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Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of the Clp protease subunits during senescence of *Phaseolus vulgaris* (L.) leaves

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Abstract. Our objective was to determine the coordination of transcript and/or protein abundances of stromal enzymes during leaf senescence. First trifolioliate leaves of *Phaseolus vulgaris* L. plants were sampled beginning at the time of full leaf expansion; at this same time, half of the plants were switched to a nutrient solution lacking N. Total RNA and soluble protein abundances decreased after full leaf expansion whereas chlorophyll abundance remained constant; N stress enhanced the decline in these traits. Abundances of ribulose-1,5-bisposphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), Rubisco activase and phosphoribulokinase (Ru5P kinase; EC 2.7.1.19) decreased after full leaf expansion in a coordinated manner for both treatments. In contrast, adenosine diphosphate glucose (ADPGlc) pyrophosphorylase (EC 2.7.7.27) abundance was relatively constant during natural senescence but did decline similar to the other enzymes under N stress. Northern analyses indicated that transcript abundances for all enzymes declined markedly on a fresh-weight basis just after full leaf expansion. This rapid decline was particularly strong for the Rubisco small subunit (*rbcS*) transcript. The decline was enhanced by N stress for *rbcS* and Rubisco activase (*rca*), but not for Ru5P kinase (*prk*) and ADPGlc pyrophosphorylase (*agp*). Transcripts of the Clp protease subunits *clpC* and *clpP* declined in abundance just after full leaf expansion, similar to the other mRNA species. When Northern blots were analyzed using equal RNA loads, *rbcS* transcripts still declined markedly just after full leaf expansion whereas *rca* and *clpC* transcripts increased over time. The results indicated that senescence was initiated near the time of full leaf expansion, was accelerated by N stress, and was characterized by large decline in transcripts of stromal enzymes. The decreased mRNA abundances were in general associated with steadily declining stromal protein abundances, with ADPGlc pyrophosphorylase being the notable exception. Transcript analyses for the Clp subunits supported a recent report (Shanklin et al., 1995, Plant Cell 7: 1713–1722) indicating that the Clp protease subunits were constitutive throughout development and suggested that ClpC and ClpP do not function as a senescence-specific proteolytic system in *Phaseolus*.

Key words: Clp (protease) – Gene expression – Leaf senescence – *Phaseolus* (senescence) – Stromal enzymes

Introduction

Leaf senescence is a complex developmental process characterized most prominently by a decline in the photosynthetic competence of the leaf (Feller and Fischer 1994; Smart 1994). Distinctive changes in gene expression during senescence have established that the process is under genetic control (Hensel et al. 1993; Gan and Amasino 1995). It has been well documented that the decline in CO_2 fixation during senescence is closely associated with declining activity and levels of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Makino et al. 1985; Jiang et al. 1993). Other thylakoid and stromal proteins have also been shown to decrease in abundance during senescence (Roberts et al. 1987; Bate et al. 1991; Crafts-Brandner et al. 1990; Kamachi et al. 1992). Declining abundances of chloroplast enzymes have generally, but not always, been associated with decreased abundances of specific transcripts (Bate et al. 1991; Kamachi et al. 1992).

Because of its importance in catalyzing CO_2 fixation and in providing an abundant N source for developing sinks, Rubisco has been the focus of many senescence studies. With few exceptions most of the information pertaining to senescence-related changes in other soluble enzymes of carbon metabolism is based on enzyme activity data. We have previously shown that phosphoribulokinase (Ru5P kinase) and Rubisco abundances

Abbreviations: ADPGlc = adenosine diphosphate glucose; *agp*, *prk*, *rbcS*, *rca* = genes encoding the large subunit of ADPGlc pyrophosphorylase, Ru5P kinase, the small subunit of Rubisco and Rubisco activase, respectively; Ru5P kinase = phosphoribulokinase; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

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decline in a coordinated manner during soybean senescence (Crafts-Brandner et al. 1990) and that ADP-glucose (ADPGlc) pyrophosphorylase activity does not decline as extensively as Rubisco activity during soybean and tobacco leaf senescence (Crafts-Brandner 1992). It has also been reported that the activities of several Calvin cycle enzymes coordinately decline during senescence (Makino et al. 1983). Understanding how protein and transcript abundances of enzymes of photosynthetic carbon metabolism are coordinated with each other during senescence may provide insights into the mechanism of degradation of stromal enzymes.

Recent reports have indicated that degradation of stromal enzymes can occur in intact-isolated-chloroplasts (Mitsuhashi and Feller 1992). However, there are only a few reports of chloroplast proteolytic activities that may use soluble proteins as substrates (Bushnell et al. 1993) and no information describing how the proteases recognize the target substrates. Recently a chloroplast-localized proteolytic system analogous to the Clp system of *Escherichia coli* (Maurizi 1992) has been shown to be constitutive in several plant species (Shanklin et al. 1995). This protease is composed of at least two subunits, ClpP which is the proteolytic subunit and is chloroplast-encoded, and ClpC (analogous to ClpA in *E. coli*) which is a nuclear-encoded ATPase. The function of this protease in plants is unknown.

In this study we have determined the coordination of the abundances of several key stromal enzymes involved in photosynthetic carbon metabolism during development of a leaf beginning at the time of maximum leaf area formation. Additionally, we determined how transcript abundances were related to changes in protein abundances and we provide the first information on transcript abundances of two subunits of the Clp protease system throughout natural and N-stress-induced senescence.

Materials and methods

Plant culture. Seeds of *Phaseolus vulgaris* L. cv. Saxa were germinated on paper towels and planted in quartz gravel. After 7 d the seedlings were transplanted into 1-L containers (four plants per container), and grown hydroponically in aerated half-strength complete nutrient solution (Hildbrand et al. 1994). At 14 d after germination the growth medium was changed to a full-strength complete nutrient solution. Nutrient solutions were changed every 5 d. Plants were grown using a 14-h photoperiod with a photon fluence rate of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. Day/night temperatures were 25°C/21°C, respectively.

Sampling. Sampling was started when first trifolioliate leaf expansion was > 95% of the maximum; plants were harvested at the end of the dark cycle. At this same time, the nutrient solution in half of the containers was replaced by a minus-N solution that contained 460 $g \cdot m^{-3}$ KH_2PO_4, 300 $g \cdot m^{-3}$ K_2HPO_4, 231 $g \cdot m^{-3}$ $CaSO_4 \cdot 2H_2O$, 200 $g \cdot m^{-3}$ $CaCO_3$, and 750 $g \cdot m^{-3}$ $MgSO_4 \cdot 7H_2O$. Micronutrients were as described in Hildbrand et al. (1994). There were no visible nodules on the roots throughout the experiment. In two independent experiments plants were sampled at 0, 4, 8, and 12 d (Expt. 1) or 0, 4, 8, 12, and 16 days (Expt. 2) after full leaf expansion. At each sampling time two replicates, each consisting of the first trifoliolate leaves from three plants, were placed on ice and taken to the laboratory for processing. Three leaf discs (one from each of the three middle leaflets) were weighed fresh and used for chlorophyll extraction. Leaf discs removed from one half of all leaflets from a replicate were frozen at − 80°C for subsequent Western analyses. The other half of each leaflet for both replicates were combined and used for RNA extraction from the fresh tissue. Results for both experiments were combined and are presented as means ± standard error of the mean (SE). For the sample taken at 16 days after full leaf expansion (Expt. 2) the protein and chlorophyll data are presented as the mean ± SE of the two replicates; for total RNA and Northern analyses there was no replication for this sample.

Western analysis. Western analysis was performed as previously described (Tijssen 1985; Fröhlich and Feller 1991) using sample aliquots that were adjusted to represent equal amounts of leaf fresh weight. Sodium dodecyl sulfate-polyacrylamide gels were used for analysis of Rubisco abundance based on the intensity of staining of the large subunit. For ADPGlc pyrophosphorylase, samples were electrophoresed in 9% native polyacrylamide gels in order to separate this protein from Rubisco. Antibodies for ADPGlc pyrophosphorylase were commercially prepared (East Acres Biologicals, Southbridge, Mass., USA) in rabbits according to procedures described in Crafts-Brandner et al. (1990), using purified tobacco enzyme. Antibodies for Rubisco activase and Ru5P kinase were prepared as previously described (Crafts-Brandner et al. 1990; Salvucci et al. 1993). Protein abundance was estimated by scanning gels and blots using an image acquisition densitometer (BioImage, Milligen/Bioresearch, Ann Arbor, Mich., USA).

Extraction of RNA and Northern analysis. Total RNA was isolated from 450 mg of fresh leaf tissue (frozen and powdered) using 5 mL of TRIzol Reagent (Gibco BRL Life Technologies, Inc., Gaithersburg, Md., USA) according to the protocol supplied by the manufacturer. Polyadenylated RNA was isolated from 80 µg of total RNA using magnetic beads conjugated with oligo-dT according to the protocol supplied by the manufacturer (Dynal Inc., Lake Success, N.Y., USA).

Samples of RNA (ranging from 2 to 9 µg total RNA) were electrophoresed through formaldehyde-agarose gels (Sambrook et al. 1989) prior to transfer to, and fixation on, positively charged nylon membranes. Antisense-RNA probes were synthesized from DNA templates using digoxigenin-UTP, hybridization was performed overnight at 58°C, and stringency washes were performed according to protocols provided by Boehringer Mannheim (Indianapolis, Ind., USA). Hybridized probes were detected using the chemiluminescent substrates CDP or CDP-Star (Boehringer Mannheim) after incubation of the blots with an anti-digoxigenin antibody conjugated to alkaline phosphatase. Northern blots for *rbcS*, *rca* and *clpC* were developed by reprobing the same blot following procedures outlined by the manufacturer (Boehringer Mannheim).

For isolating a partial cDNA for ADPGlc pyrophosphorylase, degenerate oligonucleotide primers were designed against two conserved regions of the primary structure of the protein with the number of degeneracies in each pool reduced by the alignment of nucleotide sequences of known ADPGlc pyrophosphorylase (Smith-White and Preiss 1992) and by methods as previously described (Klein et al. 1993). The gene-specific (sense) primer was 5′-TAT[A,C]GICTIATIGATATTCCI[A,G]TIAG[T,C]AA[T,C]TG (region Y209 to C219) of the primary structure of known *agp* (Smith-White and Preiss 1992). The antisense ADPGlc pyrophosphorylase-specific primer was 5′-AAIG[A,C]TTTIATIGT[T,A]CCGATGTC[T,C]TCCCA[A,G]TA (region Y460 to F470). Bracketed nucleotides refer to an equal mixture of those bases at that position. First-strand cDNA synthesis and conditions for polymerase chain reaction (PCR) were as described (Klein et al. 1993). After PCR amplification, a single 780-bp product was detected (data not shown).

A similar strategy was employed for the isolation of a partial cDNA for *clpC* from tobacco RNA. Utilizing the amino acid sequences of known Clp-like sequences (Gottesman et al. 1990), the gene specific (sense) primer 5′-GC[T,G]GGIACIAAATATC-GIGGIGA[C,G]TT[T,C]GA[G,A]GA (region A344 to E354)

was synthesized. The antisense Clp-specific primer was 5'-GTTGAA[C,G]ACGTCIGGGTG[A,C,G,T]GC[C,T]TTTTC (region E722 to N730). Conditions for PCR amplification were as described by Klein et al. (1993) except the annealing temperature was reduced to 47°C for the first 4 cycles and then increased to 51°C for the subsequent cycles. Following amplification, an 1160-bp product was detected (data not shown). For the isolation of a *clpP* cDNA, total RNA was isolated from intact tobacco chloroplasts as described by Klein and Mullet (1986) and, following DNase-treatment, first-strand cDNA was generated. The PCR primers were designed against the known nucleotide sequence of the tobacco chloroplast *clpP* gene (Shinozaki et al. 1986). The gene specific primer was 5'-ATCATATGCCTATTGGTGTTCC and the antisense primer was 5'-TTCTCGAGATAAAATCTCAAATCAC. Amplification conditions were the same as those for *clpC*. Following amplification, a 650-bp *clpP* fragment was generated (data not shown). Following cloning and transformation, recombinant clones of *clpC*, *clpP* and *agp* were selected and confirmed by restriction enzyme digestion and nucleotide sequence analysis.

The probe for *rbcS* was a 743-bp antisense RNA from tobacco and the probe for *rca* was a 1300-bp antisense RNA from tobacco (Klein and Salvucci 1995); the probe for *prk* was a 1500-bp antisense RNA from ice plant and was kindly provided by Dr. H.J. Bohnert, University of Arizona, USA.

Northerns blots were scanned using an image acquisition densitometer (BioImage, Milligen/Bioresearch, Ann Arbor, Mich., USA) to obtain a relative estimate of the mRNA abundances.

Chlorophyll and soluble protein. Chlorophyll was extracted from three leaf discs (75 mm^2 per disc) in 5 mL of methanol as previously described (Crafts-Brandner et al. 1990). Soluble protein was determined using the extracts prepared for Western analysis by the method of Bradford (1976).

Results

During the first 4 d after full leaf expansion there was a precipitious decline in leaf total RNA abundance per unit fresh weight (Fig. 1A). The amount of polyadenylated RNA was a relatively constant proportion of the total RNA (2.2 ± 0.2% when averaged for all samples), indicating that this RNA fraction declined in parallel with the total RNA. Between 4 and 16 d after full leaf expansion the total RNA abundance declined slowly and the decline was marginally enhanced by N stress.

Soluble protein abundance was maximum at the time of full leaf expansion and declined consistently thereafter, N stress enhanced the decline in soluble protein abundance (Fig. 1B). Chlorophyll abundance did not change over time for controls and was therefore not correlated with senescence (Fig. 1C). Even under N stress the chlorophyll abundance was not affected until 12 d after removing N from the growing medium. Although chlorophyll abundance is often a good indicator of the senescence process, our results were not exceptional and re-emphasize that caution is needed when using cholrophyll to monitor leaf senescence (Smart 1994).

The decline in soluble protein abundance during senescence was related to changes in several stromal enzymes (Fig. 2). The abundances of Rubisco, Rubisco activase and Ru5P kinase all were at an apparent maximum at the time of full leaf expansion and progressively declined thereafter (Fig. 2A–C). In contrast to Rubisco, Rubisco activase and Ru5P kinase, the abundance of ADPGlc pyrophosphorylase was relatively constant up to 12 d after full leaf

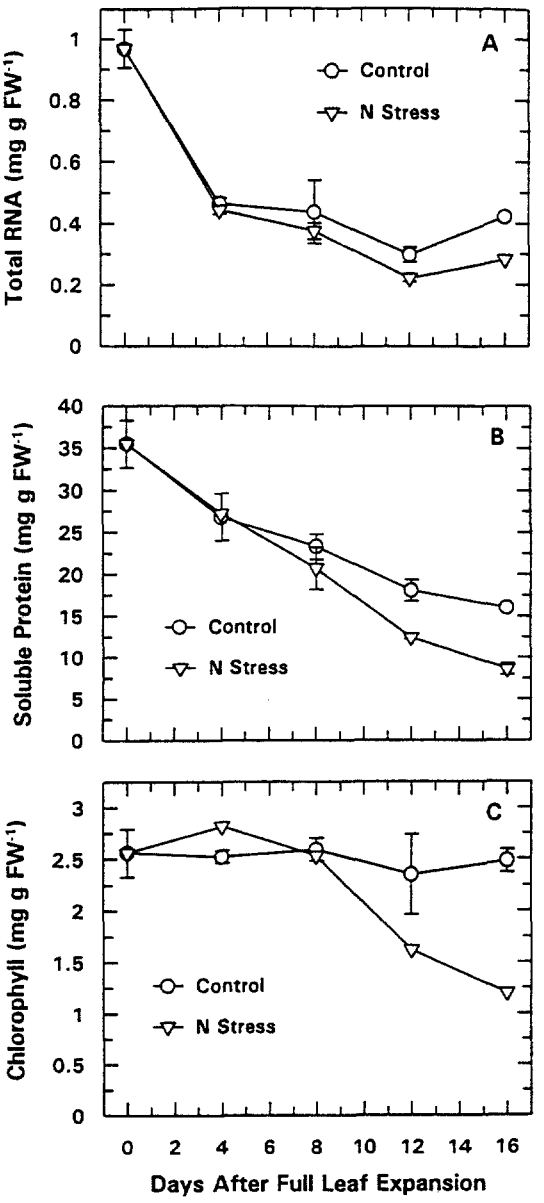

Fig. 1A–C. Abundances of total RNA (**A**), soluble protein (**B**) and chlorophyll (**C**) per unit leaf fresh weight during natural- and N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Data are presented as means ± SE

expansion (Fig. 2D, control). At 16 d, ADPGlc pyrophosphorylase abundance was still approximately 70% of the amount at full leaf expansion (Fig. 2D, control).

For N-stressed plants, all of the stromal enzymes that were analyzed declined more rapidly than for controls (Fig. 2A–D). In contrast to the situation of natural senescence, ADPGlc pyrophosphorylase was only slightly more persistent than the other enzymes during N-stress-induced senescence.

Northern blot analyses (RNA loads based on equal fresh weight) indicated a general decline in specific mRNA species beginning just after the time of full leaf expansion (Fig. 3). The decline in mRNA abundance was especially precipitious for *rbcS*, which declined 70% by four days

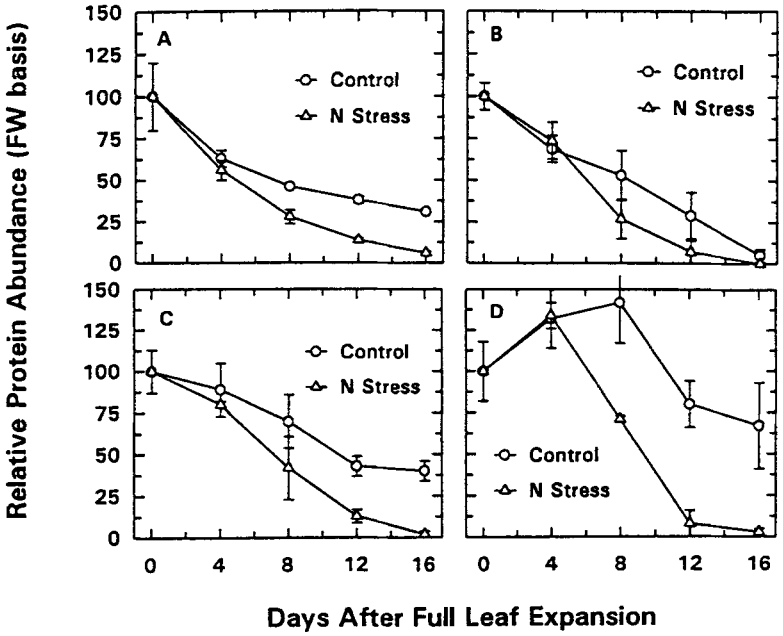

Fig. 2A–D. Abundances of stromal enzymes Rubisco (**A**, based on the Rubisco large subunit), Rubisco activase (**B**), Ru5P kinase (**C**) and ADPGlc pyrophosphorylase (**D**) per unit leaf fresh weight during natural- and N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Data are presented as relative values ± SE with the data for zero days after full leaf expansion set at 100

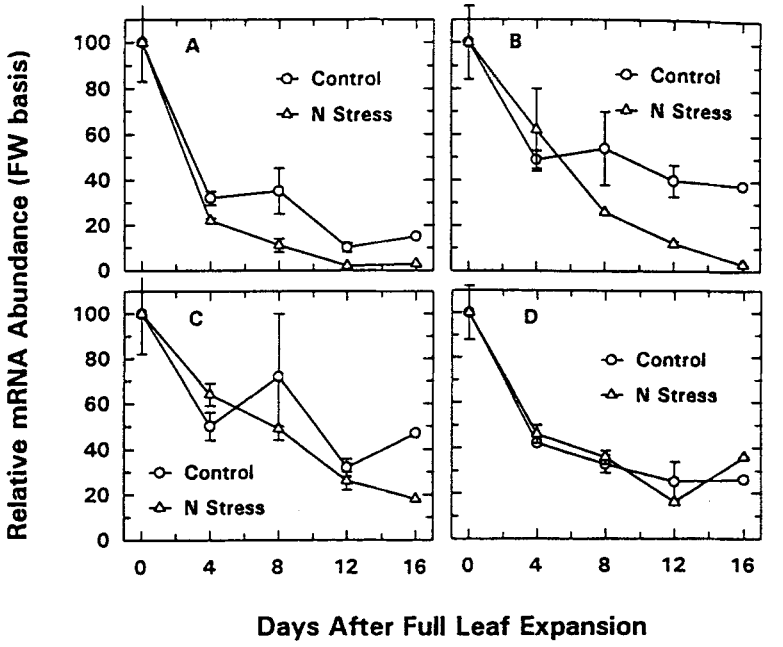

Fig. 3A–D. Abundances of mRNA for *rbcS* (**A**), *rca* (**B**), *prk* (**C**), and *agp* (**D**) per unit leaf fresh weight during natural- and N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Data are presented as relative values ± SE with the data for zero days after full leaf expansion set at 100

after full expansion (Fig. 3A). After the initial decline, transcript abundances declined slowly between 4 and 16 d after full leaf expansion (Fig. 3A–D). Comparison of Western and Northern analyses indicated a general coordination between protein and mRNA abundances for Rubisco, Rubisco activase and Ru5P kinase during natural senescence. For ADPGlc pyrophosphorylase such a relationship was not observed for controls since protein abundances did not parallel the observed decline in *agp* transcript abundance (Figs. 2, 3). Nitrogen starvation enhanced declines in mRNA abundances for *rbcS* and *rca* but had minimal or no effect on *prk* and *agp* mRNA species, respectively (Fig. 3A–D).

Abundances of mRNA species for *clpC* and *clpP* (Fig. 4) declined after full leaf expansion in a manner similar to that of the other stromal proteins examined (Fig. 3). Two relevant points with regard to the Northern analysis for *clpC* and *clpP* are: (i) the mRNA species were easily detectable throughout the development of senescence and (ii) N starvation tended to increase the abundance of *clpC* mRNA, relative to controls, during the latter stages of senescence (Fig. 4A).

The data presented in Figs. 3 and 4 were expressed on a tissue-fresh-weight basis to give as much physiological significance to the results as possible. For comparative purposes, *rbcC*, *rca* and *clpC* transcript abundances were

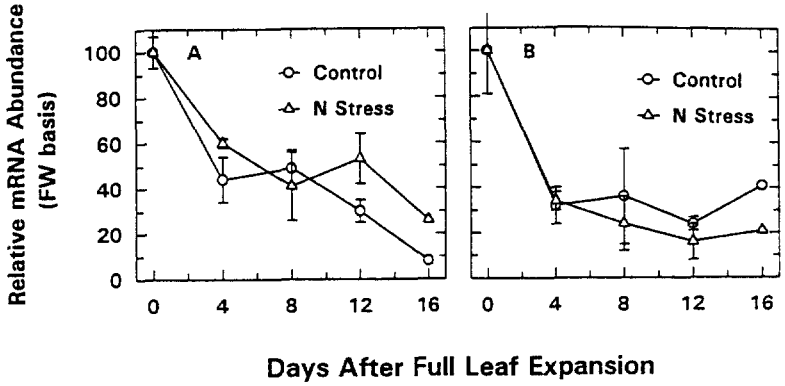

Fig. 4A, B. Abundances of mRNA for *clpC* (**A**) and *clpP* (**B**) per unit leaf fresh weight during natural- and N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Data are presented as relative values ± SE with the data for zero days after full leaf expansion set at 100

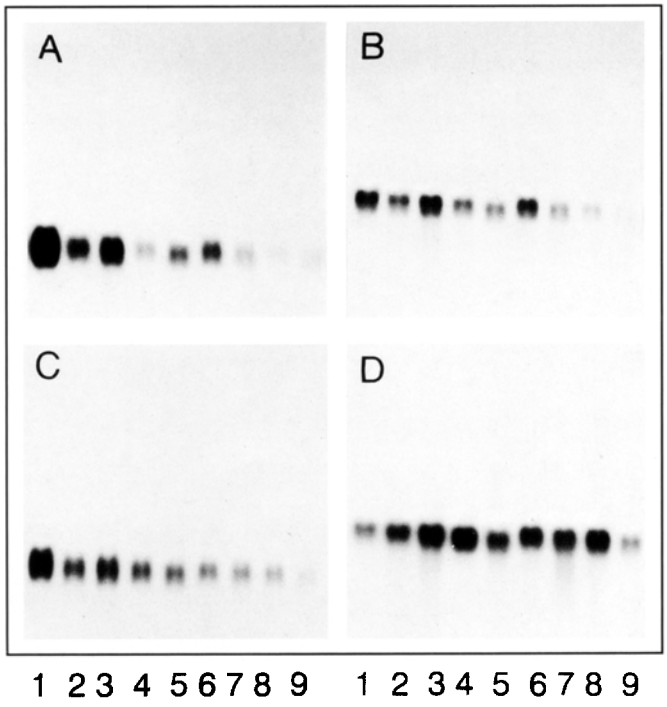

Fig. 5A–D. Representative Northern blots showing changes in abundances of *rbcS* (**A**, **C**) or *rca* (**B**, **D**) during natural- or N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Blots in **A** and **B** were based on total RNA loads corresponding to equivalent leaf fresh weight and blots in **C** and **D** were based on equal amounts (5 μg) of total RNA. *Lanes 1–5* correspond to controls at 0, 4, 8, 12 and 16 d after full leaf expansion, respectively and *lanes 6–9* correspond to the N-stress treatment at 4, 8, 12 and 16 d after full leaf expansion, respectively

quantitated on either a fresh-weight or an equal-RNA basis and representative Northerns for *rbcS* and *rca* are shown in Fig. 5. Regardless of the method, the *rbcS* mRNA declined markedly just after full leaf expansion and N starvation enhanced the decline (compare Figs. 3 and 6). The mRNA abundances for *rca*, however, actually increased after full leaf expansion for both controls and N-stressed plants when expressed on an equal-RNA basis (Fig. 6B); however, the N-starvation treatment did tend to decrease the mRNA abundances compared to controls at a given sampling time. The *clpC* mRNA abundance remained relatively constant over time for controls but was increased at any given sampling time by the N-starvation treatment (Fig. 6C). The results represented in Figs. 3–6 indicated that caution is required when making physiological inferences from Northern loaded with equal total RNA from tissues sampled over long-term developmental periods.

Discussion

It can be assumed from other reports that photosynthetic competence is at a maximum near the time of full leaf expansion (Rawson and Constable 1981; Hensel et al. 1993; Makino et al. 1985). Our results indicate that just as the first trifoliolate leaves reached full expansion, the transcript abundances of several stromal enzymes involved in photosynthetic carbon metabolism markedly declined in a manner coordinate with the decline in total RNA and soluble protein, as well as the abundances of several, but not all, specific stromal enzymes. These results demonstrate a pattern of development that may be common for annual plants. The progression of the senescence process can vary greatly depending on species, response to source-sink effects, and environmental factors (Kelly and Davies 1988; Feller and Fischer 1994; Smart 1994). In particular the effects of source-sink manipulations on senescence are highly variable and have led to much discussion about the nature of the regulatory control of senescence (Kelly and Davies 1988). However, despite the variable inferences from studies of source-sink manipulation, the overriding observations suggest that the leaves of annual plants become predisposed to senesce near the time of full leaf expansion for both vegetative and reproductive plants. From the recent work of Gan and Amasino (1995) it can be inferred that the rate and extent of senescence can be profoundly affected by endogenous cytokinin biosynthesis.

The large decline in both total RNA (largely rRNA) and in specific mRNAs indicated a loss of cellular protein synthesis capacity when the leaves reached full expansion (Fig. 2). Several reports have indicated that mRNA and protein abundances for Rubisco decline markedly during senescence (Mae et al. 1983; Bate et al. 1991; Jiang et al. 1993). Our results indicated that the progressive decline in

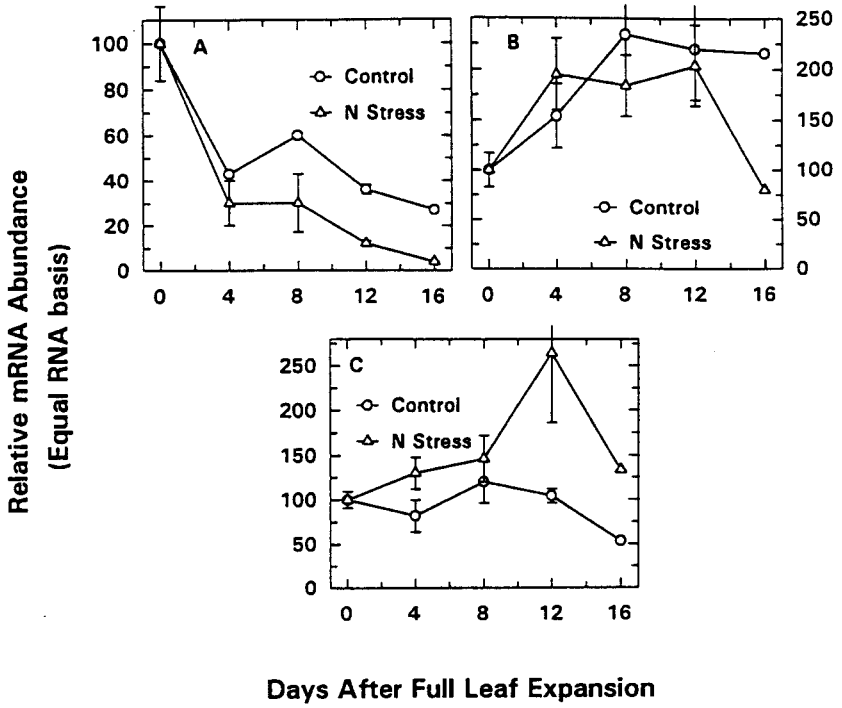

Fig. 6A–C. Abundances of mRNA for *rbcS* (**A**), *rca* (**B**) and *clpC* (**C**) per unit total RNA during natural- and N-stress-induced senescence of first trifoliolate leaves of *Phaseolus vulgaris*. Data are presented as relative values ± SE with the data for zero days after full leaf expansion set at 100

soluble protein (Fig. 1B) was closely associated with the decline in abundances of several stromal enzymes, most notably Rubisco, but also Rubisco activase and Ru5P kinase. This coordinated loss of stromal enzymes that differ greatly in abundance (Crafts-Brandner et al. 1990) indicates a highly regulated mechanism for dismantling various components of the carbon assimilation pathway. However, the stability of ADPGlc pyrophosphorylase during the time of rapid loss of other stromal enzymes indicated that the synthesis/degradation of this enzyme was regulated differently than for Rubisco, Rubisco activase and Ru5P kinase. Since all transcripts analyzed declined markedly just after full leaf expansion it is hypothesized that ADPGlc pyrophosphorylase was not as susceptible to degradation as the other stromal enzymes. Since altered solute levels may influence the stability of a protein (Mitsuhashi and Feller 1992; Feller and Fischer 1994), it is possible that increased adenylates derived from RNA catabolism stabilized ADPGlc pyrophosphorylase in our system. We also observed from stained SDS-polyacrylamide gels loaded with extract corresponding to equal amounts of leaf fresh weight that the abundance of the light-harvesting chlorophyll *a*/*b*-binding protein (LHCII) did not change appreciably during senescence (data not shown), consistent with the lack of change in chlorophyll abundance (Fig. 1C). Previously, Bate et al. (1991) and Hensel et al. (1993) have reported that LHCII transcript abundance and synthesis declined during senescence. Thus, it can be inferred that LHCII, similar to ADPGlc pyrophosphorylase, was less susceptible to proteolytic degradation during senescence than other photosynthesis-related proteins examined.

The early and large decline in both Rubisco protein and *rbcS* transcript abundances in our experiments indicated that this is a key regulatory point for the senescence process. The salient question as to what causes stromal enzymes such as Rubisco to decline during senescence remains unanswered. Whether the process can be explained on the basis of photosynthate allocation, hormonal changes, or other factors (Kelly and Davies 1988; Feller and Fischer 1994; Smart 1994) cannot be addressed by the present results. It has been demonstrated that chloroplasts isolated from healthy leaves can degrade stromal enzymes (Mitsuhashi and Feller 1992). This result suggests the possibility that individual protein species are in some manner altered prior to degradation by a pre-existing chloroplast proteolytic system. Our results have demonstrated the presence throughout senescence of transcripts for the two components of the Clp protease system and it is tempting to invoke an association of this protease system with senescence. It has recently been demonstrated that the translation products for these genes are localized in the stroma during all stages of leaf development (Skanklin et al. 1995). Although we have shown that the transcript for *clpC* marginally increases in response to N starvation (Fig. 4), our results would support the general conclusion of Shanklin et al. (1995) that Clp is constitutive in chloroplasts and probably functions in some housekeeping aspect of protein catabolism rather than as a regulatory component of the senescence process.

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