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A methyl jasmonate-induced shift in the length of the 5′ untranslated region impairs translation of the plastid *rbcL* transcript in barley

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The plant growth substance (−)-jasmonic acid methyl ester (methyl jasmonate, JaMe) affects plastid gene expression at the protein and mRNA levels when applied exogenously to detached leaf segments of *Hordeum vulgare* L. cv. Salome. Translation of the large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (LSU, *rbcL* gene products) and of the 65 and 68 kDa proteins of photosystem I (*psaA* and *psaB* gene products, respectively) ceased, whereas synthesis of the 32 kDa photosystem II protein (D1, *psbA* gene product) continued in JaMe-treated leaf tissues. These changes were not caused by corresponding alterations in transcript abundances. The loss of LSU protein synthesis, occurring within 24 h of JaMe treatment, correlated with a decline in the *in vitro*-translatable *rbcL* mRNA, but contrasted with an almost constant transcript level. The 5′ ends of the *rbcL* transcripts shifted from '−59' in freshly harvested or water-treated leaves to '−94' in JaMe-treated leaf tissues. Transcripts ending at these positions presumably arise from alternative processing of the primary transcript ending at position '−316'. The '−94' transcript contains, within the 5′ untranslated region, a 35-base motif with remarkable complementarity to the extreme 3′ terminal part of the 16S rRNA, involved in intramolecular base pairing within the ribosome and can associate with 30S but not 70S complexes *in organello*, suggesting that intermolecular base pairing impairs translation initiation, probably by competing for ribosome binding at the Shine–Dalgarno sequence. In contrast, transcripts ending at '−59' lack the 5′ terminal 'extra' sequence and are active in terms of translation initiation.

Key words: barley (*Hordeum vulgare* L. cv. Salome)/(−)-jasmonic acid methyl ester/plastid gene expression/*psaA-psaB*, *psbA* and *rbcL* transcripts/translational control

Introduction

Jasmonates are naturally occurring cyclopentanone compounds that are widespread in the plant kingdom (Demole *et al.*, 1962; Crabalona, 1967; Yamane *et al.*, 1981; Meyer *et al.*, 1984; Sembdner and Gross, 1986). Their ubiquity has been interpreted as an indication that jasmonates might have a similar function in various plant species, either as hormonal regulators or as stress factors (Parthier, 1990, 1991; S.Reinbothe *et al.*, 1992a,b). Among the pleiotropic effects observed in barley and various other plant species [see Parthier (1990, 1991) for references], the JaMe-induced tissue- and species-specific accumulation of novel abundant polypeptides, designated jasmonate-induced proteins (JIPs; Parthier *et al.*, 1987), was the most remarkable event (Weidhase *et al.*, 1987; Müller-Uri *et al.*, 1988; Herrmann *et al.*, 1989; S.Reinbothe *et al.*, 1992a,b).

JIPs have been shown to be proteinase inhibitors involved in plant defence reactions in tomato, potato and alfalfa (Farmer and Ryan, 1990, 1992; Farmer *et al.*, 1992), and vegetative storage proteins (VSPs) in soybean (Anderson, 1988; Anderson *et al.*, 1989; Mason and Mullet, 1990; Staswick, 1990; Bell and Mullet, 1991; Tranbarger *et al.*, 1991). Interestingly, VSPs from diverse plant species, such as *Sophora japonica* (Herman *et al.*, 1988), potato (Andrews *et al.*, 1988) and soybean (Bell and Mullet, 1991; Tranbarger *et al.*, 1991), possess lectin-like properties, lipid acyl hydrolase and lipoxygenase activities, respectively. In cotton and barley (S.Reinbothe *et al.*, 1992a,b), several JIPs seem to belong to the group of late embryogenesis abundant (LEA) proteins, which are normally expressed during embryogenesis to mitigate the physico-chemical constraints encountered in the dormant embryo (Baker *et al.*, 1988; Dure *et al.*, 1989; C.Reinbothe *et al.*, 1992a,b). Another barley JIP has recently been identified as a leaf thionin (Andresen *et al.*, 1992). Even if quite distinct, all of the induced proteins seem to share an involvement in a specific set of stress responses which are probably mediated by jasmonates.

Another aspect of the JaMe-induced alteration of gene expression in barley pertains to the negative effect that this compound exerts on the translation of those leaf mRNAs, which are already present before JaMe treatment (Weidhase *et al.*, 1987). This aspect is reflected in the maintenance of almost all *in vitro*-translatable mRNAs in jasmonate- and water-exposed leaf tissues, in contrast to an apparent lack of *in vivo* synthesis of the respective proteins (Müller-Uri *et al.*, 1988). In the particular case of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase), the key enzyme of photosynthetic carbon dioxide fixation (Miziorko and Lorimer, 1983), amino acid incorporation into the large (LSU) and small subunits (SSU) stops after 5–8 h of JaMe treatment (Müller-Uri *et al.*, 1988). For the RuBPCase SSU and a member of the light harvesting chlorophyll *a/b* binding protein family, recent experiments demonstrate that their synthesis is repressed at the level of translation initiation in JaMe-treated leaf tissues (Reinbothe *et al.*, 1993).

In the present study we investigate plastid gene expression with particular reference to the LSU of RuBPCase, and demonstrate that JaMe influences LSU formation primarily at the level of transcript functionality (translatability).

Results

Formation of plastid proteins

We have studied the phenomenon of restricted protein synthesis in JaMe-treated barley leaf segments by comparing the populations of total leaf and plastid proteins. As shown in Figure 1A, Coomassie-staining of the total protein patterns revealed the accumulation of novel protein species of M_r 66 000, 37 000, 30 000, 23 000 and 12 000 (marked by triangles in lane 2, compare with lane 1), which have previously been identified as abundant JIPs (Parthier *et al.*, 1987; Weidhase *et al.*, 1987; Müller-Uri *et al.*, 1988; S.Reinbothe *et al.*, 1992b). None of them could be found among plastid proteins (cf. lanes 2 and 4). Several other proteins were present in lower amounts in JaMe-treated leaf segments than in water-treated ones (cf. lanes 2 and 1). Among them were the LSU of RuBPCase, and the 65 and 68 kDa photosystem I proteins (cf. lanes 4 and 3). Their decreased content in JaMe-exposed segments compared with water-exposed ones (Figure 1C, compare lane 10 with lane 9, and lane 14 with lane 13) correlated with their lowered rates of *in organello* synthesis (Figure 1B, cf. lanes 6 and 5, filled triangles). Similarly, the formation of the nuclear-encoded SSU of RuBPCase was negatively influenced by JaMe (Figure 1C, compare lane 12 with lane 11, and lane 2 with 1). In contrast, only a weak effect was observed on the synthesis and accumulation of the plastid-encoded 32 kDa (D1) protein of photosystem II, and several other plastid-encoded polypeptides (Figure 1B and C, lanes 5–8, circles).

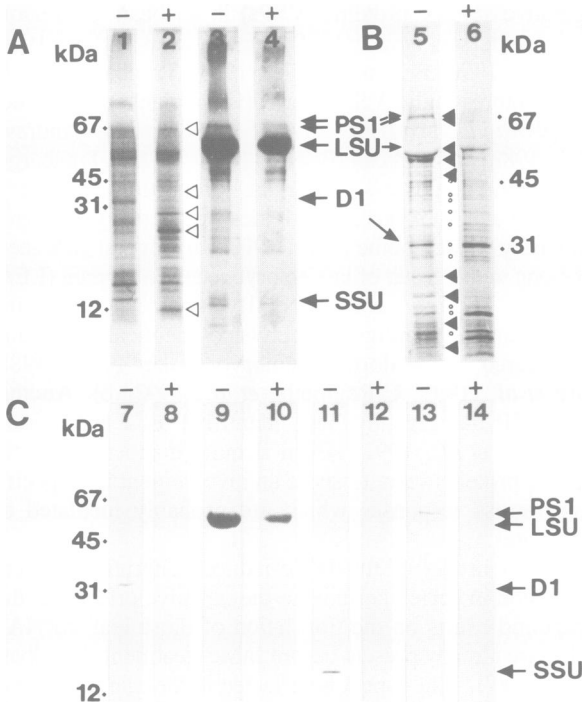

Fig. 1. Accumulation and synthesis of plastid proteins in barley leaf segments exposed to water (−) or JaMe (+). (**A**) Total leaf (lanes 1 and 2) or plastid (lanes 3 and 4) proteins were stained with Coomassie. The open triangles mark the positions of abundant JIPs among the total leaf proteins. The arrows indicate the positions of the plastid-encoded 68 and 65 kDa photosystem I proteins (PS 1), the RuBPCase LSU (LSU), the 32 kDa photosystem II protein (D1) and the nuclear-encoded SSU of RuBPCase, among plastid proteins. (**B**) *In organello* translation profiles. Proteins labelled for 1 h with [^{35}S]methionine in purified plastids of water- (lane 5) or JaMe-treated (lane 6) leaf segments were detected by autoradiography. The filled triangles mark proteins whose synthesis was lower in JaMe-treated segments than in water-treated segments. Circles mark constitutively synthesized proteins. (**C**) Western blot analyses with antisera raised against the D1 protein of photosystem II (lanes 7 and 8), LSU (lanes 9 and 10) and SSU (lanes 11 and 12) of RuBPCase and the 65 kDa protein of photosystem I (lanes 13 and 14). Molecular mass standards (bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; cytochrome *c*, 12 kDa) are indicated by dots in panels A–C.

Steady state content of plastid transcripts

In order to determine whether the lowered rates of protein synthesis observed for the LSU and the 65 and 68 kDa photosystem I proteins were caused by equivalent changes in their transcript abundances, RNA gel blots containing equal amounts of nucleic acids were hybridized with corresponding gene probes of spinach chloroplast DNA (for nomenclature of chloroplast genes, see Hallick and Bottomley, 1983). Figure 2 demonstrates that the detected *rbcL*, *psbA* and *psaA–psaB* transcript species had the expected sizes of 1.6, 1.3 and 5.8 kb, respectively, the latter transcript arising from co-transcription of the corresponding genes (Klein and Mullet, 1986). The levels of all of these transcripts changed only slightly during the first 24 h of JaMe treatment and were similar to those found in water-incubated

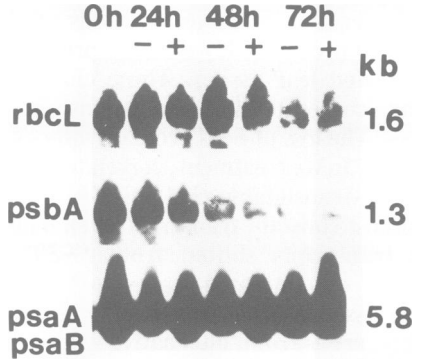

Fig. 2. Steady state level of plastid transcripts in water-treated (−) and JaMe-treated (+) leaf segments. RNA gel blots containing high molecular mass RNAs prepared from freshly harvested untreated leaves (0 h) or leaf tissues that had been exposed to water or JaMe for the indicated periods of time, were hybridized with the corresponding spinach gene probes. Nucleic acid hybrids were detected by autoradiography.

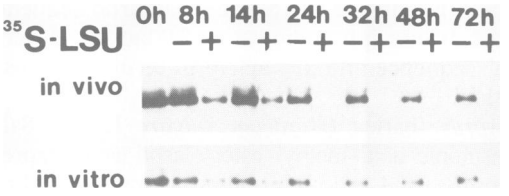

Fig. 3. LSU synthesis and *in vitro*-translatable *rbcL* mRNA content in water-treated (−) and JaMe-treated (+) leaf segments. Following a 2 h *in vivo* labelling with [^{35}S]methionine prior to harvesting of leaf tissues and ultrasonication, the RuBPCase LSU was immunoprecipitated from crude protein extracts with monospecific antibodies raised against the purified protein. The *rbcL* mRNA content was determined by *in vitro* translation of poly(A)$^-$ RNA in a rabbit reticulocyte lysate and immunoprecipitation with the anti-LSU antibodies.

leaves (Figure 2). At later stages, the transcripts accumulated differentially, however: JaMe promoted the decline of the *psbA* mRNA, and to a lesser extent of the *rbcL* mRNA, but antagonized the decline in the dicistronic *psaA−psaB* mRNA, in comparison with water-treated tissues (Figure 2).

The obviously different decay kinetics of the various transcript species (Figure 2), determined on an equal nucleic acid basis, were superimposed on a general lowering of the total RNA content on a per leaf basis in JaMe- or water-treated tissues (data not shown; see Reinbothe *et al.*, 1993).

Translatability of rbcL mRNA in vitro

Since the changes in transcript abundances occurring in JaMe-exposed segments after 24 h (Figure 2) did not correlate with the observed alterations in protein synthesis (Figure 1), a post-transcriptional type of control was implied.

We assumed that the obvious lack of synthesis of the RuBPCase LSU might be caused by a rapid and selective inactivation of the *rbcL* mRNA in JaMe-treated leaf segments. To test this idea, the rate of LSU synthesis and the *in vitro*-translatable *rbcL* mRNA content were compared in parallel for JaMe- and water-treated leaf segments in a time course experiment. For *in vitro* translation, a rabbit reticulocyte lysate was used (Pelham and Jackson, 1976) since it has been shown to translate not only eukaryotic-type mRNAs but also prokaryotic-type mRNAs, such as plastid RNAs (Camerino *et al.*, 1982). Plastid RNA was prepared from isolated chloroplasts (Klein and Mullet, 1986) or was enriched within the poly(A)$^-$ RNA fraction by binding of poly(A)$^+$ RNA to messenger affinity paper (Werner *et al.*, 1984).

Figure 3 demonstrates a rapid depression of LSU synthesis in JaMe-exposed leaf tissues, but a continued synthesis of this polypeptide in water-treated tissues. The formation of LSU ceased almost completely during the first 24 h of JaMe treatment. In contrast, LSU synthesis was reduced much less in water-exposed tissues, and remained detectable at a significant rate throughout the entire incubation period (Figure 3). In both JaMe- and water-treated leaf tissues, the changes in LSU labelling *in vivo* seemed to be caused by corresponding alterations in the *in vitro*-translatable *rbcL* mRNA level, determined with either poly(A)$^-$ (Figure 3) or plastid RNA (data not shown).

Table I summarizes our results on the expression of the RuBPCase LSU at the protein, steady state transcript and translatable mRNA levels in JaMe- and water-treated leaves. These results demonstrate that the loss of the translatable *rbcL* mRNA is the primary event down-regulating LSU formation in JaMe-incubated leaf tissues.

Table I. Expression of RuBPCase LSU in jasmonate- and water-treated barley leaf segments

Time (h)	*rbcL*					
	LSU synthesis		Transcript content		Translatable mRNA	
	JaMe	water	JaMe	water	JaMe	water
0	100	100	100	100	100	100
8	15.2	95.6	97.5	98.2	23.8	66.4
14	13.9	98.7	91.2	95.1	15.8	60.5
24	2.2	67.1	60.5	85.2	2.4	62.8
32	2.1	61.4	53.2	86.7	2.0	53.4
48	0.1	40.1	45.7	70.6	1.5	47.5
72	0.1	35.2	25.2	53.2	0.1	51.8

The rate of LSU synthesis was estimated after a 2 h pulse-labelling of total protein with [^{35}S]methionine and immunoprecipitation as described in the legend to Figure 3. The *rbcL* transcript content on an equal nucleic acid basis was determined by Northern blot hybridization with a spinach *rbcL* gene probe (see Figure 2). The translatable *rbcL* mRNA level was estimated from the amount of ^{35}S-labelled LSU formed by cell-free translation of poly(A)$^-$ RNA in a rabbit reticulocyte lysate (see Figure 3). All data are based on optical scanning of signals in the respective autoradiograms. They refer to an equal leaf number basis and are expressed as percentages of maximum level found in freshly harvested untreated leaf segments.

Mapping of the rbcL 5′ transcript ends

These results suggest that a possible mechanism for the selective inactivation of the *rbcL* mRNA in JaMe- versus water-treated leaf segments might be a modification in its 5′ non-coding region. Since covalent modifications common to eukaryotic-type mRNAs (e.g. 'capping', Shatkin, 1976; Darzynkiewicz *et al.*, 1988), have not as yet been reported for plastid messengers (Weil, 1987), a change in the length of the 5′ non-coding region was assumed to inactivate the *rbcL* mRNA in terms of protein synthesis. A method to demonstrate this is the precise mapping of the 5′ ends of the *rbcL* transcripts. Primer extension analyses and RNA sequencing reactions were carried out with plastid RNA from freshly harvested leaves and leaf tissues that had been exposed to JaMe or water for various periods of time. As shown in Figure 4, freshly harvested leaves expressed one abundant *rbcL* transcript species whose 5′ end was localized at position '−59', and a second transcript species of lower abundance ending at position '−316'. Both transcript ends are in close agreement with the data of Poulsen (1984) who suggested that the longer transcript arose from transcription initiation at '−10' and '−35' prokaryotic-type promoter elements (Jaurin *et al.*, 1981; van den Putte *et al.*, 1983),

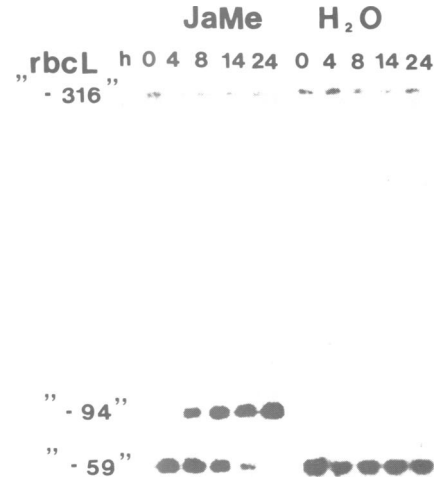

Fig. 4. Primer extension analysis of *rbcL* transcript ends. Plastid RNA from freshly harvested leaves (0 h) or leaf segments that had been exposed to JaMe or water for the indicated periods of time was used for reverse transcription using a *rbcL*-specific primer (see Materials and methods). The resulting copy DNAs were separated in urea-containing sequencing gels and detected by autoradiography. The exact location of the transcript ends was determined from the migration pattern of the 124 bp BRL ladder and was confirmed by RNA sequencing (not shown).

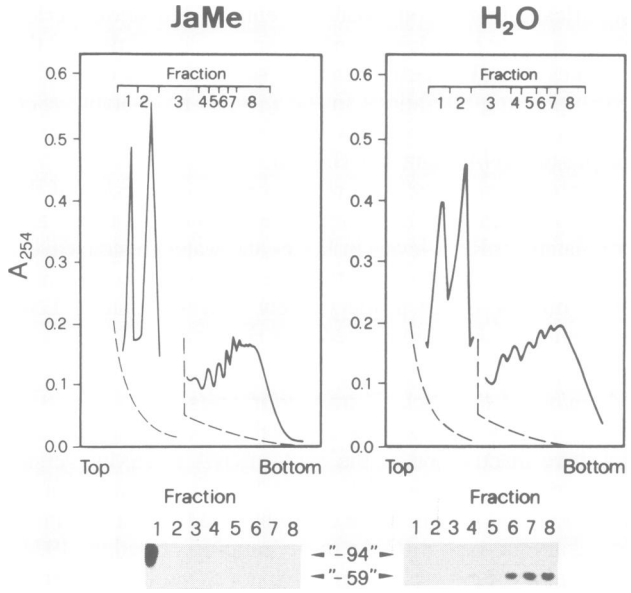

Fig. 5. Association with plastid ribosomes of '−59' and '−94' *rbcL* transcripts in leaf tissues treated for 24 h with water and JaMe, respectively. Ribonucleoprotein material, recovered from an equal number of plastids of both tissues, was separated in discontinuous sucrose gradients. During harvest of the gradients, the absorbance at 254 nm was monitored continuously. A 2.5-fold decrease in full scale absorbance is indicated by a break in the tracing of each profile. After phenol extraction, RNAs of the indicated fractions were recovered by ethanol precipitation and used for reverse transcription using a *rbcL*-specific primer. The resulting copy DNAs were separated in sequencing gels and detected by autoradiography. Further details are given in Figure 4.

and that the shorter transcript was formed by RNA processing from the longer primary transcript. This conclusion is substantiated by the observation that similar promoter elements have been found not only 5′ to the *rbcL* genes of barley (see Figure 6A) and several other plant species (Poulsen, 1984; and references cited therein) but also in other plastid genes from diverse plant species (Rosenberg and Court, 1979; Gruissem and Zurawski, 1985a,b; Kung and Lin, 1985; for reviews see Hanley-Bowdoin and Chua, 1987; Weil, 1987; Gruissem *et al.*, 1988; Sexton *et al.*, 1990). In contrast, no '−10' and '−35' sequence motifs could be found as corresponding promoter elements in the *rbcL* gene region upstream of the '−59' transcript end (Figure 6A).

The abundances of both the '−59' and '−316' transcripts did not change significantly in water-treated leaf tissues (Figure 4). In contrast, the '−59' transcript gradually disappeared upon JaMe treatment. It was replaced by a novel transcript species of intermediate size whose end mapped at position '−94' (Figure 4). By analogy to the shorter transcript species, this intermediate-sized transcript is assumed to originate from RNA processing rather than transcription initiation. The alternative interpretation, however, that the formation of the two transcripts ending at positions '−59' and '−94' arises from transcription initiation at promoter elements deviating from the typical '−10' and '−35' prokaryotic-type consensus sequences, should also be borne in mind. Interestingly, multiple promoters have been observed in maize polycistronic plastid operons (Haley and Bogorad, 1990), spinach *atpB* (Chen *et al.*, 1990) and tobacco (Yao *et al.*, 1989), pea (Woodbury

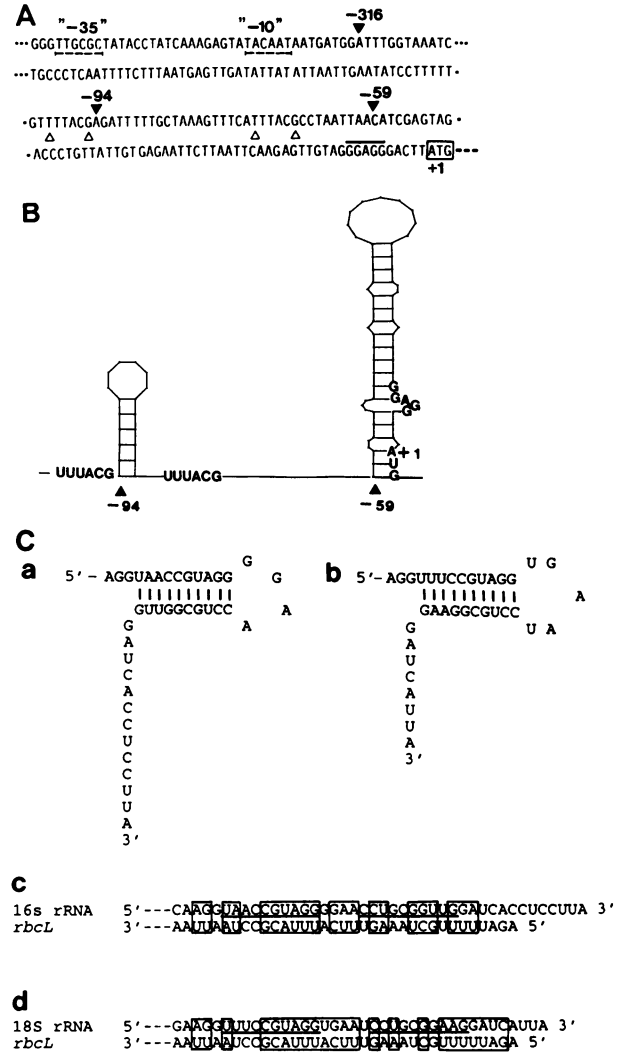

Fig. 6. *rbcL* gene, proposed RNA secondary structures within the 5′ untranslated leader and putative base pairing with the 16S and 18S rRNAs. (**A**) Nucleotide sequence of the *rbcL* promoter region as reported by Poulsen (1984). Contiguous and non-contiguous stretches of DNA are indicated by dots and dotted lines, respectively. The different *rbcL* transcript ends, as well as the '−35' and '−10' promoter elements (underlined) and the Shine−Dalgarno sequence (overlined), are indicated; the ATG triplet encoding the translation start is boxed. Triangles mark the ends of a conserved hexameric sequence element. (**B**) Predicted RNA secondary structure within the 5′ untranslated region of the *rbcL* transcript beginning at position '−100' and ending at position '+3', relative to the start site of translation initiation ('+1'). The '−94' and '−59' transcript ends are indicated. Bases of the Shine−Dalgarno sequence and of the hexameric repeat element (see panel A) are printed in full in the schematic representation, which is drawn to scale. (**C**) Intramolecular base pairing within the 3′ terminal parts of the 16S (a) and 18S rRNAs (b), contrasted with intermolecular base pairing, suggested to occur between the 35-base sequence of the '−94' *rbcL* transcript and the 16S (c) and 18S rRNAs (d), respectively. Bases involved in intramolecular base pairing are underlined in (c) and (d), whereas bases proposed to form intermolecular base pairs are boxed.

et al., 1989) and barley *psbD−psbC* (Sexton *et al.*, 1990; Christopher *et al.*, 1992), some of them having atypical structures (Sexton *et al.*, 1990; Christopher *et al.*, 1992). Thus, *in vitro* capping assays in combination with S1 nuclease mapping of transcript ends (Moss, 1987; Sexton *et al.*, 1990) will be required to answer the question on the origin of the '−94' and '−59' transcripts.

Association of the different rbcL transcripts with plastid ribosomes

If one compares the levels of the three different *rbcL* mRNAs, ending at positions '−316', '−94' and '−59', with the labelling of the LSU *in vivo* and *in vitro* (Figure 3), it is reasonable to assume that only the '−59' transcript is active in terms of translation. The decline of *in vivo* and *in vitro* synthesis of the LSU correlates remarkably with the appearance of the intermediate-sized transcript in JaMe-treated leaf tissues but contrasts with the almost constant level of the primary transcript (Figure 4). On the other hand, the slight quantitative reduction of the '−59' transcript in water-treated leaf tissues (Figure 4) is reflected in a corresponding decrease in the labelling of the LSU *in vivo* and *in vitro* (Figure 3).

We next investigated the association of the different *rbcL* transcripts with plastid ribosomes. We expected that regulation at any step of translation, including chain initiation, elongation and termination, should be reflected in a unique polysome profile (Vassart *et al.*, 1971; Lodish, 1976). Plastids were isolated from leaf tissues that had been treated with either JaMe or water for 24 h. Ribonucleoprotein material was recovered from the lysed plastids by magnesium precipitation (Palmiter, 1974) and resolved in discontinuous sucrose step gradients. As concluded from the absorbance readings shown in Figure 5, the plastid polysome profiles were similar for JaMe- and water-treated leaf tissues. The two major peaks (fractions 1 and 2) represent the 30S and 50S ribosomal subunits, whereas the several lower peaks are comprised of stromal polysomes consisting of two or more message-bound ribosomes (fractions 3−8). The *P/T* ratio (as a measure for the proportion of plastid polysomes to the sum of plastid polysomes, ribosomal subunits and monosomes) of 0.21 and 0.25 were similar for JaMe- and water-treated leaf tissues, respectively.

RNAs were recovered by phenol extraction and ethanol precipitation from the arbitrarily defined gradient fractions shown in Figure 5, and used for the primer extension analyses with the *rbcL*-specific primer described previously. As demonstrated in Figure 5, plastid polysomes (fractions 3−8) and, to a lesser extent, 30S initiation complexes (fraction 1) from water-treated leaf tissues contained *rbcL* transcripts ending at position '−59'. In contrast, *rbcL* transcripts ending at position '−94' were found exclusively in fraction 1 of a corresponding sucrose gradient separating plastid ribonucleoprotein material of JaMe-exposed leaf tissues. Interestingly, *rbcL* transcripts ending at position '−316' could not be detected in any of the gradient RNA populations of plastids from either JaMe- or water-treated leaf tissues (data not shown). However, this transcript could be recovered from the non-polysomal plastid stroma fractions of both tissues (data not shown).

Discussion

Methyl jasmonate affects plastid gene expression at the protein and mRNA levels

The plant growth substance methyl jasmonate not only induces novel abundant proteins in barley (Figure 1) but also, as we demonstrate in the present paper, affects plastid gene expression in a complex manner. We find a selective reduction in the amounts of the LSU of RuBPCase and of the 65 and 68 kDa proteins of photosystem I in JaMe-treated compared with water-treated leaf segments that was caused by a decreased labelling of these polypeptides *in organello* (Figure 1). The changes in plastid protein synthesis occurring after 24 h of JaMe treatment, however, did not correspond to equivalent alterations in the steady state contents of the respective transcripts (Figure 2), suggesting a post-transcriptional mode of control.

The differential changes in the steady state levels of individual plastid transcripts (Figure 2) implicate a specific JaMe effect either on the transcription activities of the corresponding plastid genes or on RNA stabilities. Both possibilities should be kept in mind, since they have been shown to be control points during the light-induced transformation of etioplasts to chloroplasts in barley (Mullet and Klein, 1987; Krupinska and Apel, 1989) and in other plant species (Link, 1984; Rodermel and Bogorad, 1985; Sasaki *et al.*, 1987). Differences in the absolute rates of transcription initiation in chloroplasts versus etioplasts (Reiss and Link, 1985; Baumgartner *et al.*, 1989), in combination with different promoter strengths of individual genes (Gruissem and Zurawski, 1985a,b; Deng *et al.*, 1987; Hanley-Bowdoin and Chua, 1987) and limiting RNA polymerase levels (Link, 1988; Eisermann *et al.*, 1990), have been demonstrated to be determinants for mRNA accumulation. On the other hand, post-transcriptional steps including intron splicing and transcript processing at the 5′ and 3′ ends have been shown to be involved in transcript formation (for reviews see Gruissem *et al.*, 1988; Link, 1988; Mullet, 1988; van Grinvsen and Kool, 1988; Gruissem, 1989). In several cases, constitutive transcription has been observed for the plastid genes studied, whereas the respective transcripts accumulated with significantly different time courses and to different extents, suggesting that RNA stabilities were also influenced by light (Deng and Gruissem, 1987, 1988; Deng *et al.*, 1987; Gruissem *et al.*, 1987; Mullet and Klein, 1987).

Post-transcriptional control of rbcL gene expression by differential 5′ end formation

Although we do not know as yet whether the transcription activities or mRNA stabilities or both were influenced by JaMe, the entire cascade of processing reactions seemed to occur, since mature *rbcL*, *psbA* and *psaA-psaB* transcripts of the expected sizes were found (Figure 2). Nevertheless, the differential changes in the steady state (Figure 2) and *in vitro*-translatable *rbcL* mRNA levels (Figure 3 and Table I) suggested that at least one post-transcriptional event was influenced by JaMe. This presumably led to an obvious loss of functional active (i.e. translatable) mRNA before a comparable transcript decline became evident (Table I). From previous findings demonstrating the occurrence of two different *rbcL* transcript species in barley (Poulsen, 1984), we assumed that 5′ end formation of *rbcL* transcripts might have been influenced by JaMe. We found a JaMe-induced 35-base shift in the length of the 5′ untranslated region of the *rbcL* transcript (from '−59' to '−94'), in comparison with water-treated segments (Figure 4), that correlated with the altered translatability of the *rbcL* mRNA *in organello* (Figure 1), *in vivo* and *in vitro* (Figure 3 and Table I). We assume that the two transcript species with ends at positions '−59' and '−94', respectively, originate from alternative processing events of the larger primary transcript whose 5′ end is located at position '−316' (Figure 4). Interestingly,

a common hexameric consensus motif (TTTACG) upstream from the '−59' and '−94' transcript ends can be found in the *rbcL* promoter region (Figure 6A). If one considers potential secondary structures (predicted according to Tinoco *et al.* (1973) and Boser *et al.* (1974)) in the 5′ untranslated region of the primary transcript, both consensus motifs are centred around a stem−loop (hairpin) structure between positions '−94' and '−77' (Figure 6). We hypothesize that both the consensus motifs and the stem−loop structure function as recognition and/or entry sites of an endoribonuclease whose target site specificity might be altered under the influence of JaMe. We further propose that after binding, this putative endoribonuclease (probably in concert with other proteins) scans the mRNA for potential stem−loop structures at which RNA processing ultimately occurs (Figure 6B).

Although this model is hypothetical it deserves further investigation, since neither the mechanistic details nor the enzymes involved in the generation of plastid mRNA 5′ ends are known thus far. The limited data available suggest a high variability in the pathways of 5′ end formation in mono- and dicistronic mRNAs (Crossland *et al.*, 1984; Poulsen, 1984; Mullet *et al.*, 1985; Westhoff, 1985), and do not imply an evolutionary conservation in diverse plant species (Krebbers *et al.*, 1982; Shinozaki and Sugiura, 1982; Mullet *et al.*, 1985).

The model we propose, however, shares several characteristics with concepts invoked to explain the formation of 3′ ends of plastid transcripts. For example, inverted repeat elements that can potentially form hairpin structures, together with polyuridine-rich flanking sequences, have been demonstrated to function as recognition and binding sites for diverse plastid proteins (Stern and Gruissem, 1987, 1989; Gruissem *et al.*, 1988; Stern *et al.*, 1989; Hsu-Ching and Stern, 1991a,b; Nickelsen and Link, 1991; Schuster and Gruissem, 1991). Cloning of the nuclear genes encoding these proteins from plant species such as tobacco (Li and Sugiura, 1990) and spinach (Schuster and Gruissem, 1991) has identified amino acid sequence domains common to known RNA binding proteins involved in RNA splicing (Bandziulis *et al.*, 1989), suggesting that these plastid proteins might be endoribonucleases involved in RNA processing.

A change in the length of the 5′ untranslated region renders the rbcL mRNA inactive in terms of translation initiation

With respect to the question of translational control it is interesting to note that a 35-base shift in the 5′ untranslated region of the *rbcL* mRNA was sufficient to cause an apparently complete loss of its translatability in both prokaryotic and eukaryotic protein-synthesizing systems, i.e. in plastids and rabbit reticulocyte lysates, respectively (cf. Figures 1 and 3). Even if quite similar in several respects, both systems are markedly different in terms of translation initiation (Lodish, 1976; Rhoads, 1988; Hershey, 1991; Kozak, 1991; Noller, 1991), the step that is assumed to have been influenced by the leader length of the *rbcL* mRNA.

One possibility to explain the selective inactivation of the *rbcL* mRNA in JaMe-treated leaf tissues might be to assume that base pairing within the 5′ untranslated region of '−94' transcripts gave rise to elaborate secondary structures, which in turn affected translation. Similarly, 5′ terminal secondary structures have been demonstrated to affect translation of mRNAs both in prokaryotic (Hall *et al.*, 1982; Draper, 1989; Springer *et al.*, 1989) and eukaryotic (Lawson *et al.*, 1986; Kozak, 1988, 1989; Roy *et al.*, 1990; Fu *et al.*, 1991) translation systems. Secondary structure predictions (Tinoco *et al.*, 1973; Boser *et al.*, 1974) made for the 5′ untranslated regions of the two different *rbcL* mRNAs, however, demonstrated only a limited potential of the 35-base sequence in *rbcL* transcripts ending at position '−94', in comparison with transcripts ending at position '−59', to form a separate, thermodynamically stable stem−loop structure (data not shown), which might have disturbed the formation of 30S and 40S initiation complexes in plastids and reticulocyte lysates, respectively. On the other hand, the 35-base sequence in the '−94' *rbcL* transcript has no apparent tendency to compete for base pairing with those regions of the '−59' transcript that are assumed to be required for translation initiation, such as the Shine−Dalgarno (Shine and Dalgarno, 1975) sequence (data not shown). Nevertheless, we cannot rule out the possibility that the 35-base sequence might act as a binding site for a translational repressor found both in plastids and in reticulocyte lysates. Alternatively, it might prevent binding of a translational activator at the stem−loop structure found in transcripts ending at position '−59' (Figure 6B). In this context, it is interesting to note that translational activators have recently been identified as RNA binding proteins interacting with hairpin structures within the 5′ untranslated regions of both plastid (Danon and Mayfield, 1991) and mitochondrial mRNAs (Costanzo and Fox, 1988; McMullin *et al.*, 1990).

The differences in translatability of the '−59' and '−94' *rbcL* transcripts might, however, also be explained if one considers the primary structure of the 35-base sequence. Interestingly, there is a remarkable complementarity to the extreme 3′ terminal part of both the 16S rRNA (Brimacombe *et al.*, 1988; Stern *et al.*, 1988) and 18S rRNA (Lockard *et al.*, 1982). Taking a previous paper of Azad and Deacon (1980) into account, we propose that this region of the *rbcL* mRNA is able to compete for intramolecular base pairing normally occurring within both the 16S and 18S rRNAs (Figure 6C, compare c and d with a and b). If such intermolecular RNA interaction were to occur in the prokaryotic system, one might expect that the Shine−Dalgarno sequence of the '−94' *rbcL* transcript and the corresponding region of the 16S rRNA would not be able to interact normally to mediate translation initiation (Shine and Dalgarno, 1975; Dontsova *et al.*, 1992). Indeed, '−94' *rbcL* transcripts comigrate with 30S initiation complexes in sucrose gradients separating plastid ribonucleoprotein material from JaMe-treated leaf tissues (Figure 5). However, these complexes seem to be '30S-like' complexes that have lost the ability to associate with the 50S ribosomal subunits in order to form 70S complexes, i.e. ribosomes (Figure 5), that are active in protein synthesis (Figures 1 and 3). In this context it is tempting to speculate that binding of the '−94' *rbcL* transcript to the 30S ribosomal subunit might influence the interaction of the 16S rRNA with the 23S rRNA and/or with ribosomal protein S7 (Noller, 1991; Dontsova *et al.*, 1992). In a similar way, intermolecular RNA base pairing in the eukaryotic system might impair the formation of 40S initiation complexes.

In summary, our results imply a novel mechanism of translational regulation of plastid gene expression by

differential 5′ end formation. It may result from a JaMe-induced switch in processing site selection within the primary *rbcL* transcript. We hope to isolate the suggested processing endoribonuclease by virtue of its assumed substrate specificity in order to test its direct and subtle interaction with JaMe.

Materials and methods

Plant growth

Seeds of barley (*Hordeum vulgare* L. cv. Salome) were germinated and grown under greenhouse conditions in continuous light (30 W/m^2, fluorescent bulbs) at 23°C for 7 days (Weidhase *et al.*, 1987). Primary leaves were cut from the seedlings, and 5 cm segments beginning 1 cm from the leaf tip were floated on either water or an aqueous solution of (−)-jasmonic acid methyl ester (45 μM, Firmenich, Geneva, Switzerland) for various periods of time as indicated in the text.

Plastid isolation, in organello translation and analysis of plastid polysomes

Plastids were isolated by Percoll (Pharmacia-LKB, Sweden) gradient centrifugation and further purified by flotation on Percoll cushions according to our previously published protocol (S.Reinbothe *et al.*, 1990a), with the modification that surface-sterilized leaf segments were disrupted by grinding at 4°C in the homogenization buffer. Plastid preparations yielded ~80% intact plastids as assessed by phase-contrast microscopy. For *in organello* translation with L-[^{35}S]methionine (1.87 MBq per 50 μl assay, 37 TBq/mmol, Amersham International plc, Bucks., UK), the protocol of Mullet *et al.* (1986) was used, but with intact chloroplasts at only 0.1 mg chlorophyll per ml assay. After gentle lysis of the plastids, stromal ribonucleoprotein material was enriched by magnesium precipitation according to Palmiter (1974) and separated by centifugation (60 000 r.p.m., Beckman Ti 60 rotor, Spinco L75 centifuge, 1 h, 4°C) in a discontinous sucrose step gradient, ranging in concentration between 0.5 and 2.0 M sucrose, prepared in a buffer containing 50 mM HEPES−KOH, pH 8.3, 150 mM NaCl, 5 mM $MgCl_2$, 50 μg/ml heparin, 10 mM β-mercaptoethanol. The gradients were harvested from bottom to top in a modified Beckman harvesting device with continuous monitoring of the absorbance at 254 nm (2138 Uvicord S, LKB). RNAs were recovered from the arbitrarily defined gradient fractions (cf. Figure 5) by phenol extraction and ethanol precipitation (S.Reinbothe *et al.*, 1990b).

In vivo labelling and immunoprecipitation of proteins

For *in vivo* labelling of proteins with L-[^{35}S]methionine (3.70 MBq for every ten 3 mm segments, 37 TBq/mmol, Amersham), the previously published protocol of Müller-Uri *et al.* (1988) was used. Immunoadsorption to protein A−Sepharose (Pharmacia-LKB, Sweden) was performed as described previously (S.Reinbothe *et al.*, 1990a) with monospecific antibodies raised against the 65 kDa protein of photosystem I, the 32 kDa protein of photosystem II and the LSU or SSU of RuBPCase (kindly provided by J. Lehmann, Halle/Saale, Germany), respectively.

Preparation and in vitro translation of RNA

High molecular mass leaf RNA was prepared as described previously (Müller-Uri *et al.*, 1988; S.Reinbothe *et al.*, 1990a). Poly(A)$^-$ was separated from poly(A)$^+$ RNA by messenger paper (Hybond^{TM}-mAP, Amersham) affinity chromatography as described by Werner *et al.* (1984). Plastid RNA was isolated according to Klein and Mullet (1986). *In vitro* translation in a rabbit reticulocyte lysate was performed according to Pelham and Jackson (1976). Assays contained 11.2 MBq L-[^{35}S]methionine (37 TBq/mmol, Amersham).

Electrophoretic separation and hybridization of filter-bound RNAs

For denaturing RNA electrophoresis, agarose gels (1%, w/v) containing formaldehyde (Meinkoth and Wahl, 1984) were prepared as described previously (S.Reinbothe *et al.*, 1990b). After electrophoresis, the RNAs were blotted onto nitrocellulose filters (BA-S 85, Schleicher and Schuell, Dassel, Germany) by standard procedures (Sambrook *et al.*, 1989). Plasmids containing parts of the *psaA−psaB* gene (Westhoff *et al.*, 1983), the entire *psbA* gene (Zurawski *et al.*, 1982) and the entire *rbcL* gene from spinach (Zurawski *et al.*, 1981) were isolated from bacteria by standard procedures (Sambrook *et al.*, 1989). Plasmids were labelled with [α-^{32}P]dATP (1.85 MBq per assay, 29.6 TBq/mmol, Amersham) by random priming as described by Feinberg and Vogelstein (1983). For hybridization and posthybridization washing of the filters, standard conditions were used (Meinkoth and Wahl, 1984; Sambrook *et al.*, 1989).

Primer extension analysis

A synthetic 36mer oligonucletoide primer corresponding to the first 12 amino acids of the maize *rbcL* gene (Miziorko and Lorimer, 1983) was synthesized in an Applied Biosystems DNA Synthesizer, Model 380A. The primer was purified in preparative polyacrylamide gels and the recovered probe was labelled with [γ-^{32}P]ATP at its 5′ terminus by the T4 kinase reaction (Sambrook *et al.*, 1989). For primer extension analysis, 2 μg of purified radioactive primer and AMV reverse transcriptase were used, as described by Chandler and Huiet (1991). The samples were heated and run in 42% (w/v) urea, 8% (w/v) polyacrylamide sequencing gels prepared in TBE (90 mM Tris−borate, 2.5 mM disodium ethylenediaminetetraacetic acid) according to Sambrook *et al.* (1989).

Miscellaneous

RNA sequencing was performed with the above mentioned oligonucleotide primer and reverse transcriptase using a modified version of the dideoxy chain termination method (Sanger *et al.*, 1977). Denaturing polyacrylamide gradient gels were run according to Laemmli (1970).

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