Cytokinin production from tRNA in *Saccharomyces cerevisiae*

Salme Timmusk, Anna Golovko, Elisabeth Tillberg, Björn Nicander*
Department of Plant Biology, P. O. Box 7080, Swedish University of Agricultural Sciences
S-75007 Uppsala, Sweden

* corresponding author

Summary

N^6-isopentenyladenosine is one of the modified nucleosides of the tRNA of *Saccharomyces cerevisiae* and many other organisms. In plants this compound, in the tRNA-free form, is an important cytokinin hormone. Extracts of logarithmically growing yeast cells were found to contain tRNA-free N^6-isopentenyladenosine, mainly bound as the 5'-monophosphate. The level was around 4 pikomoles per gram cells. The cells also excreted isopentenyladenine; a level of about 0.4 nM was found in the medium of mid log phase cultures. Low molecular weight isopentenyladenines could derive from degradation of tRNA, but a direct pathway bypassing tRNA has been proposed (Laten and Zahareas-Doktor 1985). A mutant yeast line, that lacks the gene for tRNA isopentenyl transferase (IPPT, E.C. 2.5.1.8), has no isopentenyladenosine in tRNA. When the culture medium of this line was analyzed, no isopentenyladenine could be detected (> 0.01 pmol per 100 ml medium from log phase culture). This indicates that the excretion of isopentenyladenine in wild type cells require the presence of tRNA isopentenyltransferase. tRNA as an intermediate in the formation of free isopentenyladenosine is further supported by studying the yeast line lacking IPPT transformed with the genes for IPPT originating from either *Homo sapiens* or *Arabidopsis thaliana*. In these, the levels of isopentenyladenosine in tRNA was lower than in the wild type. The level of isopentenyladenine excreted into the medium was also reduced, supporting that tRNA is an intermediate in the pathway to the tRNA-free isopentenyladenines in yeast.

Introduction

The cytokinins are a group of molecules with key regulatory roles in plant growth and development. The cytokinins are adenines modified at position N^6 with a short hydrophobic sidechain, usually isoprenoid. Cytokinins are also found as modified bases at position A37 in certain tRNAs that bind to codons starting with an U. The tRNA cytokinins are found in plants, animals and most microorganisms (Persson *et al*. 1994; Taller 1994) .
tRNA cytokinins are synthesized by tRNA isopentenyltransferases (IPPT) (Winkler 1998) that insert an isopentenyl group from dimethylallyl pyrophosphate into AMP residues at position 37 in certain pre-tRNA. All tRNAs with cytokinin have been found to have anticodons complementary to codons starting with U. The IPPT enzymes have been studied extensively in microorganisms, including *Saccharomyces cerevisiae* (Persson *et al*. 1994, Tolerico *et al*. 1999 and references therein). We recently cloned the first genes coding for IPPT enzymes from Homo (Golovko *et al*. 2000) and Arabidopsis (Golovko *et al*. 2002) .
A hormonal role for cytokinins has only been shown for plants, but many suggestions of functional links to tRNA cytokinin function have been made (see Letham and Palni 1983; Murai 1994). For instance, it has been suggested that the hormones are synthesized through tRNA, since tRNA degradation would release active cytokinins like isopentenyladenosine (i^6A). A link between free and tRNA-bound cytokinins has not been demonstrated, but at least part of the cytokinin pool could conceivably arise when tRNA is degraded, for instance during turnover (reviewed in Prinsen *et al*. 1997). The major source of tRNA-free cytokinins in plants are the recently discovered class of cytokinin synthase enzymes (Kakimoto 2001, Takei *et al*. 2001). Certain plant-associated microorganisms like *Agrobacterium tumefaciens* have long been known to have similar enzymes (Morris 1986) but also in this case a production from tRNA been shown (Gray *et al*. 1996).
Presumably, cytokinins acquired their hormonal role early in plant evolution. From this perspective, it was of great interest when evidence for a direct pathway to the cytokinin isopentenyladenosine (i^6A) was found in *Saccharomyces cerevisiae* and *Saccharomyces pombe* (Laten and Zahareas-Doktor 1985). Both wild-type and mutants that lack i^6A in tRNA were found to contain significant amounts of tRNA-free i^6A. However, these results must be questioned since the medium used contained a supplement of autolyzed yeast extract, a component of many media used for the growing of microorganisms. The supplement could be the source of the i^6A, since it has been shown to contain large amounts of compounds active in cytokinin bioassays (van Staden 1974). The presence of isopentenyladenine (deribosylated i^6A), i^6A and small amounts of zeatin and zeatin riboside, has been shown in yeast extract (Difco brand) using HPLC and immunoassay (Jameson and Morris 1989). We have made the first unequivocal identification of i^6A from yeast extract (Oxoid brand) employing GC-MS (Timmusk *et al*. 1999).

We have now reexamined, using unequivocal methods, the tRNA-free cytokinins in yeast.

Materials and Methods

Yeast culture

Saccharomyces cerevisiae strains used were H57 (MATa MOD5 SUP7 can1-100 ade2-1 his5-2 lys1-1 ura3-1 and MT-8 (MATa SUP7 ura3-1 his5-2 leu2-3,112 ade2-1 trp1 lys1-1 lys2-1 can1-100 mod5::TRP1 (Gillman *et al*. 1991)(gifts from Dr. Anita Hopper, Pennsylvania State University College of Medicine, Pennsylvania, USA). The yeast lines were maintained on YPD medium (Gillman *et al*. 1991). For cytokinin experiments, the cultures were grown in a synthetic complete medium (containing per liter: glucose 20 g, $(NH_4)_2SO_4$ 5 g, Yeast Nitrogen base (Difco) 1.7 g, and adenine 40 mg, uracil 40 mg, histidine 10 mg, leucine 60 mg, lysine 40 mg, methionine 10 mg, tryptophan 10 mg, isoleucine 60 mg, threonine 50 mg, tyrosine 50 mg, phenylalanine 60 mg, arginine 10 mg, aspartate 100 mg, glutamate 100 mg, serine 375 mg, valine 150 mg, at pH 6.7-6.8) at 30° C in a climate chamber on rotary shaker (130 rpm) until the first late log phase (OD 0.5). Cells were harvested by centrifugation at 7000 g for 20 min.

Extraction

The cells were extracted in methanol: formic acid: water (60:5:35) as described (Hammerton *et al*. 1996). Extractant chilled to -20° C was added to the yeast pellet, and the mixture sonicated for 20 seconds. Kinetin riboside (100 pmol) was added to serve as an internal standard. Sonication was repeated when the extract reached -10°, -5° and 0° C. Insoluble material was removed by centrifugation, and reextracted twice. The supernatants were pooled and the methanol removed by rotary evaporation. The remainder was passed through columns of insoluble polyvinylpolypyrrolidone (Nicander *et al*. 1993), then lyophilized.
Medium was extracted by the addition of ice cold methanol to 60%, and formic acid to 5%, final volume. Kinetin riboside (50 pmol) was added and the mixture was left at 0 °C for 30 min, then centrifuged as above. Methanol was removed by rotary evaporation, and the remainder lyophilized.

Immunoaffinity chromatography

Samples were dissolved in phosphate buffered saline (PBS, 50 mM phosphate, 140 mM sodium chloride, 1mM EDTA, pH 7.0). pH was adjusted to pH 7 ±0.2 with NaOH, filtered, and taken through a precolumn containing 1 ml Sepharose 6B (Pharmacia, Uppsala, Sweden). The samples were passed through an immunoaffinity column as described (Nicander *et al*. 1993), except that the column temperatures were 30-35 °C. The eluate was evaporated under vacuum until less than 300 μl remained, diluted by the addition of 2 ml PBS, then taken through the immunopurification a second time. The new eluates were evaporated under vacuum until less than 300μl remained.

HPLC

A Merck-Hitachi 655A-12 programmable pump, or two Gilson model nnn pumps were used. Samples were separated using a 125 x 4 mm column and a 75 x 4 mm column connected in series, both packed with Supersphere RP-select B (Merck-Hitachi). The gradient profile was: 0 min 1% acetonitrile; 8 min 3%; 21 min 16,6%; 25 min 40%. The column temperature was 35° C. Eluates were monitored by a scanning UV detector (Spectra-Focus, Spectra-Physics, San Jose, CA, USA).

Nucleotide degradations

The fraction containing the putative i^6A nucleotide peak was collected, evaporated to dryness and dissolved in 100 mM Tris-HCl (pH 8.0). This was mixed with MgCl2 to give 10 mM, 0.1 U of Crotalus 5'-nucleotidase (Sigma, St. Louis, Mo., USA), and 100 pmol of benzyladenosine-5'-monophosphate. The final volume was 300 μl. The sample was incubated for 30 min at 37° C. Fractions that could contain i^6A di- and triphosphates were collected and evaporated to dryness, dissolved in 100mM Tris-HCl (pH 8) and incubated with 1 U bacterial alkaline phosphatase (Sigma Chemical. CO. St. Louis, USA) and 100 pmol benzyladenosine-5'-monophosphate for 60 min at 37° C.

For treatments 1 and 2, proteins were removed by addition of cold methanol to give 60%. the samples were kept on ice for 20 min and centrifuged. The supernatants were evaporated under vacuum until 100-200 μl remained.

The antibody column flow-through non-binding fraction was also treated to aid detection of any i^6A di- and triphosphates. Benzyladenosine-5'-monophosphate (100 pmol) was added, and the solution was treated with sodium periodate followed by methylamine to release the bases from nucleotides (Garrett and Santi 1979). The samples were then neutralized, immunopurified and analyzed by HPLC as described above.

Gas chromatography and mass spectrometry

Permethylation of standards and samples was performed according to Kovak (1993), except that chloroform was used instead of dichloromethane and the organic phase was backwashed two times with water before it evaporated to dryness under a stream of nitrogen at 60° C. The samples were dissolved in 3 μl of chloroform, from which 1 μl was injected. A Fison MD 800 EI 60 mass spectrometer (Fison Instrument, UK) coupled to a gas chromatograph (model 800, Carlo Erba) was used. The mass spectrometry conditions were: EI ionizing voltage 70 eV, source temperature 300° C, interface temperature 290° C. GC-MS chromatograms were obtained by selected ion recording with a dwell time of 50 ms and a mass range span of 0.3 amu. Ions monitored were 391, 348, 216, 215, 174. The GC had a splitless injection at 300° C and a 15 m x 0.25 mm x 0.1 μm DB-5ms, (J&W Scientific, USA) column with helium as the carrier gas et 4 psi. The temperature program was 1 min at 70° C, 15 min to 290° C and 5 min at 290° C.

Results

Isopentenyladenosine production in yeast

Laten and Zahareas-Doktor (1985) reported the presence of significant amounts of tRNA-free isopentenyladenosine (i^6A) in extracts of *Saccharomyces cerevisiae*. A mutant line, where the tRNA isopentenyl transferase gene was altered, resulting in a level of i^6A in tRNA a few percent of that in the wildtype, was found to have similar amounts of tRNA-free i^6A. This led to the suggestion that *Saccharomyces* can make i^6A through a pathway bypassing tRNA (Laten and Zahareas-Doktor 1985). In view of later results, this conclusion must be questioned. The medium used in the study contained autolyzed yeast extract, a common medium supplement. Autolyzed yeast extract has later been shown to be a rich source of isopentenyladenine (Jameson and Morris 1989), which could have been the source of the i^6A.

The cell line MT-8 has had the tRNA isopentenyl transferase gene inactivated by an insertion (Gillman *et al*. 1991). This line was grown, using defined media, to mid log phase and the content of i^6A in tRNA and in the medium was analyzed (Table 1). No i^6A was detected from tRNA or medium. The wild type did contain i^6A in both tRNA and medium. This indicates that production of tRNA-free i^6A requires the presence of an active tRNA isopentenyl transferase. To further study the relation between tRNA cytokinins and free cytokinins, the line MT-8 was transformed with either the human tRNA isopentenyl transferase (Golovko *et al*. 2000) or the *Arabidopsis* IPPT (Golovko *et al*. 2002). In both lines, i^6A production resumed in tRNA and the medium (Table 1). The levels in tRNA were lower than the level in the wild-type, indicating that these enzymes are less efficient in producing i^6A-tRNA in the yeast environment than the yeast IPPT. The reduced i^6A in tRNA level was accompanied by reduced levels of isopentenyladenine in the media (Table 1), supporting the idea that the tRNA-free cytokinin arise via tRNA.

Table 1. Levels of i^6A in tRNA and medium from mid log phase cultures of *Saccharomyces cerevisiae*.

yeast line	i^6A in tRNA pmol/OD_{260} unit	i^6A* in medium pM
wild type	97	398
MT-8	0	0
MT-8, human IPPT	31	98
MT-8, plant IPPT	43	47

* in the deribosylated form (isopentenyladenine)

tRNA-free cytokinins in yeast

The tRNA of *S.cerevisiae* contain a single cytokinin, isopentenyladenosine (i^6A) (Persson *et al*. 1994). To determine if the yeast contain tRNA-free cytokinins, cells grown in defined medium were harvested in mid log phase and low molecular compounds extracted. The extract was purified with immunoaffinity chromatography using broad affinity antibodies that bind over 25 different cytokinins, including most of the active forms found in plants (Nicander *et al*. 1993). Figure 1 shows an HPLC chromatogram of the antibody-binding fraction.

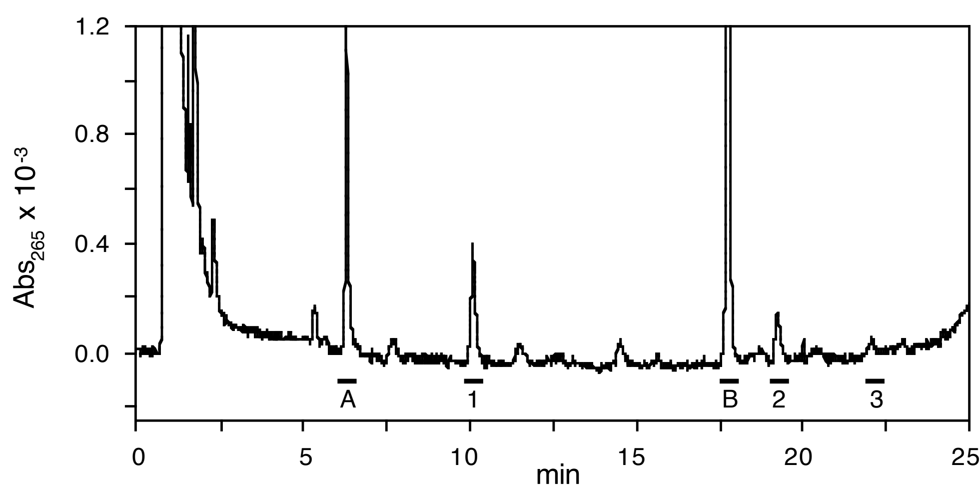

Figure 1. HPLC chromatogram of immunopurified yeast extract. Horizontal lines below the absorbance trace indicate where standards elute. A: dideoxyadenosine; added before injection for relative retention time calculations, B: kinetin riboside; added at extraction time to monitor recovery. 1: i^6A-5'-monophosphate, 2: i^6A, 3: isopentenyladenine.

Peaks are seen at the elution times of i^6A, the i^6A 5'-monophosphate, and isopentenyladenine. The mononucleotide was quantitatively the most abundant. The three compounds can all arise from tRNA by enzymatic degradation. Treatment of the fraction containing the peak labelled 1 (Figure 1) with 5’-nucleotidase, resulted in a compound that co-eluted with i^6A (not shown). The material from this peak was permethylated and analysed by GC-SIM (Fig. 2), proving that the compound was i^6A. Plant cells have been reported to sometimes contain large amounts of the di- and triphosphates of i^6A (Laloue *et al*. 1974). If these forms are present in yeast, they could hold a significant part of the total isopentenylated adenines. It was not known if cytokinin di- and triphosphates bind to the immunoaffinity columns, or if they would give peaks in the reversed-phase HPLC system used. Therefore both the antibody-binding fraction and the flow-through fraction were treated to degrade the nucleotides to nucleoside or base (see Materials and Methods). HPLC analysis of the reaction products showed that no i^6A or isopentenyladenine resulted (data not shown).

Common types of cytokinin found in plant extracts, *trans*-zeatins, *cis*-zeatins, or dihydrozeatins were not found. Quantitation of the three UV peaks showed that the total level of free isopentenylated adenines compounds amounts to about 4 pmoles per gram fresh weight cells, with about 75% as the nucleotide.

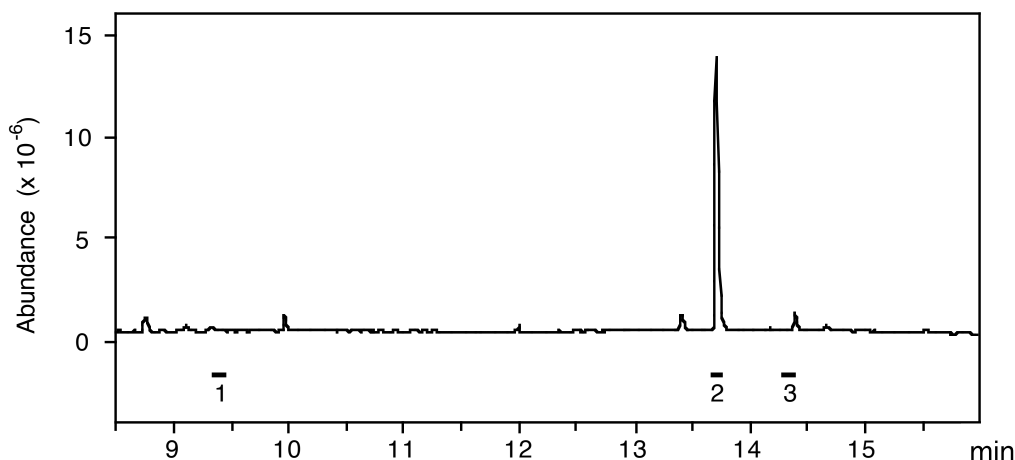

Figure 2. GC-MS chromatogram of the compound resulting from 5'-phosphatase treatment of peak 1 from Figure 1. Horizontal lines below the trace indicate where standards elute. 1: isopentenyladenine, 2: i^6A, 3: isopentenyladenine-9-glucoside. The relative abundance ratios of peak 2 were 100 (174), 82 (202), 45 (216), 44 (215), 20 (348), 17 (391). Values in parenthesis are the m/z monitored. Authentic i^6A gave the ratios 100, 73, 42, 40, 19, 17 for the same ions.

Discussion

The results here indicate that tRNA-free cytokinins in yeast originate from tRNA. They contradict the suggestion of Laten and Zahareas-Doktor (1985) that a second pathway to i^6A excist in yeast. In that work, a strain with a point mutation in the *S.cerevisiae* IPPT *MOD5* gene was found to have levels of free i^6A even higher than wild type cells. The MT-8 line used for the present report has had the *MOD5* gene inactivated by an insertion, and has no i^6A in tRNA (Gillman *et al.* 1991). The mutant used by Laten and Zahareas-Doktor (1985) still had some i^6A in its tRNA, even though the level was reduced to less than 2% of that in the wild type. This could not very well be the source of the i^6A, so a second pathway to, specifically, free cytokinin was proposed to be operating in yeast. It is more likely however that the source was the medium used, since it contained autolyzed yeast extract. This has later been shown to be a rich source of isopentenyladenine, probably arising from degradation of tRNA (Jameson and Morris 1989).
The simplest explanation for this is that the cytokinin seen in the wild type strain in Fig 1 and table 1 arise via tRNA. Free cytokinin could be released by RNase action during tRNA turnover, though other more specific mechanisms are possible. For instance, procaryotes are considered to have hardly any tRNA

turnover at all, yet in the plant pathogen *Agrobacterium tumefaciens* considerable amounts of cytokinins are released from tRNA (Gray *et al*. 1996), though it is not known if the production was sufficient to achieve a physiological effect on plants.
The idea that tRNA is the source of the free cytokinins of yeast is supported by experiments where two other eucaryotic IPPT genes, from *Homo* and *Arabidopsis*, respectively, were expressed in the MT-8 line lacking MOD5. The levels of tRNA-bound i^6A and excreted isopentenyladenine is listed in Table 1. The alien IPPT proteins complemented the mutant phenotype (Golovko *et al*. 2000, Golovko *et al*. 2002) but contained lower levels of i^6A in tRNA than the line with wild type MOD5. This could perhaps be due to a more narrow substrate specificity, since higher eucaryotes are known to have a much smaller set of i^6A-containing tRNAs (Taller 1994). The lower levels were mirrored in a lowering of the amounts of isopentenyladenine excreted to the media (Table 1).
The fact that Laten and Zahareas-Doktor (1985) found i^6A and the present report mainly the 5'-mononucleotide of i^6A is quite likely due to differences in extraction procedures. We used an acid-metanol-water mixture known to inactivate plant phosphatases (Jameson and Morris 1989, Hammerton *et al*. 1996), while Laten and Zahareas-Doktor (1985) lyzed the cells enzymatically for 2 hours at 35° C.
The results presented here does not eliminate the possibility of a second pathway to i^6A-containing compounds in yeast. For instance, such a pathway could be become activated during other physiological states, for instance starvation. i^6A has been shown to have a significant effect on sporulation in yeast (Laten 1995). A search of the *Saccharomyces cerevisiae* genome found no homologs of the MOD5 or bacterial or plant cytokinin synthases, the genes that code for three classes of enzyme that can isopentenylate adenine moieties.
Nucleotides, nucleosides and bases are rapidly interconverted in cells, and nucleotides are generally present in far higher levels than the corresponding nucleosides and bases (Brown 1991). The quantitative predominance of the nucleotide form of cytokinin in yeast is similar to the situation in many plant tissues. In most studies where extraction methods that inactivate phosphatase degradation have been used, the 5-mononucleotide forms of the cytokinins often predominate (Letham and Palni 1983; Palni et al. 1983). Unfortunately these precautions to preserve cytokinin nucleotides are not always taken when cytokinins are analyzed. Laten and Zahareas-Doktor (1985) used an immunoassay to measure crude extracts. Since the antibodies used cross-react to i^6A monophosphate, it cannot be excluded that part of what the antibodies was reacting with was in fact i^6A nucleotide. It is also possible that the nucleotide had been degraded to i^6A during the extraction.
Although cytokinins are adenine derivates and appears to be metabolised in a similar fashion as the adenines, the lack of di- and triphosphate forms indicate that yeast cannot phosphorylate the i^6A mononucleotide further.
In addition to i^6A, Jameson and Morris (1989) also found low levels of *trans*-zeatins (hydroxylated i^6A) in autolyzed yeast extract. With the amounts of yeast used in the present study, these would be undetectable.

The levels reported here are clearly lower than those commonly reported from plant extracts. Tissues with a large proportion of rapidly dividing cells, and thus are in a physiological state somewhat comparable to the yeast culture, contain several hundreds of pikomoles of cytokinins (for an example, see Palni et al. 1983). Tissues with low levels of dividing cells contains much less, but still more than the yeast cells. As an example, mature tobacco leaves contained about 20 pikomoles total cytokinin per gram (Singh *et al*. 1992). To directly compare the yeast value with cytokinin levels observed is probably misleading, for instance, a far greater proportion of the plant weight is extracellular material. Culture medium of the moss *Physcomitrella patens*, where cytokinins do play a hormonal role, contained circa 80 pM (Wang *et al*. 1984), and cultures of *Agrobacterium* contained around 260 pM (Gray *et al*. 1996). Both values are lower then the yeast excretion (Table 1). While a role for cytokinin in yeast similar to that in plants seems unlikely, the compound is present, and biosynthesis via tRNA is no argument against a regulatory role. i^6A has been shown to affect yeast spore formation, and is thus a candidate for having a role outside that as a structural component of tRNA (Laten 1995).

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