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Unassembled polypeptides of the plastidic ribosomes in heat-treated 70S-ribosome-deficient rye leaves

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Abstract. The polypeptides of the subunits of 70S ribosomes isolated from rye (*Secale cereale* L.) leaf chloroplasts were analyzed by two-dimensional polyacrylamide gel electrophoresis. The 50S subunit contained approx. 33 polypeptides in the range of relative molecular mass (M_r) 13000–36000, the 30S subunit contained approx. 25 polypeptides in the range of M_r 13000–40500. Antisera raised against the individual isolated ribosomal subunits detected approx. 17 polypeptides of the 50S and 10 polypeptides of the 30S subunit in the immunoblotting assay. By immunoblotting with these antisera the major antigenic ribosomal polypeptides (r-proteins) of the chloroplasts were clearly and specifically visualized also in separations of leaf extracts or soluble chloroplast supernatants. In extracts from rye leaves grown at 32° C, a temperature which is non-permissive for 70S-ribosome formation, or in supernatants from ribosome-deficient isolated plastids, six plastidic r-proteins were visualized by immunoblotting with the anti-50S-serum and two to four plastidic r-proteins were detected by immunoblotting with the anti-30S-serum, while other r-proteins that reacted with our antisera were missing. Those plastidic r-proteins that were present in 70S-ribosome-deficient leaves must represent individual unassembled ribosomal polypeptides that were synthesized on cytoplasmic 80S ribosomes. For the biogenesis of chloroplast ribosomes the mechanisms of coordinate regulation appear to be less strict than those known for the biogenesis of bacterial ribosomes, thus allowing a marked accumulation of several unassembled ribosomal polypeptides of cytoplasmic origin.

Key words: Ribosomal polypeptide (unassembled) – Ribosome deficiency (heat-induced) – Ribosome (70S, chloroplast) – *Secale* (plastidic ribosome polypeptide)

Abbreviations: L = polypeptide of large ribosomal subunit; M_r = relative molecular mass; r-protein = ribosomal polypeptide; S = polypeptide of small ribosomal subunit; SDS = sodium dodecyl sulfate

Introduction

The biogenesis of chloroplasts depends on the coordinate expression of proteins that are coded for and translated inside the organelle and on a majority of additional proteins that need to be imported from a nucleo-cytoplasmic site of synthesis (Ellis 1981; Bottomley and Bohnert 1982; Parthier 1982; Herrmann et al. 1983; Dyer 1984; Feierabend et al. 1984; Shinozaki et al. 1986). The organellar translation proceeds on the plastidic 70S ribosomes. The plastidic ribosomes are in many respects similar to procaryotic ribosomes. Like those of bacteria, the plastidic 70S ribosomes contain 50–60 ribosomal proteins (Ledoigt and Freyssinet 1982; Subramanian 1985). These are also of dual origin. Some of the plastidic ribosomal polypeptides (r-proteins) have been shown to be synthesized within the chloroplasts (Freyssinet 1978; Eneas-Filho et al. 1981; Ledoigt and Freyssinet 1982; Schmidt et al. 1983; Dorne et al. 1984; Posno et al. 1984; Hachtel 1985). Gene sequences for 19 r-proteins have been localized on the chloroplast DNA of *Marchantia* and tobacco (Ohyama et al. 1986; Shinozaki et al. 1986) by their homologies to r-proteins from *Escherichia coli*. Thus far, it has been assured for 12 of these genes that they are expressed in the chloroplasts. The majority of the plastidic r-proteins appear, however, to be synthesized in the cytoplasm. As far as has been investigated, precursor polypeptides are synthesized on

80S ribosomes that are post-translationally processed to their mature sizes during transport into the organelle (Schmidt et al. 1984; Schmidt et al. 1985; Gantt and Key 1986).

Information about the mechanisms ensuring a concerted expression of the rRNAs and of the different r-proteins during the biogenesis of plastidic 70S ribosomes is still lacking. From the scattered distribution of the genes and the involvement of two separated compartments in the translation of the polypeptides it is to be expected that control mechanisms might be even more complex and conceivably less strict or effective than those known to govern the biogenesis of the bacterial ribosome (Nomura et al. 1984; Lindahl and Zengel 1986).

In the present investigation we used the interference of elevated growth temperatures with the formation of plastid ribosomes in higher plants as an approach to gain some insight into the extent of coregulation or independence in the expression of plastidic r-proteins. Elevated growth temperatures are known to be non-permissive for plastid ribosome formation in higher plants (for reviews see Feierabend 1982; Feierabend et al. 1984). By growing rye seedlings, as used in this investigation, at 32° C the formation of plastid ribosomes can be specifically blocked and 70S-ribosome-deficient leaves generated. The 70S-ribosome-deficient tissue lacks chloroplastic translation products and remains achlorophyllous, but many proteins of cytoplasmic origin are synthesized and even accumulate to considerable extents (Feierabend and Schrader-Reichhardt 1976; Feierabend et al. 1984; Feierabend 1986). With the aid of antibodies raised against the individual isolated subunits of 70S ribosomes we have now investigated whether plastidic r-proteins of cytoplasmic origin were made in heat-induced 70S-ribosome-deficient leaves and able to accumulate to detectable amounts, or whether mechanisms of coregulation, such as feedback controls or post-translational mechanisms, must be expected to be effective and prevent the appearance of individual unassembled r-proteins.

Material and methods

Plant material and growing conditions. Leaves of 5- or 6-d-old seedlings of winter rye (*Secale cereale* L. cv. Petkus "Halo", F. v. Lochow-Petkus GmbH, Bergen, FRG) were used. For the preparation of chloroplasts or 70S ribosomes, seedlings were grown at 22° C in the light on Vermiculite or, for the preparation of ribosome-deficient plastids, at 32° C in glass-covered plastic boxes on filter paper (Schleicher & Schüll, Dassel, FRG; No 598) moistened with an enriched Knop's nutrient solution (for details see Feierabend 1982). For the preparation of cell-free leaf extracts, seedlings were grown in the light at either 22° C or 32° C solely in the presence of distilled H_2O in plastic boxes on filter paper. Continuous irradiation with white light was provided by fluorescent tubes (Philips, Eindhoven, The Netherlands; TL 40W/32 Warmton de Luxe and TL 40W/47 in alternating sequence) giving an incident energy fluence rate of approx. 10.5 $W \cdot m^{-2}$ (5000 lx).

Preparation of cell-free extracts. For the preparation of cell-free extracts, only the lower halves of primary leaves from 6-d-old rye seedlings were used. The leaf tissue was homogenized under ice-cold conditions with mortar and pestle in a medium (1 ml · g^{-1} fresh weight) containing 50 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine (Tricine)-KOH, pH 7.5, 10 mM $MgSO_4$ and 4 mM dithioerythritol (DTE). The homogenates were centrifuged for 30 min at 17500 · *g* and the supernatants used for further analysis.

Isolation of intact plastids. Chloroplasts or ribosome-deficient plastids were isolated, lysed, and fractionated as described (Höinghaus and Feierabend 1985). The supernatant fractions were used for further analysis.

Isolation of 70S ribosomes and their subunits. About 1200 g leaves were collected from rye seedlings, grown at 22° C for 81 h in continuous white light and for subsequent 15 h in darkness, and again illuminated for 30 min immediately before use. Homogenates were prepared at about 4° C either in a 1-gallon (3.79 l) Waring blendor or in a blendor equipped with razor blades (Kannangara et al. 1977). Per 1 g of leaf tissue, 4.5 ml grinding medium (0.35 M sucrose, 0.15 M Tricine-KOH, 10 mM KCl, 3 mM sodium ethylenediaminetetraacetate (EDTA), 1 mM $MgCl_2$, 0.01% (w/v) bovine serum albumin, 4 mM 2-mercaptoethanol, pH 7.5) was added. The homogenates were passed through four layers of muslin and one layer of Miracloth (Calbiochem, La Jolla, Calif., USA) and centrifuged for 5 min at 3000 · *g*. All centrifugations were performed at 4° C. The sediment was resuspended in 100 ml grinding medium for which the concentration of $MgCl_2$ had been raised to 10 mM. The suspension was centrifuged for 1 min at 180 · *g* and chloroplasts were sedimented from the supernatant for 5 min at 3000 · *g*. The chloroplast pellet was lysed in 90 ml of 40 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris)-HCl, 10 mM $MgCl_2$, 20 mM KCl, and 2% (v/v) Triton X-100, pH 8.5. After stirring for 20 min the suspension was centrifuged for 20 min at 27000 · *g*. The supernatant was layered onto a cushion of 1 M sucrose (ribonuclease-free for all steps of the purification procedure) in 40 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM KCl, pH 8.5, (15 ml per tube) and the crude ribosomal pellets obtained after 4.5 h centrifugation at 96000 *g* in a Beckman (München, FRG) rotor 35 were collected.

In order to dissociate ribosomes into subunits the crude ribosomal sediment was resuspended in a medium with a high ratio of K^+ to Mg^{2+} and dialysed against the same medium for 40 min at 4° C. For ribosomal-subunit preparations that were used for antibody production or one-dimensional electrophoresis 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.7 M KCl and 14 mM 2-mercaptoethanol, for those used for all two-dimensional electrophoretic separations 10 mM K-phosphate, pH 7.0, 1 mM Mg-acetate, 0.1 M KCl, and 7 mM 2-mercaptoethanol (Bartsch 1985) were applied as dissociation buffers. After dialysis the suspensions were centrifuged for 10 min at 27000 · *g*. The supernatants were layered onto gradients consisting of 24.5 ml 17.5% (w/v) and 25.0 ml 5% (w/v) sucrose in dissociation buffer (50 units of 260 nm absorbance per gradient) and centrifuged in an ultracentrifuge in a Beckman type 25.2 rotor for 7 h at 77000 · *g*. Gradients were fractionated with an ISCO (Instrumentation Specialities Company, Lincoln, Nebr., USA)

density-gradient fractionator, and the absorbance at 254 nm was recorded by an ISCO UA-2 absorbance monitor (5 nm light path). The peak fractions (with an absorbance greater than 50% of maximum) from three gradients were pooled and, after the addition of 2-mercaptoethanol (20 mM final concentration) and Mg-acetate (10 mM final concentration), centrifuged for 16 h at 250000·*g*. The pellets of isolated ribosomal subunits were resuspended in 0.5–1.0 ml dissociation medium (see above) and adjusted to a final concentration of 2% (w/v) sucrose. The resulting suspensions of ribosomal subunits from the first gradient centrifugation were separately further purified by a second sucrose gradient centrifugation, fractionated and collected as described above.

Isolation of cytoplasmic 80S ribosomes. Green leaves (50–150 g) of 5-d-old rye seedlings were homogenized in the cold in a blendor equipped with razor blades (Kannangara et al. 1977). The grinding medium (2–3 ml per g fresh weight) contained 0.2 M sucrose, 0.1 M Tris-HCl, 10 mM $MgCl_2$, 10 mM KCl, and 4 mM DTE, pH 7.6. After filtration through four layers of muslin and one layer of Miracloth, the homogenates were centrifuged for 10 min at 10000·*g*. The supernatant was adjusted to a final concentration of 2% (v/v) Triton X-100. After standing in ice for 5 min, the extract was centrifuged for 20 min at 27000·*g*. The supernatant was layered onto a cushion of 9 ml 1 M sucrose in 40 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM KCl (9 ml per tube, Beckman ultracentrifuge rotor 35) and centrifuged for 3 h at 96000·*g*. The resulting crude ribosomal pellets were resuspended in 40 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 20 mM KCl, loaded onto sucrose gradients contained in the same buffer medium and centrifuged for 6 h at 65000·*g* in a Beckman rotor 25.2. The gradients consisted of a cushion of 3 ml 34% (w/v) sucrose and a gradient made of 25.5 ml 10% (w/v) and 24.5 ml 34% sucrose. Per gradient, 200–400 units of 260 nm absorbance were applied. The gradients were fractionated with an ISCO density gradient fractionator (see above). Peak fractions of 80S ribosomes were collected. The ribosomes were sedimented by 2 h centrifugation at 200000·*g* in a Kontron (Hannover, FRG) TFT 65.13 rotor, resuspended in 40 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 10 mM KCl and repurified by a second gradient centrifugation, as described. The 80S ribosomes obtained after the second gradient centrifugation were stored in 40 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 10 mM KCl and 14 mM 2-mercaptoethanol at −20° C.

Extraction of RNA. Total RNA was extracted from 6-d-old green leaves according to Leaver and Ingle (1971).

Electrophoretic separation of rRNA. Ribosomal RNAs were separated on slab gels (110·110·1.5 mm^3), consisting of a basal 2 cm 7.5% (w/v) polyacrylamide supporting gel and a composite 0.5% (w/v) agarose–2.4% (w/v) polyacrylamide resolving gel (Peacock and Dingman 1968). According to Bishop et al. (1967), 40 mM Tris-HCl, 20 mM Na-acetate and 1 mM sodium-EDTA, pH 7.2, were used as both gel and reservoir buffer. The electrophoretic separation was performed at 13° C and 40 mA and terminated 1.5 h after the front zone had left the gel. Purified ribosomal fractions were submitted to electrophoresis without removal of proteins. To 6 µl of sample, 1 µl of a solution containing 50 mM DTE, 18% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) sucrose, and 0.02% (w/v) bromophenol blue was added and kept at room temperature for 15 min before electrophoresis. Gels were stained with 0.0005% (w/v) 1-ethyl-2-[3-(1-ethylnaphto[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphto[1,2-d]thiazolium bromide (Stains all) in 50% formamide.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One-dimensional separations of ribosomal polypeptides were performed in the presence of 0.4% (w/v) SDS with the buffer system of Laemmli (1970) on slab gels (100·130·1.5 mm^3) consisting of a 12.5–17.5% (w/v) polyacrylamide gradient resolving gel and a 6% polyacrylamide stacking gel. To 30-µl samples, 5 µl 18% (w/v) SDS and 50 mM DTE were added. After boiling for 2 min and subsequent cooling the samples were adjusted to final concentrations of 10% (w/v) sucrose and 0.015% (w/v) bromophenol blue. For molecular-weight determinations the Pharmacia (Uppsala, Sweden) low-molecular-weight calibration kit was used.

Two-dimensional electrophoresis of ribosomal proteins. Purified ribosomal fractions and leaf or plastid extracts were extracted by the acetic-acid procedure of Kaltschmidt and Wittmann (1972), as modified by Schmidt et al. (1983), prior to electrophoresis of proteins. The two-dimensional electrophoresis was performed according to Schmidt et al. (1983), except that tube gels were used for the first dimension (3 mm diameter, 8.5 cm length) and a polyacrylamide concentration of 15% (w/v) was applied for the slab gels (130·111·3 mm^3) of the second dimension. Gels were stained for protein with 0.035% (w/v) Serva-Blue G 250 and 0.035% Serva-Blue R 250 in 45.4% methanol and 9.2% acetic acid.

Immunological assays. The preparation of antisera against purified 50S and 30S ribosomal subunits and standard double-diffusion immunoprecipitation tests followed previously described procedures (Kurzok and Feierabend 1984). For immunoblotting, proteins were transferred from the polyacrylamide gels to nitrocellulose paper (HAHY 304 FO, 0.45 µm pore size; Millipore, Molsheim, France). After binding of antibodies the locations of antigens were visualized by peroxidase-conjugated goat-anti-rabbit antibodies using *o*-dianisidine and H_2O_2 as substrates for the peroxidase (Towbin et al. 1979).

Results

Characterization of rye 70S ribosomal polypeptides and of antisera. The polypeptides of isolated 50S and 30S subunits of chloroplast 70S ribosomes purified from rye leaves were separated by two-dimensional polyacrylamide gel electrophoresis (Fig. 1 a, c). In the first dimension, proteins were separated in the presence of 8 M urea according to charge, and in the second dimension, in the presence of 0.1% SDS, according to their size. With appropriate protein loads, aprox. 33 polypeptides were observed for the 50S subunit and 25 polypeptides for the 30S subunit. The molecular weights of the 50S r-proteins ranged between relative molecular mass (M_r) 13000 and 36000, those of the 30S r-proteins between M_r 13000 and 40500. Not all of these polypeptides were detected with lower protein loads. In some instances it could not be decided with certainty whether two or three protein spots were overlapping (e.g. L7, 9, 12; L15, 19; L20, 23). No information is yet available whether some protein spots represented only modified or cleaved forms of r-proteins. Some, only faintly

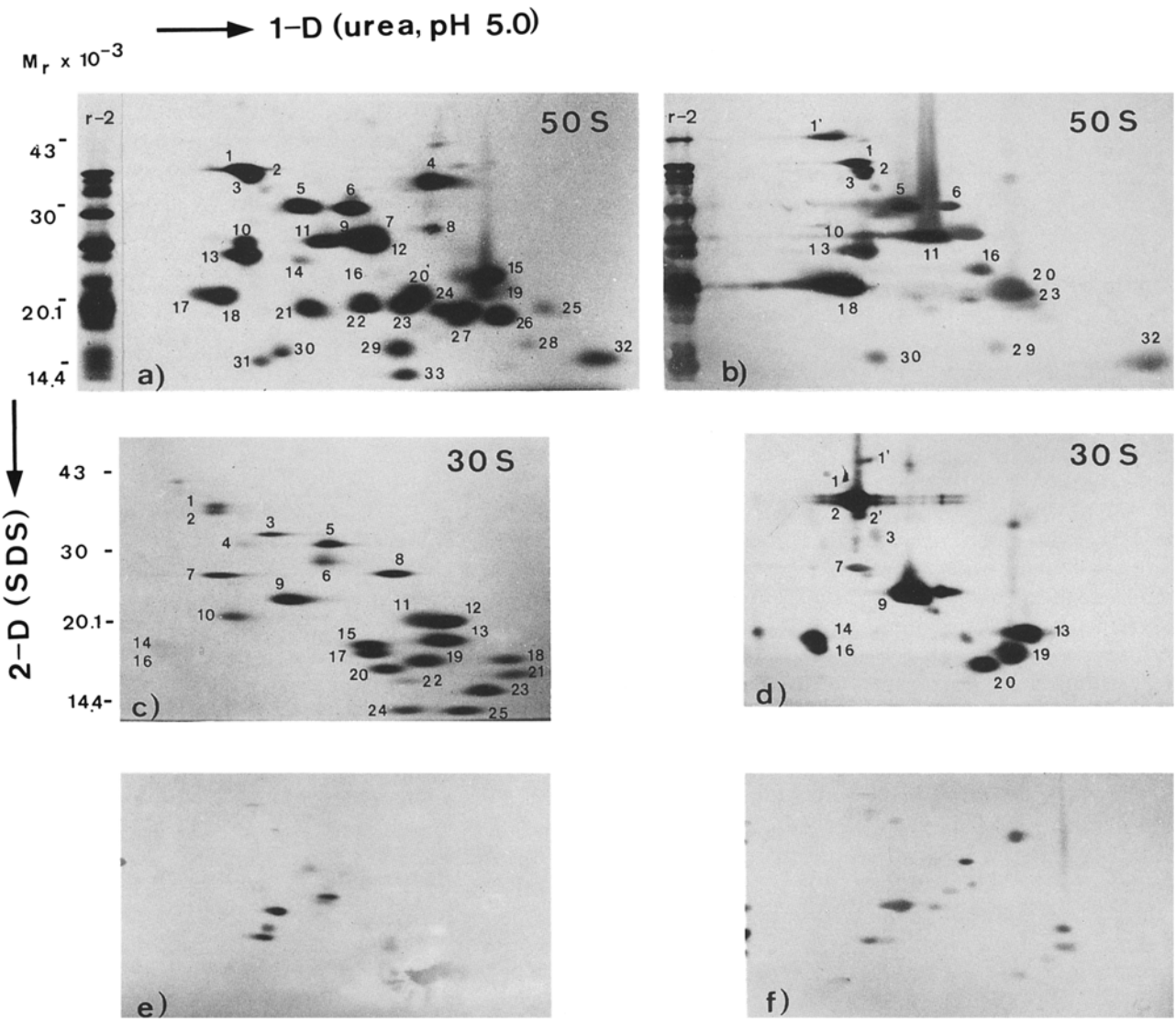

Fig. 1 a–f. Two-dimensional polyacrylamide gel electrophoresis of the polypeptides of isolated 50S (**a**, **b**, **f**) and 30S subunits (**c**, **d**, **e**) of chloroplast ribosomes from rye leaves. **a**, **c** Protein stain with Serva-Blue G 250/R 250. **b**, **e** Immunoblotting of 50S (**b**) or 30S subunits (**e**) with anti-50S-serum; **d**, **f** Immunoblotting of 30S (**d**) or 50S subunits (**f**) with anti-30S-serum. *r-2*, sample of isolated 50S ribosomal subunit separated in the second dimension only

stained, spots (e.g. L16, S14, S16) were included and numbered because they exhibited a strong antigenic reaction with antisera (see below). The numbering of the polypeptides in Fig. 1a and c serves only for an operational identification of the plastidic r-proteins from rye; homologies to r-proteins from *E. coli* are not yet known.

Antisera against the total 50S or 30S subunits of isolated rye chloroplast ribosomes were raised in rabbits. Immunoblotting of the two-dimensional separations of purified ribosomal subunits showed that the anti-50S-serum reacted with about 17 of the 33 50S r-proteins, and the anti-30S-serum reacted with 10 out of the 25 30S r-proteins (Fig. 1b, d). In the immunoblots, some additional antigens were visualized that were not to be detected as stainable spots in the r-protein separations (e.g. next to S9 and those designated as L1′, S1′, and S2′; Fig. 1b, c). The latter three were numbered because they will be regularly detected in immunoblots of leaf or chloroplast extracts (see below).

For the ribosomal subunit preparations used for the immunoblots it was specifically ascertained by analysis of their rRNAs that they were not mutually cross-contaminated. The 50S subunit preparations contained only the 23S rRNA and fragments, the 30S subunit preparations only 16S

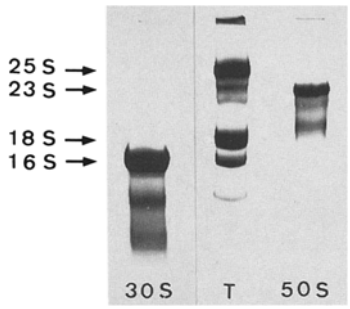

Fig. 2. Electrophoretic separation of high-molecular-weight RNAs from the purified 30S or 50S subunits of the chloroplast 70S ribosomes from rye leaves. *T*, total RNA from rye leaves

rRNA and RNA fragments. Both the rRNAs from cytoplasmic 80S ribosomes (25S and 18S) and the rRNA of the respective other plastid ribosomal subunit were not detectable in the 50S and 30S subunit preparations used (Fig. 2). Nevertheless, some, though relatively weak, reactions were obtained when the 30S subunit polypeptides were immunoblotted with anti-50S-serum (Fig. 1e) or the 50S subunit polypeptides with anti-30S-serum (Fig. 1f). All signals detected with the anti-50S-serum in the 30S preparations appeared to correspond clearly to some 50S r-proteins (in particular L11, L13, L18). The pattern of signals detected by the anti-30S-serum in separations of r-proteins from 50S subunits did not, however, at all resemble the pattern of 30S r-proteins; at least all major spots clearly corresponded to 50S r-proteins, including some that were not or hardly detected by the anti-50S-serum (e.g. L4). The cross-reactivities observed do not indicate that any of the plastidic r-proteins are common to both subunits. Though the rRNA analysis seemed to exclude cross-contaminations between the 50S and 30S subunits the immunoblotting technique was, because of its greater sensitivity, obviously capable of detecting even minor contaminations of the 30S-subunit preparations with some individual 50S r-proteins that were presumably lost from the 50S particles during centrifugation. Contamination of the 50S subunits with 30S r-proteins seems to be negligible. However, the anti-30S-serum also appears to contain some antibodies against 50S r-proteins that had presumably contaminated the 30S-subunit preparations used for raising the antiserum.

Detection of plastidic r-proteins in normal and 32° C-grown 70S-ribosome-deficient leaves. We examined whether plastidic r-proteins could be identified, by immunoblotting in crude leaf or plastid extracts, with the antisera described above. The two-dimensional electrophoresis applied for r-protein separations turned out to be much more difficult for leaf or plastid extracts than for purified ribosome fractions, because part of the bulk of acidic leaf proteins precipitated at the top of the first-dimension gel and also tended to trap major portions of the r-proteins. However, when care was taken not to overload the gels, clear results were, nevertheless, obtained. In extracts from green leaves or supernatants from isolated chloroplasts (not shown) the major r-proteins detected by our anti-50S- and anti-30S-serum were clearly and specifically visualized by immunoblotting (Figs. 3a, 4a). The streaks on the left sides of the two-dimensional separations in Fig. 4a and b (marked as P) result from precipitations of bulk acidic leaf proteins on top of the first dimension gel. Particularly, more acidic r-proteins, such as S14, were heavily trapped in this protein-precipitation zone (Fig. 4a). The polypeptide S16 which had charge properties identical to those of S14 but is slightly smaller in size was not detected by immunoblotting of leaf or chloroplast extracts and might represent a cleavage product or modification of S14 generated in vitro in 30S-subunit preparations.

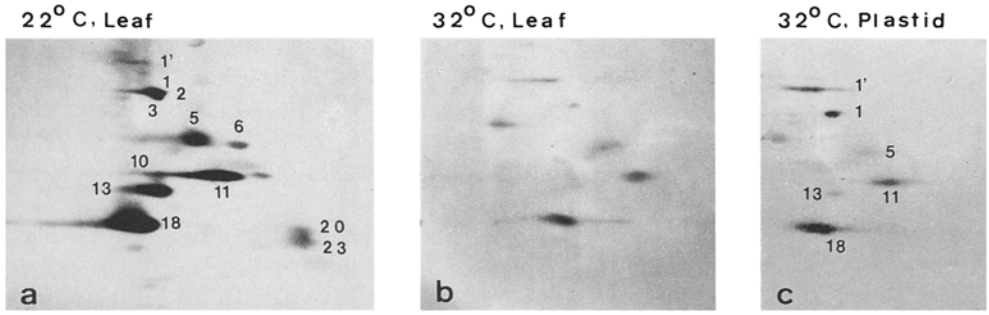

Fig. 3a–c. Two-dimensional electrophoresis and immunoblotting with anti-50S-serum of total soluble protein extracts of 22° C-grown green rye leaves (**a**), total soluble protein extracts from the bleached 70S ribosome-deficient tissue of 32° C-grown rye leaves (**b**), and total soluble supernatant proteins of ribosome-deficient plastids from 32° C-grown rye leaves (**c**)

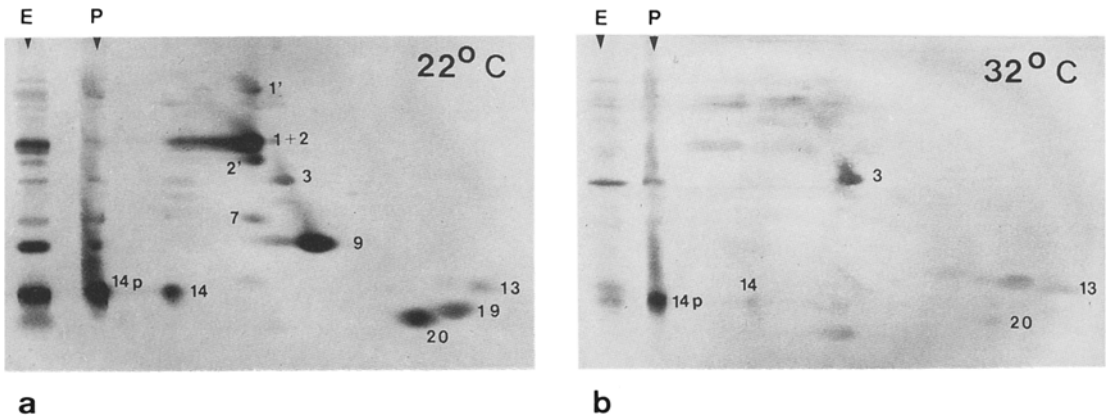

Fig. 4a, b. Two-dimensional electrophoresis and immunoblotting with anti-30S-serum of total soluble protein extracts of 22° C-grown green rye leaves (**a**), or total protein extracts from the bleached 70S ribosome-deficient tissue of 32° C-grown rye leaves (**b**). *E*, extract samples separated in the second dimension only; *P*, protein precipitation at the top of the first dimension gels; *14 p*, reaction of the S14 polypeptide trapped in the protein precipitation on top of the first-dimension gel

In immunoblots of 2-dimensional separations of extracts from 32°-C-grown 70S-ribosome-deficient leaves or plastids with anti-50S-serum, major signals could be clearly attributed to the r-proteins L1′ (M_r 42500), L1 (M_r 38000), L11 (M_r 26000), and L18 (20300). Weaker signals were attributable to L5 (M_r 30000) and L13 (M_r 24500; Fig. 3b, c). In immunoblots with anti-30S-serum, strong signals were obtained for S3 (M_r 32000) and S14 (M_r 17500) in separations from 70S-ribosome-deficient leaves or plastids (Fig. 4b). As in separations from normal leaves, a considerable portion of S14 was usually trapped in the precipitate on top of the first-dimension gel (Fig. 4b). Whether some of the weaker signals in immunoblotting of ribosome-deficient plastids with anti-30S-serum represented the r-proteins S1, S13 and S20 is not unequivocally clear (Fig. 4b). In immunoblots of protein extracts from 70S-ribosome-deficient leaves, a few spots appeared which were not seen in extracts from normal leaves. We have no information yet whether these represent unspecific reactions or, conceivably, precursors of r-proteins that were not processed under these conditions (compare Schmidt et al. 1985).

Several control reactions were performed with one-dimensional electrophoretic separations (Fig. 5) in order to ensure the validity and specificity of the results and conclusions obtained from immunoblotting of 70S-ribosome-deficient leaf extracts after 2-dimensional analysis. The major signals detected by the anti-50S- and anti-30S-antisera in 70S-ribosome-deficient leaves were not the consequence of unspecific reactions since they were not seen when pre-immune sera were applied (Fig. 5d, e). It can also be excluded that these immunoreactions resulted from cross-reactions of the 80S cytoplasmic ribosomes. Anti-30S-sera did not cross-react with isolated 80S ribosomes. Anti-50S-serum gave some weak reactions with 80S ribosomes which corresponded to the pattern of major 50S polypeptides and appeared to result from minor contaminations of our 80S-ribosome preparation with 50S r-proteins. If true immunoreactions with 80S r-proteins had occurred, one would have expected these to have been strongest in total leaf extracts but missing or hardly detectable in extracts from purified plastids. The finding (Fig. 5b, c) that those r-proteins reacting with our antibodies were, as in total leaf extracts, easily detected in extracts from purified chloroplasts as well as from ribosome-deficient plastids provides further evidence that the immunoreactions observed were not related to r-proteins of the 80S ribosomes.

Discussion

From chloroplast ribosomes of rye leaves, similar to findings for other plant species, approx. 60 polypeptides were separated. Antisera raised against isolated 30S and 50S subunits of rye chloroplast 70S ribosomes allowed us to detect a major number of plastidic r-proteins with great specificity after 2-dimensional electrophoresis and subsequent immunoblotting, even in crude extracts from leaves or isolated plastids. By these procedures, individual r-proteins of both the 30S and 50S subunit were clearly demonstrated to be present also in 32° C-grown 70S-ribosome-deficient rye leaves. Because they were extracted only from the completely 70S-ribosome-deficient lower halves of 32° C-grown leaves that were totally formed de

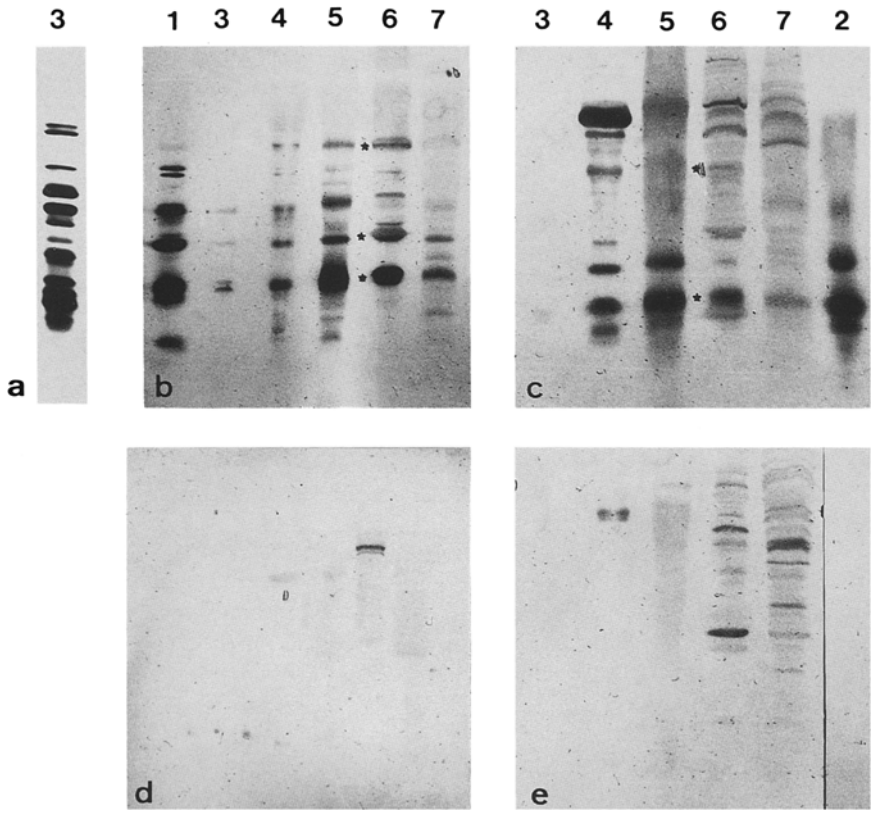

Fig. 5a–e. One-dimensional electrophoresis in the presence of SDS. *Lane 1*, isolated 50S subunits of rye chloroplast ribosomes; *lane 2*, isolated 30S subunits of rye chloroplast ribosomes; *lane 3*, isolated cytoplasmic 80S ribosomes from rye leaves; *lane 4*, supernatants from isolated rye chloroplasts; *lane 5*, extracts from 22° C-grown green rye leaves; *lane 6*, supernatants from isolated ribosome-deficient plastids from 32° C-grown rye leaves; *lane 7*, extracts from 70S-ribosome-deficient 32° C-grown leaves. **a** Protein stain with Serva-Blue G250/R250; **b** immunoblotting with anti-50S-serum; **c** immunoblotting with anti-30S-serum; **d** immunoblotting with pre-immune serum taken before immunization with the 50S subunit; **e** immunoblotting with pre-immune serum taken before immunization with the 30S subunit. *Small asterisks* indicate major protein bands reacting with the antisera in ribosome-deficient plastids, that are further resolved in **Figs. 3** and **4**

novo under the non-permissive conditions and unable to synthesize proteins within the plastids (see Feierabend and Schrader-Reichhardt 1976; Feierabend 1982; Feierabend et al. 1984; Biekmann and Feierabend 1985), the presence of plastidic r-proteins in this tissue is conclusive evidence for their cytoplasmic origin. The inverse conclusion is, however, not justified. Those plastidic r-proteins that are antigenic to our antisera and detected in normal leaves but missing in 70S-ribosome-deficient leaves are not necessarily all of chloroplastic origin since mechanisms of coregulation are to be expected preventing the accumulation of r-proteins when they are not assembled into functional ribosomal subunits.

Very strict mechanisms of coregulation are known to control the biogenesis of ribosomes in *E. coli* where the formation of the rRNAs and r-proteins is finely tuned to such an extent that usually no appreciable buildup of pools of free components is observed (Nomura et al. 1984; Lindahl and Zengel 1986). Also in eucaryotes, ribosomal constituents appear to be coordinately expressed (e.g. Dreisig et al. 1984; Teem et al. 1984; Warner et al. 1985). In *E. coli*, unassembled r-proteins would immediately repress their further translation or even the transcription of the operon in which the respective gene is organized. Since the plastidic ribosomes have otherwise retained many procaryotic properties (Ledoigt and Freyssinet 1982; Subramanian 1985), it is, therefore, rather remarkable, as compared to bacterial ribosomes, to find so many plastidic r-proteins accumulated to appreciable and easily detectable amounts in the 70S-ribosome-deficient tissue of rye. However, for plastidic r-proteins of cytoplasmic origin the situation is markedly different from that in a bacterial cell, because the mature polypeptides are separated from their site of translation or transcription by compartmentation. Direct feed-back mechanisms are thus excluded. Only for the chloroplast-coded r-proteins does it remain to be examined whether direct translational or transcriptional feed-back controls, comparable to those in *E. coli*, exist, particularly for those eight genes which are clustered in an operon-like structure and of which at least some appear to be cotranscribed polycistronically (Tanaka et al. 1986).

Though the methods applied do not yet allow adequate quantitative estimations of the unassembled plastidic r-proteins in 70S-ribosome-deficient leaves, some of them appear to accumulate to surprisingly high levels, relative to the amounts of unassembled subunits observed for other multi-

meric proteins of the chloroplast (e.g. ribulose-1,5-bisphosphate carboxylase or the coupling factor of photophosphorylation CF_1) that are also composed of polypeptides of different site of synthesis. Under the same conditions where the presence of unassembled r-proteins was easily documented, free polypeptides of cytoplasmic origin, e.g. of ribulose-1,5-bisphosphate carboxylase, its small subunit, or of the subunits γ and δ of the coupling factor CF_1would not have been detectable simply by immunoblotting of extracts, unless the sensitivity of detection could have been greatly increased by enrichment of the antigens or by the use of radioactive labeling (Feierabend and Wildner 1978; Biekmann and Feierabend 1985). Unassembled individual polypeptides of ribulose-1,5-bisphosphate carboxylase or the coupling factor CF_1 cannot accumulate to larger extents, because they are removed by degradation, as long as they are not stabilized by integration into the holoprotein-complex (Schmidt and Mishkind 1983; Biekmann and Feierabend 1985). Rapid turnover of excess free r-proteins is also regarded as a means by which a balanced stoichiometry is achieved in the 80S ribosomes of yeast (Warner et al. 1985). Since the proportions of plastidic r-proteins present in 70S-ribosome-deficient tissue differed considerably (e.g. those of L1, L11 or L18 were much greater than those of L5 or L13) it is quite conceivable that at least part of the free r-proteins was also subject to degradation. It would be meaningful, and a hypothetical possibility that needs further examination, if those unassembled r-proteins with greater stability that accumulated in relatively high amounts (L1, L1′, L11, L13, S3, S14) represented r-proteins involved in ribosome assembly. In that case a finely tuned and stoichiometric supply of r-proteins would be achieved if the amounts of all other plastidic r-proteins were adjusted to those of the assembly proteins by feed-back controls or post-translational mechanisms. Thus a concerted expression of all polypeptides would be guaranteed and the assembly proteins of cytoplasmic origin would determine the capacity for ribosome formation in the chloroplast. The use of the high-temperature-induced 70S-ribosome deficiency may serve as an initial approach to further unravel regulatory interrelationships in chloroplast ribosome biogenesis.

The results of this investigation further complement our knowledge about the action of the non-permissive elevated temperature on plastid ribosome formation. Previous work has shown that the plastidic DNA is not affected (Herrmann and Feierabend 1980), which implies that the genes for rRNA and plastid-coded r-proteins must be available, and that the plastids from heat-treated leaves were also capable of transcription (Bünger and Feierabend 1980). Several properties of the heat-induced ribosome deficiency appeared to indicate that the heat treatment did not primarily block the synthesis of any specific ribosomal components but that the latter were unable to assemble at the non-permissive temperature (Feierabend 1982; Feierabend et al. 1984). The occurrence of plastidic r-proteins of cytoplasmic origin under under non-permissive conditions described in the present work is also in close accordance with such an hypothesis, though the latter cannot yet be conclusively verified.

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