

## 2D-PAGE as an effective method of RNA degradome analysis

Martyna Nowacka · Paulina Jackowiak · Agnieszka Rybarczyk ·  
Tomasz Magacz · Pawel M. Strozycki · Jan Barciszewski ·  
Marek Figlerowicz

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**Abstract** The continuously growing interest in small regulatory RNA exploration is one of the important factors that have inspired the recent development of new high throughput techniques such as DNA microarrays or next generation sequencing. Each of these methods offers some significant advantages but at the same time each of them is expensive, laborious and challenging especially in terms of data analysis. Therefore, there is still a need to develop new analytical methods enabling the fast, simple and cost-effective examination of the complex RNA mixtures. Recently, increasing attention has been focused on the RNA degradome as a potential source of riboregulators. Accordingly, we attempted to employ a two-dimensional gel electrophoresis as a quick and uncomplicated method of profiling RNA degradome in plant or human cells. This technique has been successfully used in proteome analysis. However, its application in nucleic acids studies has been very limited. Here we demonstrate that two dimensional electrophoresis is a technique which allows one to quickly and cost-effectively identify and compare the profiles of 10–90 nucleotide long RNA accumulation in various cells and organs.

**Keywords** RNA degradome · 2D-PAGE · Small non-coding RNA · RNA biomarkers

### Introduction

The maturation, quality control and turnover of all types of transcripts are inseparably connected with RNA degradation. It secures a balance between RNA synthesis and RNA decay and provides cell homeostasis. For fulfilling its role RNA degradation has to be confined to a specific set of molecules, those unnecessary at given conditions, dysfunctional, or defective. On the other hand the RNA molecules essential for proper cell functioning should stay intact. This appears to be accomplished by strict and complex control mechanisms. Another inherent feature of RNA degradation is its high efficiency, which often precludes the identification of any intermediary products [1]. A rapid removal of any redundant RNA fragments is clearly advantageous since they have a potential to interfere with gene expression pathways [2, 3]. However, there is accumulating evidence that the degradation of several types of mature RNAs (among them tRNA, rRNA and snoRNA) can yield stable intermediates [4–6]. What is more, it has been shown that at least some of these intermediates display regulatory functions [7, 8]. These findings promote further research of stable intermediates of RNA degradation. All together these intermediates collectively constitute the RNA degradome. In consequence, demand for new methods of RNA degradome analysis is still increasing. Certainly, numerous approaches used for non-coding RNA characterization can be employed for this purpose. They cover a variety of techniques including the cloning and sequencing of RNA or hybridization-based methods such as northern blot and microarrays [9]. At present, next generation sequencing appears to

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M. Nowacka · P. Jackowiak · T. Magacz ·  
P. M. Strozycki · J. Barciszewski · M. Figlerowicz (✉)  
Institute of Bioorganic Chemistry, Polish Academy of Sciences,  
Noskowskiego 12/14, 61-704 Poznan, Poland  
e-mail: marekf@ibch.poznan.pl

A. Rybarczyk · M. Figlerowicz  
Institute of Computing Science, Poznan University  
of Technology, Piotrowo 3A, 60-965 Poznan, Poland

prevail as the method of choice due to the great potential offered by available platforms [10]. Unfortunately, massive sequencing methods are laborious and expensive. They are perfect for a very detailed analysis of RNA degradome but too sophisticated for fast screening or comparison of several RNA degradomes. The latter can be done with DNA microarrays, provided that either all components of examined RNA degradomes were earlier cloned and sequenced or tiling microarrays covering the whole genome are available. Here we showed that two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) can be employed for analyzing high copy number RNA degradants isolated from plant and human cells. The collected data indicate that one can use 2D-PAGE to determine whether and to what extent the pattern of RNA degradant accumulation is tissue- or cell-specific. What is also important, the described method is relatively simple, quick and cost-effective. It seems that 2D-PAGE can also be used instead of one-dimensional electrophoresis to facilitate the fractionation of RNA degradome for more profound sequencing studies.

## Materials and methods

### Plants growth

*Arabidopsis thaliana* (ecotype Columbia-0) were grown in peat-filled pots at 22°C during the day and 20°C at night in a 8/16 h light/dark photoperiod [11]. Leaf and root samples collected from 5-week-old plants were immediately frozen in liquid nitrogen and stored at −80°C. *Lupinus luteus* L. cv. Ventus were grown in perlite-filled pots at 24/26°C during the day and 21/23°C at night in a 16/8 h light/dark photoperiod. A detailed description of growth conditions, lupin *Bradyrhizobium* inoculation and medium contents was previously published by Strozycki and coworkers [12, 13]. Non-infected, ~5 cm long lupine roots were collected 9 days after germination. Symbiotic nodules were collected from lupine roots 9 days post-infection with *Bradyrhizobium*. Both root and nodule samples were immediately frozen in liquid nitrogen and stored at −80°C.

### HeLa and HL60 cell line cultivation

HeLa cells were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma) and gentamycin (100 µg/ml). Cells were harvested in the logarithmic growth phase by trypsin/EDTA treatment and counted using Coulter Counter. They were then centrifuged, washed with PBS, immediately frozen in liquid nitrogen (10<sup>6</sup> cells per tube) and stored at −80°C. HL60 cells were cultured at 37°C with 5% CO<sub>2</sub> in RPMI 1640 medium (Gibco) supplemented with 10%

fetal bovine serum (Sigma) and gentamycin (100 µg/ml). Cells were harvested in the logarithmic growth phase and counted using Coulter Counter, then centrifuged, washed with PBS, immediately frozen in liquid nitrogen (10<sup>6</sup> cells per tube) and stored at −80°C.

### RNA extraction

RNA samples enriched in molecules shorter than 200 nt were isolated from (i) *A. thaliana* leaves, (ii) *L. luteus* roots and nodules, and (iii) HeLa or HL60 cells using the *mirVana* miRNA Isolation Kit (Ambion). Subsequently, 1 µg of RNA was labeled with  $\gamma$  <sup>32</sup>P ATP using T4 PNK Kinase (Fermentas) and subjected to electrophoresis in polyacrylamide gel.

### 2D-PAGE of small RNAs

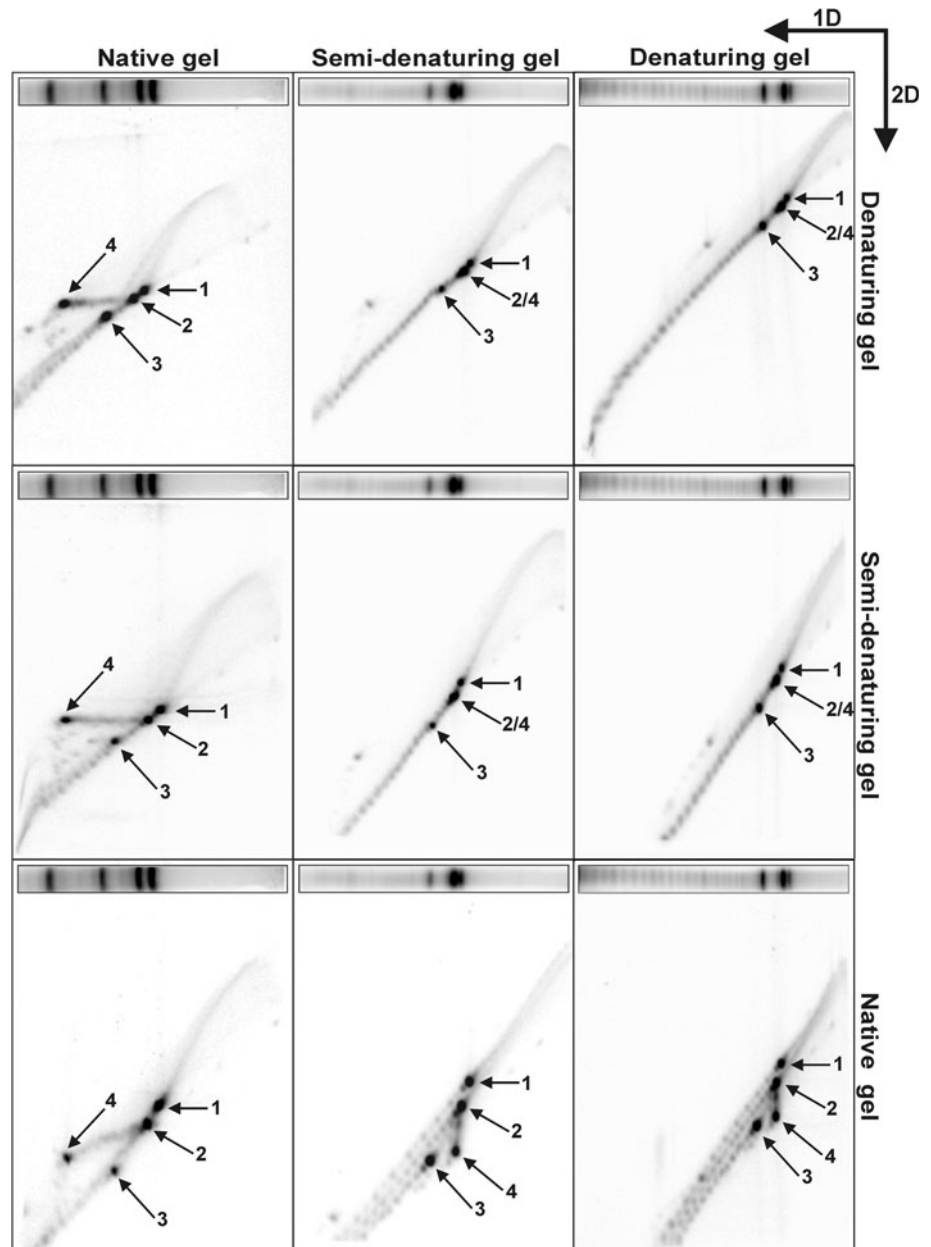
2D-PAGE was performed under: non-denaturing conditions (without urea), semi-denaturing conditions (4 M urea) or denaturing conditions (7 M urea). With minor modifications, we followed the procedure elaborated by Fradin et al. [14] (see also Online Resource Fig. 1). A 10% polyacrylamide gel (10% of acrylamide:bisacrylamide 19:1 solution; 1× buffer TBE pH = 8.3; 0/4/7 M urea, respectively; 0.1% APS; 0.1% TEMED) was used for first dimension electrophoresis (1D-PAGE). The size of the gel was 60 × 30 × 0.04 cm. 1D-PAGE was performed in 1× TBE buffer (pH = 8.3) at 300 V for 24–28 h at room temperature. A ~25 × 2.5 cm gel slice containing RNAs ranging in size between ~12 and ~120 nt was cut out from the gel (the length of RNA molecules was estimated based on the location of bromophenol blue and xylene cyanol), placed between new electrophoresis glasses, parallel to their shorter edge, and embedded in a freshly prepared 10% polyacrylamide gel (up to ~1 cm below the slice). The remaining space between electrophoresis glass plates was filled with a freshly prepared 20% polyacrylamide gel (20% of acrylamide:bisacrylamide 19:1 solution; 1× buffer TBE pH = 8.3; 0/4/7 M urea, respectively; 0.1% APS; 0.1% TEMED). The size of the 2D gel was as follows: 40 × 30 × 0.04 cm. Second dimension electrophoresis was performed at 500 V for 23–24 h at room temperature. The gel was exposed to a phosphor screen which was subsequently scanned by a Fuji FLA-5100 imaging system.

### Cloning of RNA extracted from a gel

After 2D-PAGE, RNA molecules accumulating to highest levels were visualized by autoradiography. Selected spots were excised from the gel, gel fragments were collected in



**Fig. 2** 2D-PAGE of Oligo1–4 mixtures. Electrophoreses were performed under denaturing, semi-denaturing, and native conditions. Location of particular oligonucleotides are indicated with *numbers* (1–4) and *arrows*. Directions of the first dimension electrophoresis (1D) and the second dimension electrophoresis (2D) are indicated by *black arrows*

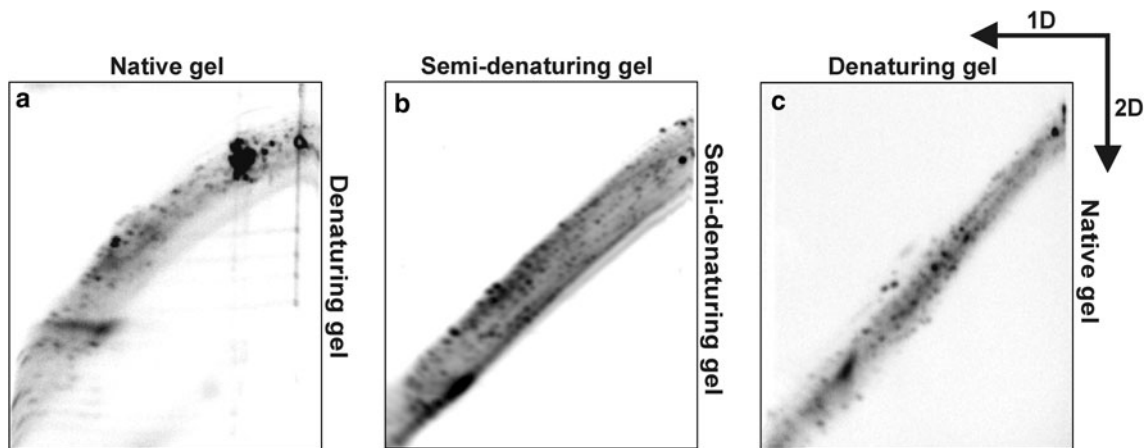


native gel for electrophoresis in the first or second dimension permitted us to distinguish among oligonucleotides with the same molecular mass but different secondary structures.

In the next series of experiments we attempted to determine whether 2D-PAGE enables an effective analysis of complex mixtures containing short RNA molecules. Taking into account the above observations we used three combinations of gels: native/denaturing, denaturing/native and semi-denaturing/semi-denaturing. The fraction of RNA enriched in molecules shorter than 200 nt was isolated from leaves of *A. thaliana*. After 5'-labelling with  $^{32}\text{P}$  (exchange reaction) the RNA samples were subjected to 2D-PAGE. The obtained patterns of separation were markedly different and highly reproducible (Fig. 3). In contrast to the

results obtained for Oligo1–4, 2D electrophoresis in native/denaturing and denaturing/native gels did not produce expected highly resolved autoradiograms (Fig. 3a, c). Instead, the gels contained a small number of well-defined spots. The other spots were poorly split and often fuzzy. The analyzed mixtures of short RNAs were best separated when semi-denaturing gels were applied for electrophoresis in both dimensions. In the corresponding autoradiograms the highest number of well separated spots was detected (Fig. 3b).

The data presented above suggested that 2D-PAGE can be used for the analysis of complex mixtures composed of short RNA molecules. Unfortunately, we did not know whether the observed RNAs were indeed the products of



**Fig. 3** 2D-PAGE-based analysis of RNA (fraction enriched in RNA shorter than 200 nt) isolated from *A. thaliana* leaves. Electrophoreses were performed under native/denaturing (a), semi-denaturing/semi-

denaturing (b) and denaturing/native (c) conditions in the first and second dimension, respectively. The direction of electrophoresis in the first and second dimensions is indicated by *arrows*

degradation. Recent studies indicated that the fraction of RNA between 30 and 80 nt can be especially rich in this type of molecules (longer than siRNA/miRNA and shorter than tRNA) [4, 6, 18–25]. Accordingly, we attempted to determine the nature of the selected RNA visualized in 2D gels. The RNAs extracted from the 20 most intense spots (Fig. 3b) were mixed together and amplified by RT-PCR. The obtained cDNA was concatamerized, cloned and sequenced. The sequencing of 150 clones let us to identify 20 RNAs. Their length ranged from 19 to 86 nt but half of them (10) were 28–38 nt long. A computational analysis involving *A. thaliana* genome database revealed that the majority of the sequenced cDNA clones (19) represented the products of tRNA (9), rRNA (8), mRNA (1) and snRNA (1) degradation (Table 1). One molecule was classified as an unknown transcript of the intergenic region. Several mismatches to the *A. thaliana* genomic sequence were detected during BLAST search. Since they appeared only in tRNA-derivatives, it is highly plausible that these mismatches reflect modified bases in tRNA.

Finally, we tried to determine whether 2D-PAGE can be used to identify specific profiles of RNA degradation in various cells/tissues/organs or in the same type of cells/tissues/organs but growing in different conditions. For this purpose, fractions of RNA enriched in molecules shorter than 200 nt were extracted from different plant organs and from human cell lines and subjected to 2D-PAGE under semi-denaturing conditions.

The exemplary autoradiograms presented in Figs. 3, 4 and 5 suggest that the generated patterns of RNA degradant accumulation are cell- or organo-specific. In Fig. 4 we compared RNA extracted from *L. luteus* roots (Fig. 4a) and from young nodules forming on *L. luteus* roots infected with symbiotic bacteria (Fig. 4b). In Fig. 5 RNA isolated from HeLa and HL60 cell lines were compared. In both

cases significant qualitative and quantitative differences in individual RNA accumulation were observed. In general, the obtained results indicate that 2D-PAGE is a very effective fast and cheap technique that can be applied for the analysis of high copy number RNA degradants. It can be an interesting alternative to other classic techniques such as northern blot hybridization or standard sequencing.

## Discussion

Although developed over 30 years ago and successfully applied for fractionation of tRNA and structural analysis of viral RNA [14, 26–29] the 2D-PAGE technique had not found many applications in RNA-related areas. Recently it has been shown that this method is suitable for the analysis of oligonucleotide mixtures [9, 30, 31]. In this study, we demonstrated that 2D-PAGE can be successfully applied for analyzing RNA degradants ranging in size from ~10 to ~90 nt.

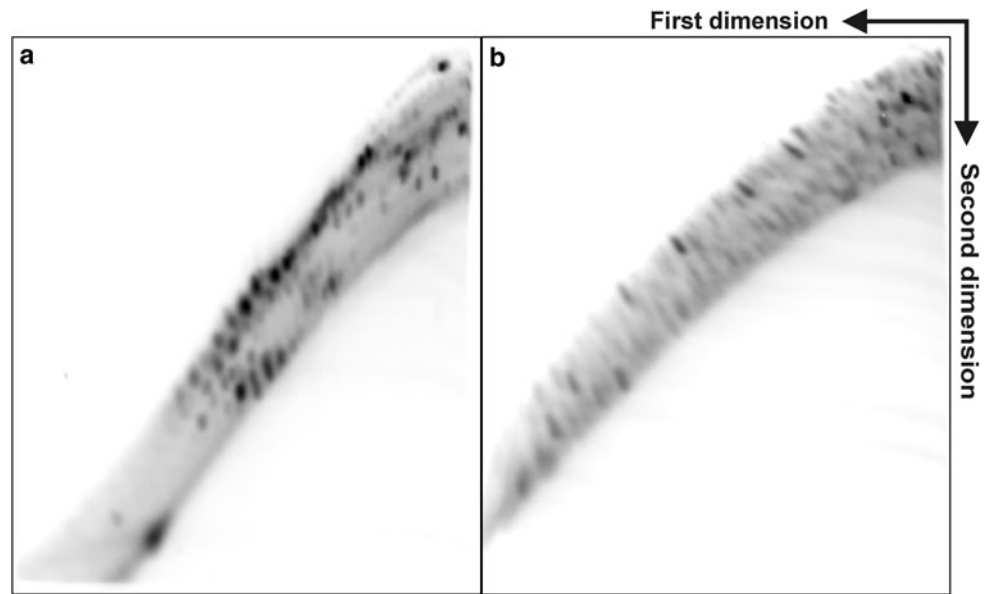
The experiments carried out in distinct electrophoretic conditions revealed that 2D-PAGE enables an effective fractionation of RNA molecules even if they have the same length but different base composition or secondary structure. Our results indicate that the application of native gels in the first or second dimension is the best choice for analyzing a limited number of small-sized RNA molecules. We also observed that 2D-PAGE under semi-denaturing/semi-denaturing conditions is the best option if one wishes to examine very complex and diversified pools of RNA extracted from cells/tissues/organs. The electrophoresis in native gels preserves the intra- and intermolecular interactions. As a result, the analyzed RNAs are not well resolved because they can adopt various conformations or form multimolecular complexes. In contrast, under semi-

**Table 1** RNA identified in the samples isolated from *A. thaliana* leaves

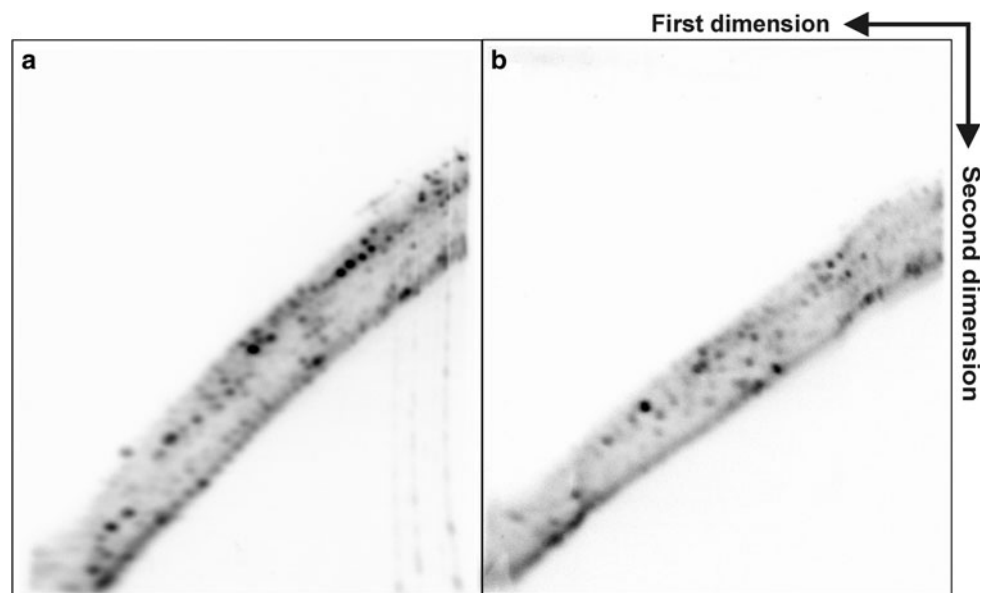
No.	Sequence (5'–3')	Length (nt)	tRNA
1	CCAGAGAUUCUGGGUUCGGU <u>CCCCAGUGAAGUCUGCCA</u>	38	Arg/ACG
2	AGAUUGGGUUUCGU <u>UCAACCCGUGGUCGCCA</u>	33	Arg/CCG
3	UCCUUAGCUCAGUGGU <u>AGACAAUUGACUGC</u>	31	Cys/GCA
4	UAGAAUAGU <u>ACCCUGCCACGGUACAGACCCGGGUUCGUUCCCGGCUUGGUCACCA</u>	56	Gly/GCC
5	AGUGGUCUAGUGGCAUGAUAGUACC	25	Gly/GCC
6	UCGCUGGUU <u>CGUUCCGGCAGGUCGGACCA</u>	30	Tyr/GTA
7	CGUGGGU <u>UCAU</u> UCCACAGUUGUCACCA	29	Leu/TAG
8	CAGGAUACUGGCUCACCCGAGA <u>GGCCCGGGUUCGUUCCCGGCAACGGAAACCA</u>	56	Glu/CTC
9	CGAGACU <u>CAAAAUCUCGUGCUA</u> AAGAGC	28	Leu/CAA
No.	Sequence (5'–3')	Length (nt)	rRNA
10	CGAUUCGGGGCGUGGACCGACGGGAU <u>ACGGUGGGGCCUAAGCCCCGGGCC</u>	52	25S
11	AGAAGCGUCCUCAGCGACGGACCGGGCC	28	25S
12	CGGGAGUAA <u>CUAUGACUCUC</u>	20	25S
13	CGCAAGUGAAAUACCACUA	19	25S
14	CAUUCAAUUUCUGCCCUA <u>UCAACUUUCGAUGGUAGGAUAGUGGCCUA</u>	48	18S
15	CUAUGGGUGGUGGU <u>CAUGGCCGUUCU</u> AGUUGGU	35	18S
16	UGAAGU <u>UUCGGAUCGCGGGCACGUGGGUGGU</u>	32	18S
17	UCUCAUGGAGAGU <u>UCGACCCGGGUCAGGAUGAACGCUGGGCGGCAUGC</u>	48	16S
No.	Sequence (5'–3')	Length (nt)	Others
18	AGAAAGCCCAAAAGAUAGCUUGCUAUCUGGGCUUUCACGAGUCGCCCAUGCGUU <u>GCACUACUGCACGGGCCUUGGCUCAACCCGCC</u>	86	U2.3 ncRNA
19	AAAGGAGGAAGAAACAAAGCAGUCUCUGUUCAUUUUU <u>UCCUUUC</u>	49	mRNA
20	UAGACUCUAAAAAUACCCUUGGUACUUUA	29	AT4G39140 Intergenic

Mismatches between the cloned species and the genomic sequence are underlined

**Fig. 4** Comparative 2D-PAGE analysis of short RNA isolated from *Lupinus luteus* roots (a) and nodules forming on the *Lupinus luteus* roots 9 days-post-infection with symbiotic bacteria (b). The direction of electrophoresis in the first and second dimensions is indicated by arrows



**Fig. 5** Comparative 2D-PAGE analysis of short RNA isolated from HeLa (a) and HL60 (b) cells. The direction of electrophoresis in the first and second dimensions is indicated by arrows



denaturing conditions only very stable structures can be preserved. As a result the majority of RNA molecules migrate as individual well-separated spots.

Recently, it has been shown that some of RNA degradation intermediates can accumulate in cells instead of being hydrolyzed to single nucleotides [4, 6, 18–25]. Accordingly, we hypothesized that a large fraction of RNA molecules observed in 2D gels (especially, molecules shorter than tRNAs and longer than miRNAs) might be products of RNA degradation. Cloning and sequencing of RNAs extracted from the most intensive spots confirmed our assumption. It turned out that out of 20 analyzed RNAs, 19 could be classified as degradants. They were identified as fragments of tRNA, rRNA, mRNA and snRNA. The obtained results also suggest that the

composition of RNA degradome can be cell/tissue/organo-specific. It seems that the profiling of RNA degradant accumulation can provide some information about physiological or pathological processes occurring in examined cells or organisms. Therefore, RNA degradome components could be considered as potential biomarkers in monitoring developmental processes, stress responses, or host–pathogen interactions.

In general, it seems that 2D-PAGE is an effective tool for analyzing high copy number RNA degradants. The undertaken studies disclosed many advantages of this technique. It permits fast comparison of RNA degradomes isolated from cell lines, tissues or organs. Unlike northern blots or DNA microarrays it does not require sequencing of individual degradants prior to their analysis. In addition, it restricts the

number of analyzed individuals to those accumulating to relatively high levels. 2D-PAGE also allows us to quickly monitor changes occurring in RNA degradome under the influence of endogenous or exogenous factors. Finally, this method can be used to identify novel classes of small RNAs analogous to the already found sno-derived RNA degradants [5] or tRNA-derived RNA degradants [8, 32].

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