point in every experiment. Only leaves from the central axis were harvested, and only the topmost three-leaf pairs were harvested from each plant. Multiple plants were used for all time points ranging from 12 plants per time point for 3-week-old plants to four plants per time point for 7-week-old plants. Leaves were harvested at 2 PM for all time points to avoid any influence of diurnal cycling on gene expression. Tissue collected was divided into two portions: 20 g for RNA isolation and 40 g for nuclei isolation. Leaf tissue harvested for RNA isolation was frozen in liquid nitrogen and stored at −70°C until used. Leaf tissue harvested for nuclei isolation was not frozen, but used immediately after harvesting for nuclei isolations.

Nuclei isolations were done in a cold room with all solutions, glassware, mortar and pestle, centrifuge tubes, and centrifuge precooled. The protocol used was a modified version of a previous ice plant nuclei isolation protocol (Cushman et al., 1989). Leaf tissue was ground thoroughly 20 g at a time in 40 mL of 1 × nuclei isolation buffer (NIB) (10 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 34.2% sucrose, and 10 μM 2-mercaptoethanol) using a mortar and pestle and then filtered through four layers of cheesecloth into a beaker. The filtrate was brought to 0.5% Triton X-100 to lyse chloroplasts by slowly adding 10% Triton X-100 dropwise while swirling. The filtrate was distributed into four 30-mL Corex tubes and centrifuged for 10 min at 1000*g* and 4°C to pellet nuclei. Each nuclei pellet was gently resuspended using a paint brush in 20 mL of 1 × NIB, 0.5% Triton X-100, 10 μM of 2-mercaptoethanol, loaded onto an 80% Percoll pad (100% Percoll diluted to 80% using 2 × NIB) in a 30-mL Corex tube, and centrifuged for 20 min at 1000*g* at 4°C. Nuclei were removed from the tops of the Percoll pads with a Pasteur pipette and transferred to two 30-mL Corex tubes and diluted in 20 mL of 1 × NIB, 0.5% Triton X-100, 10 μM of 2-mercaptoethanol to remove Percoll. Nuclei were pelleted for 15 min at 1000*g* at 4°C. After carefully aspirating off the supernatant, nuclei from both tubes were resuspended in a total of 250 μL of nuclei storage buffer (20 mM Hepes, pH 7.2, 500 μM $MgCl_2$, 200 μM DTT, 50% glycerol) using a 1-mL Pipetman (Rainin, Lake Woebegone, MI) with the tip of the disposable tip cut off. Resuspended nuclei were distributed in 50-μL aliquots into 1.5-mL Eppendorf tubes, frozen in liquid nitrogen, and stored at −70°C.

Nucleic Acid Probes

The double-stranded DNA probes specific for ribulose-1,5-bisphosphate carboxylase/oxygenase genes *RbcS-1*, *RbcS-2*, *RbcS-3*, and *RbcS-4* and the general *RbcS* probe used for RNA blot analysis were the same as described previously (DeRocher et al., 1993). Hybridization signals of the gene-specific probes were normalized as described previously (DeRocher et al., 1993). A soybean 18S rRNA clone (provided by Dr. R.B. Meagher, University of Georgia, Athens, GA; Eckenrode et al., 1985), ice plant genomic actin and tubulin clones (C.B. Michalowski and H.J. Bohnert, unpublished data), and a previously characterized full-length ice plant chlorophyll *a/b* binding protein (*cab*) cDNA clone (E.J. DeRocher, unpublished data) were also used as probes for RNA blot analysis. Probes were prepared by excising inserts from the clones with appropriate restriction digests (BamHI/EcoRI for 18S rRNA, EcoRI for actin, EcoRI/SalI for tubulin, and HindIII for *cab*), isolating the inserts from low-melting agarose gels, and labeling by the random primer method (Feinberg and Vogelstein, 1983).

RNA Blot Analysis

Total RNA for all time points of each experiment was slot blotted (8 μg per slot) in five duplicate sets. Each duplicate set was hybridized with one of the *RbcS* gene–specific probes or the *RbcS* general probe. Each probe was hybridized (42°C, overnight) to RNA from all time points simultaneously to allow direct comparison of hybridization signals between experiments. After washing at 55°C in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), filters were exposed to x-ray film. Hybridization signals were quantitated as counts per min directly from the filters using a Betascope (Betagen, Waltham, MA). Averages of duplicate hybridization signals were used in generating graphs presented in the results. Four of the sets of duplicate filters were stripped and reprobed with 18S rRNA, actin, tubulin, and *cab* probes. The results presented here reproduce the results of two previous independent experiments for NaCl stress, development and combined NaCl stress, and development experiments.

Nuclear Run-On Transcription Assays

Run-on transcription assays were initiated by adding a 50-μL aliquot of isolated nuclei immediately upon thawing to a 50-μL assay solution containing 20 μL of 5 × transcription salts (100 mM Hepes, pH 7.9, 50 mM $MgCl_2$, 500 mM $[NH_4]_2SO_4$, autoclaved), 10 μL of glycerol (treated with diethyl pyrocarbonate [DEPC] and autoclaved), 1.0 μL of 0.1 M DTT, 1.5 μL of 100 mM ATP, GTP, and CTP, 1.8 μL of 40 units per mL RNasin, 5.7 μL of H_2O (treated with DEPC and autoclaved), and 10 μL of 3000 Ci/mmol α-^{32}P-UTP (100 μCi). Reactions were incubated 20 min at 30°C. At 20 min, 5 μL of RNase-free DNase RQ1 (Promega) was added and incubated 5 min at 30°C to remove the template. Reactions were stopped by adding 200 μL of stop solution (1% SDS, 20 mM EDTA, 100 mM LiCl, 10 mM aurin tricarboxylic acid) and 300 μL of phenol/chloroform and vortexing for 30 sec. Reactions were centrifuged for 10 min at 4°C, and supernatants were transferred to fresh tubes with 75 μL of 10 M NH_4OAc and 940 μL of ethanol. Labeled transcripts were precipitated 30 min at −70°C and centrifuged 30 min at 4°C. Pellets were resuspended in 500 μL of DEPC-treated H_2O and diluted into 5 mL of hybridization solution. Incorporation of radioactivity was monitored by removing duplicate 2-μL samples at the start of each reaction and at 20 min and spotting the labeled probe onto DE-81 filters. Filters were washed five times for 5 min each in 5 mL per filter of 5% Na_2HPO_4 (dibasic) followed by two H_2O rinses and two ethanol rinses. Filters were then air dried and radioactivity was determined.

Plasmids containing the *RbcS-1*, *RbcS-2*, *RbcS-3*, and *RbcS-4* 3′ noncoding regions, 18S rRNA, actin, tubulin, and *Cab* were linearized, phenol extracted, precipitated, resuspended, and quantitated using a spectrophotometer. The plasmids were then denatured and slot blotted to nitrocellulose membranes (5 μg per slot). Plasmids containing other ice plant gene probes were also included. These were specific for the following genes: *Ppc1* (Crassulacean acid metabolism form of phosphoenolpyruvate carboxylase-1; Cushman et al., 1989), *Ppc2* (C3 form of phosphoenolpyruvate carboxylase-2; Cushman et al., 1989), *Ppdk* (pyruvate orthophosphate dikinase; E. J. DeRocher and H. J. Bohnert, unpublished data), *Mdh1* (malate dehydrogenase-1; Cushman, 1992), *Mod1* (malic enzyme-1; Cushman, 1993), *Gpd1* (glyceraldehyde-*P*-dehydrogenase-1; Ostrem et al., 1990), *Fnr1* (ferredoxin $NADP^+$ oxidoreductase-1; Michalowski et al., 1989b), and *Prk1* (phosphoribulokinase-1; Michalowski et al., 1992). pBluescript SK+ was slot blotted as a control for cross-hybridization to vector sequences. No vector hybridization was detected. Lowest backgrounds were obtained by prehybridizing blots at 42°C for at least 24 hr. Hybridizations of labeled run-on transcripts performed at 42°C for at least 48 hr in a shaking water bath. Blots were washed 1 hr at room temperature in 1 × SSC and 1 hr at 55°C in 0.1 × SSC. Hybridization signals were quantitated directly from the blots using a Betascope. Run-on transcription assays were performed twice for the NaCl stress, development, and combined NaCl stress/development experiments. The values for the 18S rRNA hybridization signals, after subtracting background, were used to calculate normalization factors setting the 18S rRNA signals equal to each other. These normalization factors were used to calculate the relative hybridization signals for the *RbcS* genes (minus background). Values obtained from duplicate sets of assays were averaged and used to generate graphs presented in the Results section. The 18S rRNA normalization factors were also used to calculate exposure times to give normalized autoradiograms. Calculation of the relative *RbcS* hybridization signals from normalized actin or tubulin hybridization signals gave results similar to those obtained using 18S rRNA as a basis for normalization.

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