ISSN 0026-8933, Molecular Biology, 2013, Vol. 47, No. 1, pp. 112–119. © Pleiades Publishing, Inc., 2013.
Original Russian Text © E.A. Lysenko, A.A. Klaus, V.V. Kuznetsov, 2013, published in Molekulyarnaya Biologiya, 2013, Vol. 47, No. 1, pp. 124–132.

GENOMICS. TRANSCRIPTOMICS

UDC 581.1

Analysis of Intron-Containing Pre-mRNA and Spliced mRNA in Maize Chloroplasts by RT-PCR

E. A. Lysenko, A. A. Klaus, and V. V. Kuznetsov

Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, 127276 Russia;
e-mail: genlysenko@mail.ru

Received August 13, 2012; in final form, September 11, 2012

Abstract—Chloroplast RNA splicing is usually studied by complicated methods, such as Northern blot hybridization, RNAse protection, or primer extension assays. The use of a simpler RT-PCR technique frequently results in the underestimation of unspliced pre-mRNA levels. Five protein-coding genes from the maize plastome were studied to analyze which factors can lead to an apparent reduction in the pool of intron-containing transcripts compared to mature RNA. It was found that the accurate determination of the unspliced RNA level should take into account the DNase inactivation mode, the cDNA synthesis temperature, and the type of DNA polymerase. For one gene, the portion of unspliced RNAs decreased with increasing number of PCR cycles. It was found that intron-containing and intron-free strands of amplicons can form heteroduplexes after PCR. A simple and effective technique of investigating chloroplast spliced mRNA and unspliced pre-mRNA is suggested based on the obtained data.

DOI: 10.1134/S002689331301007X

Keywords: chloroplasts, RNA, splicing, RT-PCR, reverse transcriptase, DNA polymerase, maize

INTRODUCTION

Many eukaryotic and prokaryotic genes contain noncoding regions called "introns;" introns are classified in four major groups, i.e., introns of groups I and II, eukaryotic spliceosomal introns, and a special type of introns characteristic of archaea and eukaryotic tRNAs [1]. Presumably, spliceosomal introns descend from group II introns [2]. Eukaryotic cell genomes contain introns of all four types. Energy-producing organelles, such as mitochondria and plastids, have retained residual genomes of their bacterial ancestors, along with certain specific features of the organization of genetic material. Contemporary plant plastomes contain about two dozen introns, one of which belongs to group I (*trnL*), while the rest are of group II [3]. These introns lie in the genes of plastid proteins and tRNAs. Algal plastomes also contain introns in rRNA genes [4].

The process of RNA maturation involves intron removal by splicing. In plants, more than a dozen proteins have been found to participate in the splicing of plastid introns [3]. Knock-out mutants by many of these splicing factors are nonviable because they cannot efficiently support long-term functioning of the photosynthetic apparatus. Such mutants have mainly been studied in maize, which possesses a large kernel capable of supporting heterotrophic seedling growth for up to two weeks.

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction.

RNA splicing in chloroplasts is usually analyzed using Northern blot hybridization, RNAse protection assay, or primer extension assay [5–8]. It is possible that the complexity of these techniques limits their application for many research purposes. For example, little is known about how the splicing efficiency in chloroplasts changes over the course of ontogenesis, in response to stress, or varies among different species. To our knowledge, there have only been two studies where plastid RNA splicing was analyzed using the relatively less complicated technique of RT-PCR [6, 9]. In the present study, we used this technique to analyze mRNA splicing for several chloroplast genes.

Our preliminary data were suggesting that maize chloroplasts did not contain unspliced pre-mRNAs, or they were present in trace amounts. However, an analysis of the published data showed that significant amounts of unspliced mRNA could be detected in maize chloroplast when other methods of detection were used. In particular, these results were obtained for *petB*, *petD* [5], and *atpF* [7]. Interestingly, in wild-type barley plants, unspliced *rpl2* pre-mRNA was detected by RNase protection assay, but not by RT-PCR [6]. Apparently, the use of RT-PCR bears the risk of underestimating the portion of unspliced RNAs and, consequently, may result in wrong conclusions concerning the ratio of spliced and unspliced RNA forms, as well as the incorrect evaluation of splicing efficiency. However, in a study by Ueda et al., significant amounts of unspliced *rps16* mRNA were detected by RT-PCR in rice and soya chloroplasts [9]. Therefore,

the shortcomings of this approach might be overcome. The aim of our study was to determine the factors that interfere with the detection of unspliced pre-mRNA molecules in chloroplasts and to develop a method for correctly evaluating spliced and intron-containing chloroplast mRNAs using five genes of the maize plastome.

EXPERIMENTAL

Material. Maize plants (*Zea mays* L., cultivar Luchistaya) were grown for 9 days at 25°C, with a light intensity of 200 μM photons per m^2 per second and a photoperiod of 16 h of light and 8 h of darkness under constant aeration in modified Hoagland's medium containing 2 mM MES buffer, pH 6.5, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 1 mM $FeSO_4$, 0.5 mM $MgSO_4$, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 1 μM KCl, 0.1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$.

Chloroplast isolation. All procedures were performed at +4°C in shaded light. Maize leaves (10–15 g) were homogenized in 60 mL of buffer A (50 mM Tris-HCl, pH 8.0, 0.4 M sorbitol, 15 mM NaCl, 2 mM EDTA, 5 mM β-mercaptoethanol). The homogenate was filtered through one layer of cheesecloth and two layers of miracloth (Calbiochem–Behring, United States) and centrifuged in a K-23 centrifuge for 1 min at 3000 rpm (1400 g at the bottom of the tube). The organellar pellet was gently resuspended in 15 mL of buffer A and fractionated in a two-step (40/70%) Percoll gradient (GE Healthcare, United States) in a K-23 centrifuge for 10 min at 5500 rpm (4000 *g* at the fractionating level). Intact chloroplasts were collected at the layer interface and washed by adding buffer A to the total volume of 1.5 mL with subsequent centrifugation at 3800 rpm for 1 min (in this and further procedures, a Heraeus Biofuge fresco centrifuge, +4°C). The supernatant was carefully removed with an automated pipette.

DNA was obtained from isolated chloroplasts using the technique described in [10] with minor modifications.

RNA was isolated according to the protocol described in [11] with the modifications suggested on <molbiol.edu.ru> and according to the authors' own experience. Chloroplast pellet was dissolved on ice in 5 to 10 volumes of buffer B (20 mM Tris-HCl, pH 7.0, 4 M guanidine thiocyanate, 20 mM EDTA, 0.7% lauroyl sarcosin, 100 mM β-mercaptoethanol), mixed with an equal volume of acidic phenol–chloroform mixture (1 : 1), vortexed for 1 min, and centrifuged for 3 min at 13000 rpm. The aqueous phase was transferred into a fresh tube; mixed with 0.1 volumes of 3 M sodium acetate, pH 6.0, and 0.8 volumes of isopropanol; and incubated at −20°C for 1 h. Following 5 min centrifugation at 13000 rpm, the pellet was washed with 75% ethanol and centrifuged again for 2 min at 13000 rpm. The residual ethanol was carefully removed on ice, and the pellet was dissolved in a small volume of deionized water to the concentration of 2–6 μg/μL.

Residual DNA was removed by DNase I treatment at 37°C for 30 to 60 min. The reaction mixture (10 μL) comprised 1 unit of DNase I (Fermentas, Lithuania), 10 units of RiboLock RNase inhibitor (Fermentas), and 1 μg chloroplast RNA. The reaction was stopped by inactivating DNase I using one of the following techniques: (1) heating the mixture to +70°C for 10 min; (2) heating the mixture to +70°C for 10 min with prior addition of 2 μL 25 mM EDTA; (3) diluting the mixture with water and adding an equal volume of phenol–chloroform (1 : 1) with subsequent precipitation, as described for RNA isolation. The latter approach resulted in a loss of approximately 15% RNA.

cDNA synthesis and PCR. The first cDNA strand was synthesized with reverse transcriptases RevertAid, RevertAid Premium (Fermentas), or ThermoScript (Invitrogen, United States) using gene-specific antisense (AS) primers (see below); for each gene, cDNA was synthesized separately. The reaction mixture (10 μL) comprised 0.5 μL revertase, 1 μL 10 mM dNTP (Fermentas), 10 units RiboLock RNAse inhibitor (Fermentas), 1 μL primer (35–50 pM), and 2 μL of DNase-treated RNA specimen. Reverse transcription was performed for 40–50 min at 42°C (for RevertAid) or at 50°C (for RevertAid Premium and ThermoScript). In several experiments, higher reaction temperatures were used (55–65°C), which is specifically indicated in the text and in figure legends. The reaction was terminated after adding 10 μL deionized water via the heat inactivation of the enzymes according the manufacturers' instructions.

PCR was performed with the following DNA polymerases: *Taq* DNA polymerase (SibEnzyme, Russia), DreamTaq (Fermentas), and Encyclo (Evrogen, Russia). The reaction mixture (20 μL) comprised 0.25 μL DNA polymerase, 2 μL 2 mM dNTP (Fermentas), 0.5 μL (17–25 pmol) of each primer (Litech, Russia), and 2 μL cDNA preparation. In all reactions, the elongation stage was performed for 90 s at 72°C. Primer sequences (5' → 3') were as follows: atpFi-S, TCTAACTGTAGTGGTTGGTGT; atpFi-AS, CAACTATTCAGAGTTCCTAGAG; ndhA-S, CGATACAACAACGTATTGGTC; ndhA-AS, ACTGTGCTTCAACTATATCAAC; petD-S, AAGGCGGATTATGGGAGT; petD-AS, CAATACCTAACCAAAGAGCTAC; rps12-S, GTTGCCAGAGTACGATTAAC; rps12-AS, TACCTCGACGTGACATGA; ycf3-S2, TTATGAAGCTACGCGACTAGA, ycf3-AS, AACCAGTTCTGTGCTTCAATA.

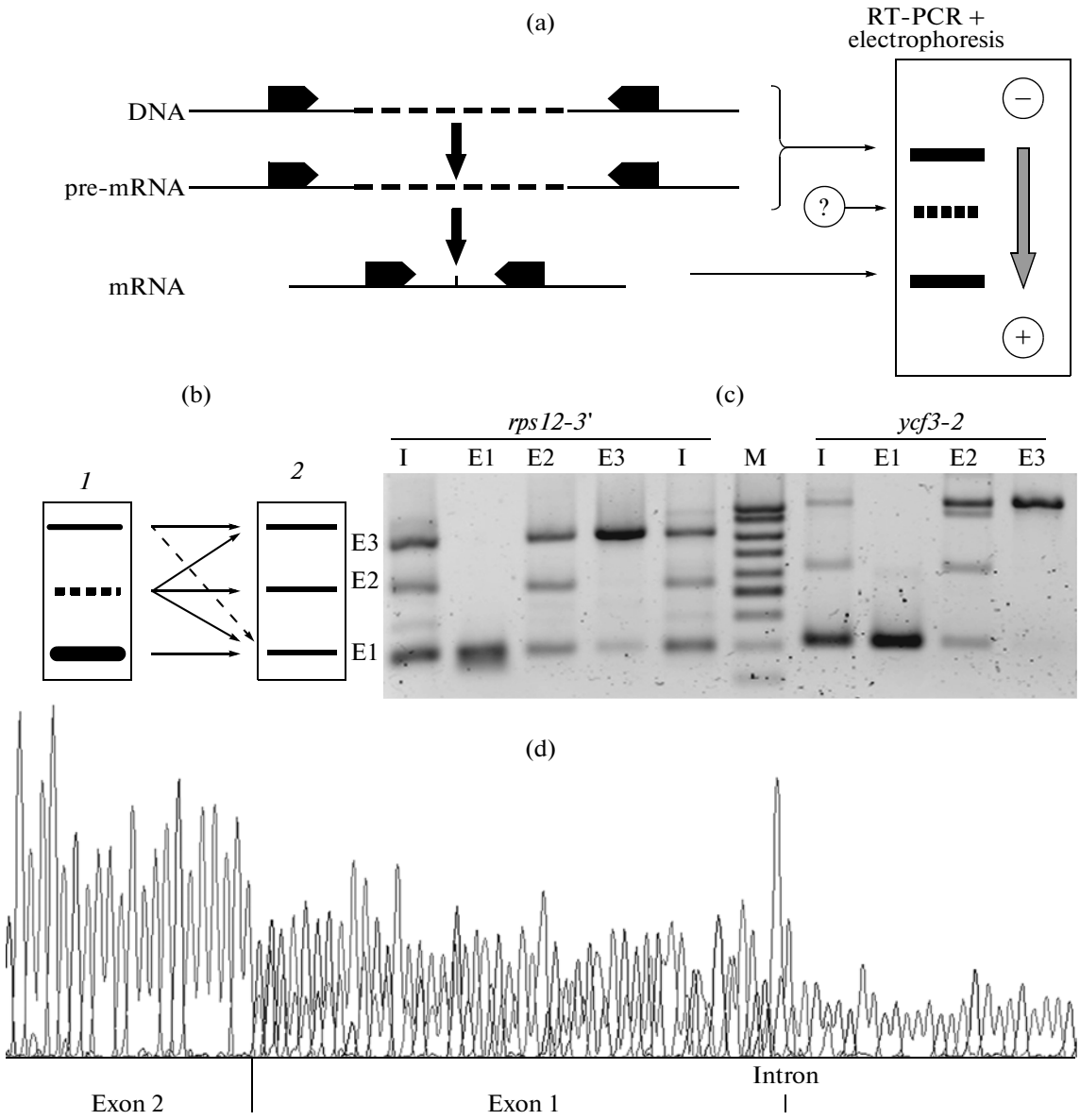

Fig. 1. Primer design for RT-PCR and general specification/description of the reaction product pool (electrophoretic bands). (a) Primer positions relative to the intron (dashed) and the expected electrophoretic pattern. (b) Transformation of initial bands (*1*) in the repeated electrophoresis (*2*). Comments are given in Results and below. (c) Initial products of cDNA amplification (I) were separated by electrophoresis, excised from the gel, and each eluted fragment (E1, E2, E3, in the order of decreasing mobility) was analyzed again. E1, E2, and E3 amounts were adjusted to obtain similar band intensities. (M), 100 bp ladder marker. (d) A fragment of the sequencing pattern of the intermediate band (E2) obtained for *atpF* mRNA. The left peak zone represents exon 2. In the middle, a double peak zone with high peaks corresponding to exon 1 (the highest peak at the end is an extra-template A added by *Taq* polymerase) and lower peaks representing the intron. At the right end, the intron sequence continues.

Our basic technique involved the inactivation of DNase I without EDTA, as well as the use of RevertAid reverse transcriptase and SibEnzyme *Taq* DNA polymerase in 40 cycles of PCR. This is the technique that was used unless specified otherwise.

PCR products were analyzed by electrophoresis in a 1.5% agarose gel in TAE buffer (40 mM Tris-HCl, pH 7.5, 20 mM Na acetate, 1mM EDTA), with 100-bp or 1-kb DNA ladder markers (SibEnzyme). Gels were stained with ethidium bromide and results were registered using a Typhoon Trio+ scanner (GE Healthcare).

Sequencing. PCR products were separated by electrophoresis, product bands were excised from the gel, and DNA fragments were eluted using a DNA Extraction Kit (Fermentas) as suggested by the manufacturer. Specimens were sequenced by Sintol (Moscow, Russia). The results were analyzed using the Sequence Scanner v1.0 program (Applied Biosystems, United States). All specific PCR products were sequenced.

RESULTS AND DISCUSSION

General Specification/Description of PCR Product Pools

In each pair primers were designed complementary to both exons flanking an intron so that fragments amplified from plastid DNA and unspliced pre-mRNA have to include an intron sequence and be size of 830–1450 bp whereas amplicons from spliced mRNA, 300–450 bp (Fig. 1a, table). However, when RT-PCR products were analyzed by electrophoresis, a third band of intermediate mobility was observed in addition to the two expected fragments. Following electrophoresis, bands were excised from the gels, DNA was eluted, and subjected to another electrophoresis. It was found that the contents of the short and the long band migrated in the second experiment at the same rate as in the first one, although the upper band sometimes contained a minor admixture of short amplicons. In contrast, the contents of the intermediate band produced in the second electrophoresis the same three bands as were seen in the first gel (Figs. 1b, 1c). Thus, it seems logical that this band contained heteroduplexes composed of short (spliced) and long (intron-containing) DNA strands.

The sequencing of the intermediate band material revealed the following pattern: a range/row of peaks that corresponds to the first exon was followed by a series of double peaks, the lengths of which was equal to the amplified fragment of the other exon; next, the intron sequence resumed. In the double-peak zone, the higher peaks usually corresponded to the sequence of the second exon, while lower peaks represented an equally long (precise up to one or two nucleotides) intron fragment. This is illustrated by the sequencing of the intermediate band obtained in RT-PCR of *atpF* (Fig. 1d). The shortest amplicon, *rps12-3'*, was sequenced completely; consequently, the sequence comprised a fragment that corresponded to the first exon, a double peak zone with the predomination of the second exon (including the reverse primer sequence at the end), an intron fragment, and the second exon (including the reverse primer sequence at the end) (not shown). This result confirms that bands of intermediate mobility represent heteroduplexes of the two target PCR products.

For the two genes studied (*rps12-3'* and *ycf3-2*), intermediate bands were observed constantly and clearly, while for three other genes (*atpF*, *ndhA*, and *petD*), they were weaker and not always present. Amplicons of *rps12-3'* and *ycf3-2* were shorter than those of the other genes, which is not likely to increase the stability of the heteroduplex. The *ycf3-2* intron is as long as the *petD* intron, and only slightly shorter than the *atpF* intron. However, amplicons of *rps12-3'* and *ycf3-2* differed from the others in that they included rather long exon sequences at both sides from the splicing site; i.e., both parts were ≥100 bp, while in other amplicons, one of the flanking exons was represented by a short fragment (46, 43, and 18 bp; table). Apparently, heteroduplexes are composed of double-stranded fragments formed by complementary exon sequences of the two products, with a loop formed by the intron sequence. Most likely, the stability of the structure depends on the sufficient length of the fragments on both sides of the loop. Therefore, to minimize the probability of heteroduplex formation, primers should be designed so that at least one of them lies as close to the exon border as possible, ideally next to the splicing site, since this was the case with *petD* (table, Figs. 2–5).

Amplicon sizes (bp)

Gene	Amplified region			PCR product	
	5' exon	intron	3' exon	short	long
atpF	46	831	332	378	1209
ndhA	383	1023	43	426	1449
petD	18*	744	428	446	1190
ycf3(*-2*)	181	731	128	309	1040
rps12-3'	193	540	103*	296	836

* Includes the 5' or 3' untranslated region. Long PCR fragments are amplified based on plastid DNA or on pre-mRNA-derived cDNA; short fragments are amplified from cDNA derived from mature mRNA. For *atpF*, *ndhA*, and *petD*, amplicons contain their single introns between exons 1 and 2; for *ycf3*, the PCR fragment includes intron 2 between exons 2 and 3; for *rps12-3'*, the single intron between exons 2 and 3 (the separately located exon 1 (*rps12-5'*) is transcribed independently and joined to mRNA by trans-splicing [8]).

DNase I Inactivation

It is known that, at increased temperatures, Mg^{2+} ions promote RNA hydrolysis and EDTA chelation is beneficial for RNA stability in such conditions [12]. In preliminary experiments, RNA specimens were heated twice, i.e., first, to inactivate DNase after enzymatic digestion of residual DNA in chloroplast RNA specimens, and later on to denature the secondary RNA structure prior to cDNA synthesis. Several techniques of DNase inactivation were tested: heating in the absence or presence of EDTA, heating in the presence of EDTA immediately before cDNA synthesis (to simultaneously inactivate DNase and denature RNA secondary structure), and deproteinization with phenol–chloroform with subsequent RNA precipitation and without heating prior to cDNA synthesis (Fig. 2a). None of these variants differed in the yield of PCR products of spliced RNA; however, the yield of unspliced RNA products depended on the technique of DNase inactivation. The lowest yield of long amplicons was always observed after heating specimens in the absence of EDTA, while the presence of EDTA increased the portion of long amplicons. Omitting the heating step by using phenol–chloroform to remove

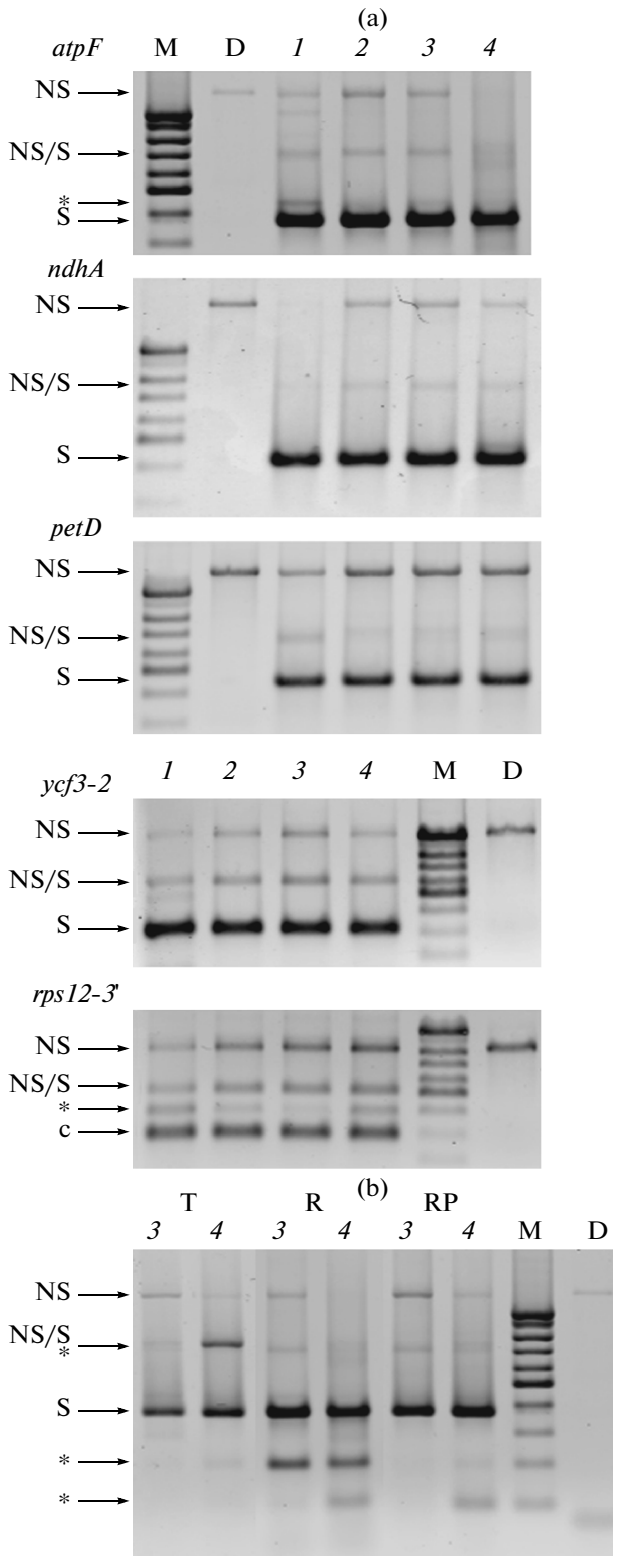

Fig. 2. Effect of different DNase I inactivation techniques on the relative yield of high molecular weight PCR products. (a) Effect of DNase inactivation techniques studied for the five genes. (*1*) Two-step heating of chloroplast RNA specimens in the absence of EDTA (10 min at 70°C for DNase I inactivation and 10 min at 70°C for denaturation of RNA secondary structure immediately before cDNA synthesis); (*2*) same in the presence of EDTA; (*3*) single-step RNA heating (DNase I treatment immediately before cDNA synthesis, 10 min incubation at +70°C to inactivate DNase I and to denature RNA secondary structure prior to cDNA synthesis); EDTA was added in advance; (*4*) Phenol–chloroform deproteinization with subsequent RNA precipitation without heating, even prior to cDNA synthesis. (b) Unfavorable effects of method 4 (phenol–chloroform deproteinization without heating) demonstrated for *atpF* RNA. D, PCR with plastid DNA; *1–4*, PCR with cDNA. M, 100 bp DNA ladder marker. Reverse transcriptases: R is RevertAid; RP is RevertAid Premium; T is ThermoScript. Here and in Figs. 3–5, the bands correspond to PCR products of the following RNA forms: NS is unspliced; NS/S is heteroduplex of spliced and unspliced bands; S is spliced; * is nonspecific. Each line was loaded with 5 μL of PCR product of cDNA, or 1–2 μL of PCR product of plastid DNA.

DNase did not improve the results; moreover, in some cases, it also reduced the yield of some amplicons from unspliced mRNA. In addition, phenol–chloroform treatment without RNA heating sometimes resulted in nonspecific synthesis in RT-PCR (Fig. 2b). The difference between the techniques using one or two heating steps was not large; however, our observations suggest that, in general, one-step heating is preferable.

cDNA Synthesis: Reaction Temperature and Enzyme Type

It is known that group II introns give rise to numerous secondary structures [3]. If cDNA is synthesized at low temperatures (37–42°C), the renaturation of these structures may interfere with reverse transcriptase activity and prevent cDNA synthesis from intron-containing RNA fragments. An increase in cDNA synthesis temperature is expected to facilitate the reading of intron-containing RNA templates by reverse transcriptases. We studied the effects of reverse transcription temperature on the amplicon yield. It was found that the amount of intron-containing amplicons produced at 42°C was indeed lower than the amount obtained at 50°C. However, this may result, not only from the increase in temperature, but also from using a different enzyme. (Fig. 3a). RevertAid Premium and ThermoScript thermophilic reverse transcriptases provided the highest yield of high-molecular-weight fragments at 50°C; the yield decreased with a further increase in the reaction temperature. At 50°C, the difference between the activities of the two enzymes was inconspicuous. At the same time, the activity of RevertAid Premium dropped rapidly with increasing temperature, as can be seen by decreasing yield of high molecular weight amplicons at 55°C and low molecular weight amplicons at 60°C (Fig. 3a). The decrease in the activity of ThermoScript was less dramatic; considerable amounts of PCR products from nonspliced *rps12-3'* and *petD* RNA were obtained even after the reverse transcription performed at 65°C (Fig. 3a).

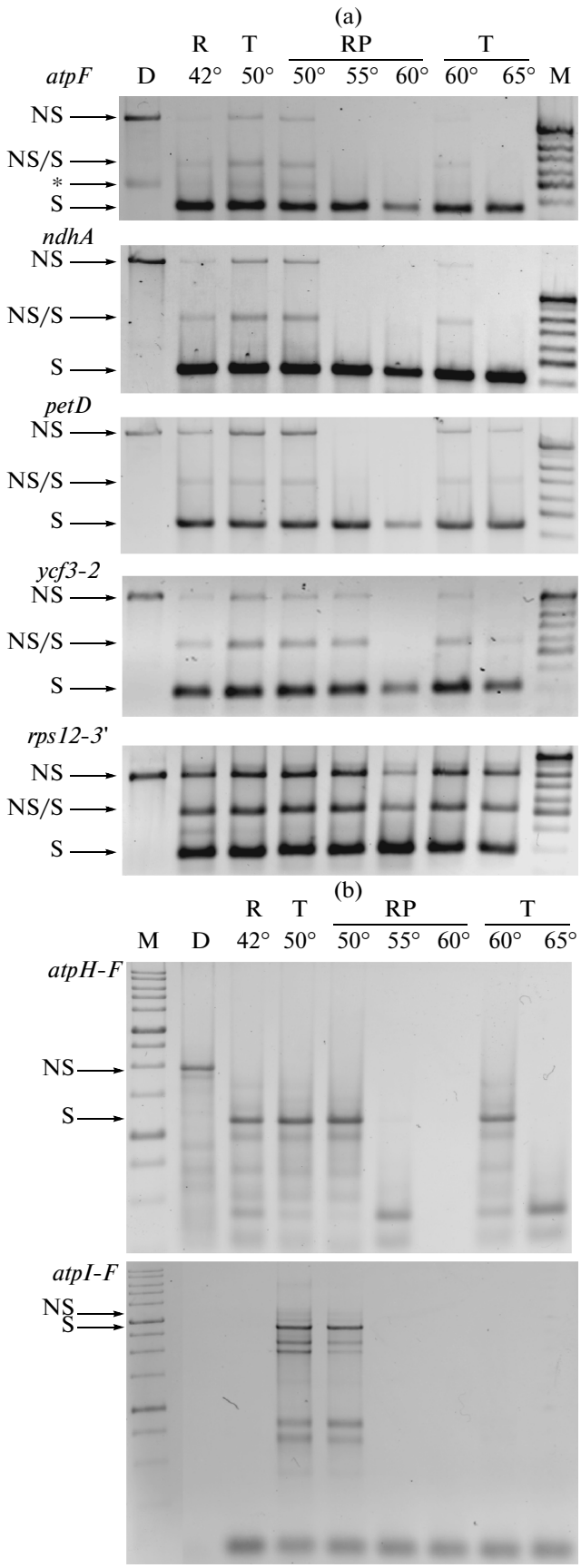

Fig. 3. Effect of enzyme used and cDNA synthesis temperature on relative yield of high-molecular-weight PCR products. (a) Effect of cDNA synthesis conditions for the five given genes. (b) Effect of cDNA synthesis conditions for longer amplicons, in this case for the *atpIHFA* operon. D is PCR with plastid DNA. Reverse transcriptases: R is RevertAid; RP is RevertAid Premium; T is ThermoScript. 42–65°C, the temperature of cDNA synthesis (°C). M is (a) 100-bp or (b) 1-kb DNA ladder marker. Fragment of plastid DNA was amplified using either standard (90 s) or prolonged (150 s) elongation step (72°C).

To find out whether the enzyme activity was the limiting factor in the analysis of long RNA molecules, we studied an extended amplicon region using primers to different genes of the *atpIHFA* operon (Fig. 3b). These primers had not been optimized for combined use, so in most cases, a large number of additional bands were observed. No PCR products were obtained from intron-containing molecules following an increase in the lengths of the analyzed fragments. However, the same trend was also observed for long, spliced RNA molecules. At 50°C, the activities of RevertAid Premium and ThermoScript did not differ, but an increase in reaction temperature impaired the enzyme activity, especially for RevertAid Premium (*atpH-F*). At 50°C, both enzymes could synthesize fragments of 2500–3000 bp (*atpI-F*), while the longest product obtained with RevertAid (42°C) was only 1200 bp long. The lack of amplicons that contain plastid DNA (*atpI-F*, reproducible result) is probably due to the inability of the DNA polymerase used (SibEnzyme) to synthesize fragments more than 3000 bp long, even for 2.5 min.

PCR: DNA Polymerase

For the reason stated above, the next step was to analyze whether the ratio between high- and low-molecular-weight amplicons in the product pool depended on the DNA polymerase used (Fig. 4). For all genes studied, the *Taq* DNA polymerase produced by SibEnzyme, which we generally use, gave a lower yield of long amplicons than the other two enzymes. In comparison, the DreamTaq polymerase synthesized the same amounts of short amplicons and significantly higher amounts of long amplicons. The Encyclo polymerase was most efficient in the synthesis of both short and long PCR products. In some cases, the use of Encyclo also allowed us to get rid of nonspecific reaction products (Fig. 4, *atpF* and *rps12-3'*). However, with regard to the ratio of PCR products for unspliced and spliced RNA forms, DreamTaq was the optimal enzyme for four of the five genes studied, and Encyclo only produced a higher portion of high molecular weight amplicons for *ycf3-2*.

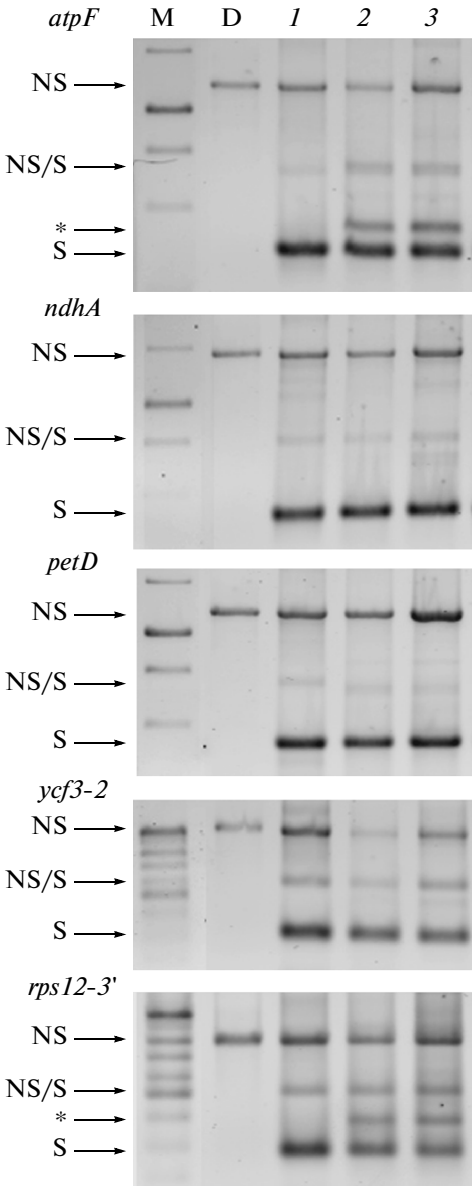

Fig. 4. Effect of DNA polymerase brand on the relative yield of high molecular weight PCR products. Here and in Fig. 5, DNase inactivation by single-step heating in the presence of EDTA (see comments to Fig. 2 and Results); cDNA synthesis with RevertAid Premium at 50°C. D, PCR with plastid DNA. DNA polymerases: (*1*) Encyclo; (*2*) *Taq* DNA polymerase (SibEnzyme); (*3*) DreamTaq. M is 1-kb DNA ladder marker (*atpF*, *ndhA*, *petD*) or 100-bp DNA ladder marker (*ycf3-2*, *rps12-3'*).

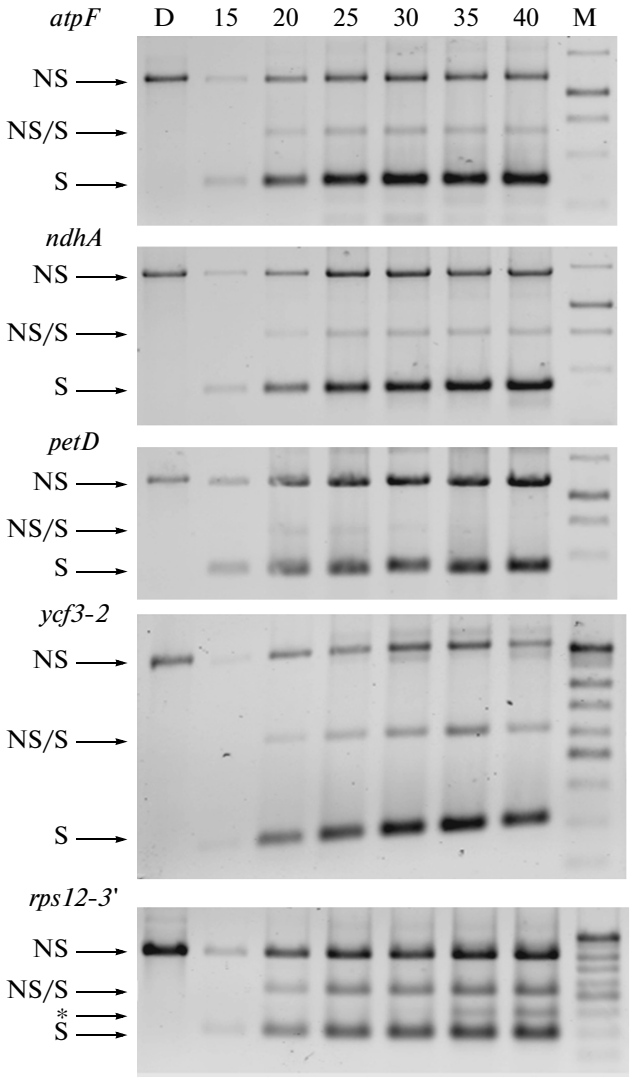

Fig. 5. Changes in the PCR product pool with increasing number of PCR cycles. DreamTaq DNA polymerase. D, PCR with plastid DNA. Number of PCR cycles in cDNA amplification is 15–40. M is 1-kb DNA ladder marker (*atpF*, *ndhA*, *petD*) or 100-bp DNA ladder marker (*ycf3-2*, *rps12-3'*).

PCR: Number of Cycles

If short amplicons are more easily synthesized in PCR than longer ones, the relative abundance of short products is expected to increase in each cycle. We analyzed the ratio between the two product types obtained in PCR of 15 to 40 cycles. No significant differences were observed for four mRNAs. For *rps12-3'*, high-molecular-weight bands were more intense than bands for shorter products after a small number of PCR cycles (15–20); however, the intensities were very similar after 35 or 40 cycles (Fig. 5). Apparently, the number of reaction cycles is only important for some amplicons; however, it seems preferable to use smaller numbers of RT-PCR cycles (in this case, approximately 20 cycles).

Recommendations

The above analysis identified the factors that affect the relative yield of high-molecular-weight amplicons corresponding to unspliced forms of RNA. Based on these data, we modified the investigation technique, optimized the reaction conditions, and were thus able

to significantly increase the portion of long amplicons in the product pool (e.g., see Fig. 2a, lane 1 and Fig. 5, lane 25). The resulting recommendations can be summarized as follows.

1. Primers should be designed to flank intron splicing site such that spliced and unspliced RNAs can be analyzed simultaneously. Preferably, one of the primers should lie as close as possible to the intron–exon border so as to diminish the probability of stable heteroduplex formation between the spliced and intron-containing strands.

2. DNase should be inactivated by heating in the presence of EDTA. It is advisable that enzymatic DNA be immediately followed by cDNA synthesis, so that a single RNA heating serves both to inactivate DNase and to denature secondary RNA structures prior to cDNA synthesis.

3. cDNA synthesis should be performed at 50°C, which is the optimal temperature for RevertAid Premium and ThermoScript reverse transcriptases. The first one is considerably less expensive.

4. Different available commercial brands of DNA polymerase should be compared to identify those that provide the highest yield of long PCR products relative to short ones. In our work, DreamTaq polymerase was optimal for most genes studied, except for *ycf3-2*, which was best analyzed using Encyclo.

5. Some nonspecific amplification can be avoided by optimizing the choice of enzymes, both reverse transcriptase (Fig. 2b) and DNA polymerase (Fig. 4).

6. It is reasonable to perform PCR with a small number of cycles; in our particular case, approximately 20.

The technique optimized using the above recommendations provides a means of successfully analyzing both spliced and unspliced mRNA forms of protein-coding genes in maize chloroplasts. Thus, it can be applied to analyze the relationship between different forms of mRNA while studying the changes in the splicing efficiency in response to various internal and external factors.

ACKNOWLEDGMENTS

The authors are grateful to E.S. Pozhidaeva for her valuable advice. The study was supported by the Russian Foundation for Basic Research (projects nos. 10-04-90052-Bel and 10-04-00594).

REFERENCES

1. Haugen P., Simon D.M., Bhattacharya D. 2005. The natural history of group I introns. *Trends Genet*. **21**, 111–119.
2. Koonin E.V. 2006. The origin of introns and their role in eukaryogenesis: A compromise solution to the introns-early versus introns-late debate? *Biol. Direct*. **1**, 22. doi10.1186/1745-6150-1-22
3. de Longevialle A.F., Small I.D., Lurin C. 2010. Nuclearly encoded splicing factors implicated in RNA splicing in higher plant organelles. *Mol. Plant*. **3**, 691–705.
4. Herrin D.L., Chen Y.F., Schmidt G.W. 1990. RNA splicing in *Chlamydomonas* chloroplasts. Self-splicing of 23 S preRNA. *J. Biol. Chem*. **265**, 21134–21140.
5. Rock C.D., Barkan A., Taylor W.C. 1987. The maize plastid *psbB-psbF-petB-petD* gene cluster: Spliced and unspliced petB and petD RNAs encode alternative products. *Curr. Genet*. **12**, 69–77.
6. Hess W.R., Hoch B., Zeltz P., Hübschmann T., Kössel H., Börner T. 1994. Inefficient *rp12* splicing in barley mutants with ribosome-deficient plastids. *Plant Cell*. **6**, 1455–1465.
7. Jenkins B.D., Kulhanek D.J., Barkanl A. 1997. Nuclear mutations that block group II RNA splicing in maize chloroplasts reveal severa1 intron classes with distinct requirements for splicing factors. *Plant Cell*. **9**, 283–296.
8. Schmitz-Linneweber C., Williams-Carrier R.E., Williams-Voelker P.M., Kroeger T.S., Vichas A., Barkan A. 2006. A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-mRNA. *Plant Cell*. **18**, 2650–2663.
9. Ueda M., Nishikawa T., Fujimoto M., Takanashi H., Arimura S., Tsutsumi N., Kadowaki K. 2008. Substitution of the gene for chloroplast RPS16 was assisted by generation of a dual targeting signal. *Mol. Biol. Evol*. **25**, 1566–1575.
10. Kolodner R., Tewari K.K. 1972. Molecular size and conformation of chloroplast deoxyribonucleic acid from pea leaves. *J. Biol. Chem*. **247**, 6355–6364.
11. Krapp A., Hofmann B., Schäfer C., Stitt M. 1993. Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates: A mechanism for the 'sink regulation' of photosynthesis? *Plant J*. **3**, 817–828.
12. Wiame I., Remy S., Swennen R., Sági L. 2000. Irreversible heat inactivation of DNase I without RNA degradation. *Biotechniques*. **29**, 252–256.