Studies of the role of tRNA-isopentenyl-transferases in the formation of cytokinins

Grzegorz Furman

Uppsala BioCenter
Department of Plant Biology and Forest Genetics
Faculty of Natural Resources and Agriculture Sciences
Swedish University of Agricultural Sciences

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Studier av tRNA-isopentenyltransferasers roll i bildandet av cytokininer

Grzegorz Furman

Supervisor: **Björn Nicander, SLU**
Department of Plant Biology and Forest Genetics

Examiner: **Johan Meijer, SLU**
Department of Plant Biology and Forest Genetics

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Abstract

Plant tRNA isopentenyltransferases (tRNA-IPT, EC 2.5.1.8) have two roles, modification of tRNA and contribution to cytokinin synthesis. tRNA-IPT is among the best studied of the tRNA-modifying enzymes, but little biochemical information is available and none for the plant enzymes. Therefore two sets of experiments were conducted.

The first one aimed to determine the kinetics and substrate specificity of the tRNA-IPT *AtIPT2* from the higher plant *Arabidopsis thaliana*. The second project aimed to study the role of IPTs from the moss *Physcomitrella patens* in the production of cytokinin hormones.

In moss only tRNA-IPTs but no adenylate IPTs are present (Schwartzenberg et al.2007). Adenylate IPTs are the major source of cytokinins in higher plants. The moss also differs by having more tRNA-IPTs than any other organism known. The first goal was to determine if these IPTs actually modify tRNA, or if one or more are in fact cytokinin synthases. For these studies a yeast mutant (MT8) lacking the intrinsic IPT gene was used. The mutant was transformed with the *IPT* from *P. patens*. The tRNA obtained from MT8 was used for *in vitro* assays to study the substrate specificity of the *Arabidopsis AtIPT2*.

Keywords: *Arabidopsis thaliana, Physcomitrella patens, Saccharomyces cerevisiae,* tRNA isopentyltransferases, cytokinin biosynthesis

Sammanfattning

tRNA isopentenyltransferaser (tRNA-IPT, EG 2.5.1.8) har två funktioner, modifiera tRNA och bidra till cytokinin syntes. tRNA-IPT är bland de bäst studerade av alla tRNA enzymer, men trots detta finns endast lite biokemisk information tillgänglig, och ingenting är känt om växtenzymerna. Därför utfördes två experiment.
Det första var att bestämma kinetik och substratspecificitet för tRNA-IPT formen AtIPT2 från *Arabidopsis thaliana* .
Det andra projektet var att studera vilken roll olika IPT från mossan *Physcomitrella patens* har för produktionen av cytokinin hormoner.
I mossa finns bara tRNA-IPT men inte adenylat-IPT (Schwartzenberg et al.2007). AdenylatIPT är den största källan för cytokininer i högre växter. Mossan skiljer sig också genom att ha många fler tRNA-IPT gener än någon annan känd organism. Det första målet var att bestämma om dessa IPT faktiskt kan förändra tRNA, eller om en eller flera av dessa i själva verket är cytokinin syntaser. För dessa studier användes en jäst mutant (MT8) som saknar IPT genen. Jästmutanten transformerades med *IPT* från *P. patens*. Erhållen tRNA från MT8 användes för *in vitro* tester för att studera substrat specificitet för AtIPT2 från *Arabidopsis*.

Nyckelord: *Arabidopsis thaliana, Physcomitrella patens, Saccharomyces cerevisiae,* tRNA isopentyltransferases, cytokinin biosyntesis

Contents

Aim of the study

In order to determine the role of tRNA-IPTs in cytokinin formation two subprojects were outlined. One approach was to study the role of tRNA-IPT in cytokinin synthesis by establishing an *in vitro* assay to study the substrate specificity of *Arabidopsis thaliana* AtIPT2. The second project addressed whether the *Physcomitrella patens* IPT actually are encoding tRNA-IPTs. This study used complementation analysis of a *Saccharomyces cerevisiae* mutant lacking the intrinsic IPT gene with IPTs from *P. patens*.

Introduction

Cytokinins – important regulators of plant development

Cytokinins (CK) have a crucial role in regulating the proliferation and differentiation of plant cells and control a variety of processes in plant development such as shoot and root balance control (Werner et al., 2001; Werner et al., 2003), nutritional signalling (Samuelson and Larsson, 1993; Sakakibara, 2005) and senescence (Gan, 1995; Mok, 1994). Rice plants genetically modified for increased cytokinin levels showed significantly higher productivity (Ashikari et al., 2005).

Cytokinins were discovered in the 1950s. The first discovered cytokinin, trans-zeatin (tZ), which occurs naturally in plants, was isolated in the 1960s from immature maize endosperm (Letham, 1963). Since then several other cytokinin-active molecules have been discovered in various plants (Mok and Mok, 2001; Strnad, 1997). There are two types of cytokinins: aromatic and isoprenoid. Some of the commonly occurring CKs in the aromatic group are: ortho-topolin (oT), meta-topolin (mT), benzyladenine (BA), ortho-methoxytopolin (MeoT) and meta-methoxytopolin (MemT) cytokinins (Sakakibara, 2006). To the isoprenoid CK group belongs N^6-(▲^{2}-isopentenyl) adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DZ) (Sakakibara, 2006).

Different cytokinins have different functions within groups. For example meta-topolin from the aromatic group of CK was used as an inducer of axillary bud proliferation (Holub et al., 1998).Another CK, methoxytopolines were tested in different bioassays, investigating tobacco callus growth stimulation, degradation of chlorophyll in excised wheat leaves and induction of betacyanin synthesis in *Amaranthus caudatus* (Tarkowska et al., 2003). tZ isoprenoid CK found in plant transpiration seems to be important for nutrient signal communication (Corbesier et al., 2003). Another type of isoprenoid CK, iP affects morphogenetic budding process (Schwartzenberg et al., 2007). The function of iP could be proven by creation of cytokinin deficient *Physcomitrella*. Zeatin found in coconut milk induce plant growth (growth of auxillary stems, callus initiation in combination with auxin) also it was found *in vitro* that the CK have anti-aging capability on skin fibroblasts (Rattan and Sodagam, 2005; Mok and Mok, 1994).

Biosynthesis of plant cytokinins

The first biosynthetic pathway forming isoprenoid cytokinins like iP or tZ, called *de novo* biosynthesis, starts with prenylation of adenylic nucleotides such as adenosine 5'-phosphatase (AMP, ADP and ATP) at the N^6 position. The reaction occurs with dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP). The reaction is catalyzed by adenylate-isopentenyltransferase (adenylate-IPT) (fig.1 A) (Sakakibara, 2006; Natalya et al., 2008). The substrate specificity for the enzymes catalyzing the reactions depends on the species and origin of enzymes (Sakakibara, 2006).

The second pathway forms cytokinins by tRNA degradation. tRNAs contains cytokinins, some of which are hyper-modified.The cytokinins are attached to adenine (A37) which is linked to the anticodon (Yevdakova et al., 2008). The dimethylallyl side chain used in the reaction is obtained from the mevalonate pathway or the

methylerythritol phosphate pathway. The reaction is catalyzed by tRNA-isopentenyltransferase (tRNA-IPT) (fig.1 B) (Kasahara et al., 2004).

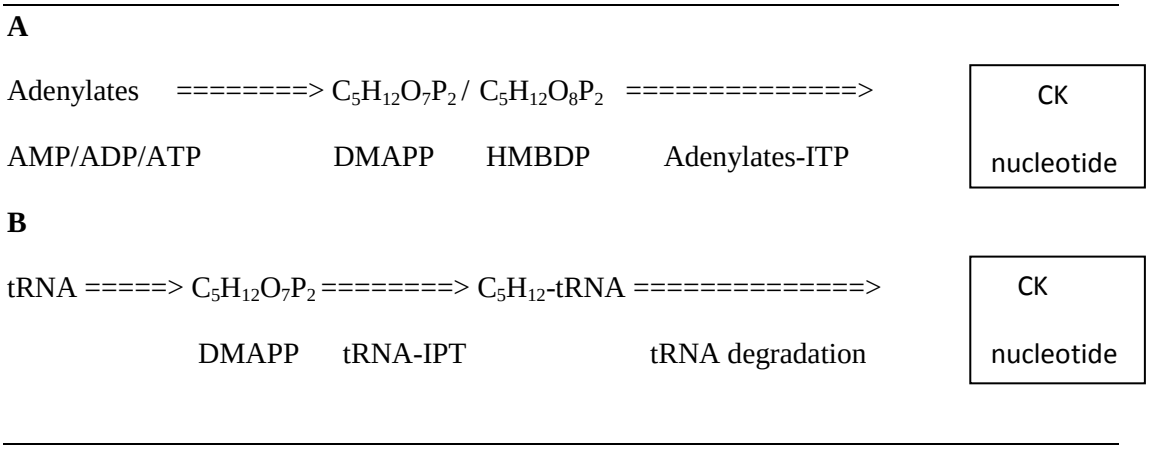


Fig. 1. The cytokinin biosynthetic pathways involving IPT enzymes. **A.** Cytokinin synthesis by adenylate-tRNA; **B.** Prenylation of adenine (N^6) at tRNA anticodon position A37 catalyzed by tRNA-ITP, followed by tRNA degradation leading to free cytokinins.

The adenylate isopentenyltransferases

IPT enzymes catalyze the transfer of the 5-C isoprene unit from DMAPP to the N^6 of adenine. These enzymes are divided into three types.

The first type of adenylate-IPTs is found in higher plants, where they constitute the first step in the synthesis of plant hormones in the cytokinin group. These enzymes catalyze the prenylation of the adenylic nucleotides ADP and ATP at the N^6 position. The isoprene unit is transferred from DMAPP, see fig.1 A (Sakakibara, 2006; Natalya et al., 2008). The substrate specificity for the enzymes catalyzing the reactions depends on the species and origin (Sakakibara, 2006).

A second type of enzymes is AMP-IPTs found in bacteria *Agrobacterium tumefaciens* and slime mold *Dictyostelium discoideum*. The isoprene source for these enzymes is DMAPP or hydroxymethylbutenyl diphosphate (HMBDP). *D. discoideum* was the first organism in which the substrate of isoprenoid CK was identified (Abe et al., 1976). Mold IPT use AMP or ADP and not ATP or cAMP as side-chain acceptor (Ihara et al., 1984; Taya et al., 1978). An *IPT* gene was first characterized in the gall-forming bacterium *A. tumefaciens* (Akiyoshi et al., 1984; Barry et al., 1984). This bacterium has two *IPT* genes, *Tmr* and *Tzs* (John and Amasino, 1988; Powell et al., 1988). Both gene products use DMAPP/HMBDP as isoprene donors and they only utilize AMP (Blackwell and Horgan, 1993; Krall et al., 2002 ;Sakakibara et al., 2005).

The third enzyme type is tRNA-IPTs. All eukaryotes, including humans and plants contain tRNA-IPT. These enzymes are also found in most bacteria (Edmonds et al., 1991). Only tRNA with the motif A36-A37-A38 serve as a substrate, i.e., certain tRNAs binding to codons beginning with U. The modified adenine, isopentenyladenine (i^6A) is always in position 37 (Björk,1995, Yevdakova et al., 2008). The dimethylallyl side chain used in the reaction is obtained from the mevalonate pathway or the methylerythritol phosphate pathway, see fig.1 B (Kasahara et al., 2004). The amino acid sequences of the tRNA-IPTs are highly conserved

throughout evolution. Recently the 3D structure of some tRNA-IPTs have been determined. Figure 2 shows results from the yeast tRNA-IPT bound to a substrate tRNA (Zhou and Huang, 2008). The best conserved areas have been shown to be in or near the catalytic centre (Fig. 2, left).

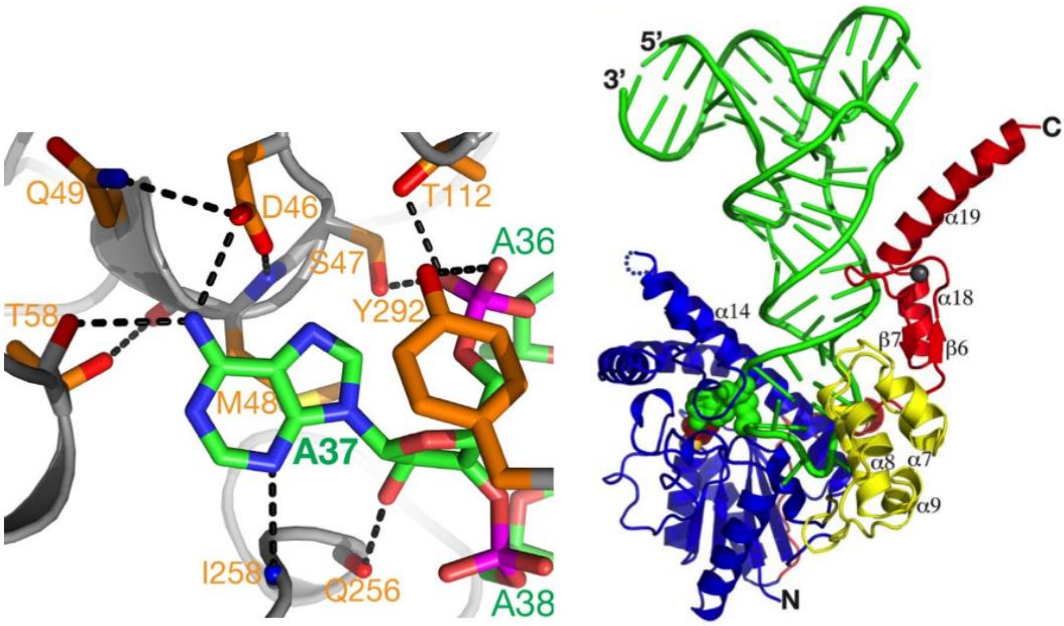


Fig. 2. The left drawing shows adenine at tRNA position 37 as part of the catalytic site of yeast tRNA-IPT. The right drawing shows tRNA (green) attached to yeast tRNA-IPT (Zhou and Huang, 2008).

The anticodon loop of the tRNA interacts with alpha helix14 and the sequences colored yellow in Fig. 2. Eucaryotic tRNA-IPTs have a long extension at the C-terminal that was also shown to interact with tRNA (red in Fig. 2 right). This includes a single Zn-finger motif (Golovko et al., 2000).

Cytokinin biosynthesis via tRNA

Ever since cytokinins were discovered in tRNA it has been speculated if they have a role in the formation of the cytokinin hormones (reviewed in Letham and Palni, 1983). The tRNA-IPTs modify a base at position A37 in certain tRNAs with an isopentenyl group (Björk, 1995). If the tRNA is degraded, the released modified base is a cytokininthat can thus contribute to cytokinin hormones. *Arabidopsis* mutants lacking tRNA-IPTs lacked all *cis*-zeatin cytokinins (Miyawaki et al., 2006). In primitive plants like moss, a BLAST search showed that only the tRNA-type of IPT was present (Schwartzenberg et al., 2007).Accordinglythe tRNA pathway appears to be the only way to form cytokinins in this plant. In *Dictyostelium*, cytokinins play a role in sporulation. A mutant lacking the AMP-IPT but retaining tRNA-IPT still produced cytokinins, but at a lower rate, and eventually also sporulated (Anjard and Loomis, 2008). These cytokinins thus appear to have arisen from the pathway starting with tRNA-IPT.

IPT in mosses

Moss experimental systems was used early on for studies of cytokinins (see Schwartzenberg, 2006). The sensitivity to growth substances and simple

developmental differentiation make moss useful in such studies as homeostasis (Cove, 2005, Decker et al., 2006). The first bioassay using moss was developed by Hahn and Bopp (1968) where the aim was to estimate the concentration of cytokinins. The other moss *Physcomitrella patens* was used by Perry and Cove (1986). They compared wild type with the mutant type *ove* which should have higher production of cytokinins, but did not found evidence for a difference in cytokinin production between these two types. Other studies by Schwartzenberg (2006) showed that the *ove* mutants have higher production of cytokinins. The IPT enzyme is encoded by the *PpIPT* gene. Several such genes were found, but only one of the genes have been cloned and expressed in yeast lacking tRNA- IPT. Bioinformatic analysis of the *P. patens* genome performed by Sakakibara et al. (2006) showed that no adenylate-IPT genes were found in moss. In the study by Yevdakova and Schwartzenberg (2007), *PpIPT1* and *PpIPT2* showed important homologies with tRNA-IPTs. The studies showed that the tRNA isopentenylation is restored in adenine free medium, the presence of cytokinin riboside (iPR) was confirmed by HPLC and UV spectra confirmed that iPR was formed by *PpIPT1.* Another study by Yevdakova et al. (2008) supported these results and showed that the tRNA dependent pathway to form cytokinins is of great importance. The study showed that the cZ and iP cytokinins dominate in extracts from tRNA, *PpIPT1* transcription increases in moss mutants and no genes for adenylate-IPT was found in the moss.

To test for tRNA-IPT function, the yeast strain MT8 was used in these studies. The MT8 strain contains three genetic modifications (Gillman et al., 1991). The first is a disruption of *MOD5*, the yeast tRNA-IPT gene. This results in a complete lack of the i^6A modification in tRNA. The second modification is several UAA nonsense mutations, including one in *ADE2* (adenine biosynthetic gene). Mutations were also introduced in *LYS1*and *CAN1*. This leads to an absolute requirement for adenine and lysine for MT8 growth. The third modification was a mutated tRNA, *SUP7,* which can suppress UAA nonsense mutations by inserting tyrosine. *SUP7* needs to be isopentenylated for this to happen (Laten et al., 1978). The MT8 strains do not contain a functional tRNA-IPT gene and therefore the MT8 strain cannot grow on medium lacking adenine and lysine. MT8 transformed with a functional tRNA-IPT can grow on a medium without adenine, though.

Materials and methods

Probing the substrate binding of tRNA isopentenyltransferases by functional complementation in *Saccharomyces cerevisiae*

Yeast strains

The *S. cerevisiae* strain MT8 (Dihanich et al., 1987) transformed with the plasmid pfl61 containing one of various IPT genes were used. The strains were obtained from Dr. Gunvant Patil. The MT8 strains were transformed with IPT genes from *P. patens*: *PpIPT1, PpIPT2, PpIPT4* or *PpIPT5*. The yeast strains were grown at 28°C on synthetic complete medium lacking adenine (SC-ade medium) (Dihanich et al., 1987).

Cultivation of yeast

Starter cultures with 40 ml SC-ade medium were inoculated with each of the yeast strains and incubated at 28°C with shaking overnight. The overnight cultures were added to Erlenmeyer flasks with 500 ml of SC-ade medium. The untransformed MT8

cultures were grown in SC medium with adenine. The cultures were further incubated at 28°C with shaking. The growth was monitored by regular sampling and measuring the absorbance at 600 nm. When the OD_{600} reached late log phase (approximately OD_{600}: 0.8 - 0.9) the cultures were harvested by centrifugation at 4000 rpmfor 5 min at 4°C. MT8 with *PpIPT2* was grown by direct inoculation to 500 ml of SC-ade medium. The OD_{600} was higher than for other cultures and reached 1.3 - 1.4.

tRNA isolation

The method of Buck and Ames (1983) was followed with some modifications. Yeast cells was suspended in a mixture containing 4.5 ml of buffer A; 0.5 ml 10% SDS and 0.2 g glass beads (0.2 mm). Thereafter the cells were lysed by disruption during 30 seconds using an Ultra-Tunax T25 (Janke&Kunkel IKA-labortechnik). The mixture was transferred to a Falcon tube containing 10 ml buffer equilibrated phenol. The samples were shaken vigorously for 60 min at 8°C, then centrifuged for 20 min to separate the phases. The aqueous phase was transferred to a new Falcon tube with 10 ml phenol as above, and shaken vigorously for 20 min at 8°C. After a second centrifugation the aqueous phase was transferred to a new Falcon tube, and 0.1 volume of calcium acetate (buffer B) was added, followed by dropwise addition of 0.2 volumes 12 M LiCl with vigorous vortexing. The samples were left overnight at 8°C.

sRNA fractionation

High molecular weight nucleic acids were removed by centrifugation. The supernatant was mixed with 3 volumes of 99.5 % ethanol and transferred to Corex tubes. Samples were stored overnight at -20°C and RNA collected by centrifugation. Corex tubes were dried by inverting them on paper and then lyophilized briefly to remove residual ethanol. The pellets were dissolved in 2 ml 0.1 M Tris-HCl, pH 7.5.

DEAE cellulose chromatography

Polypropylen columns (6 ml) containing 2.5 ml DEAE-Cellulose (GE Healthcare, Uppsala, Sweden) were prepared. The column was primed by washing first with 15 ml milliQ-water, then with 3 ml 0.1 M Tris, 2 M NaCl, pH 7.5, followed by 10 ml 0.1 M Tris pH 7.5. The samples were added, and the columns washed with 2 ml 0.1 M Tris and 5 ml 0.2 M NaCl (pH 7.5). Thereafter 10 ml of the Tris buffer with 1 M NaCl was added to elute the tRNA (Buck and Ames, 1983). The concentration of the eluates were measured by absorbance at 260 nm. Thereafter 1 ml of KAc (pH 4.5) and 3 volumes of 99.5% ethanol were added to the samples in Corex tubes. The tRNA was precipitated overnight at -20°C.

tRNA concentration measurement

The overnight samples were centrifuged and the tubes dried as described above. The pellets were dissolved in 1 ml of milliQ-water and the concentration measured as Abs_{260}.

Sample desalting

Sample desalting was achieved by using PD-10 Desalting Columns (GE Healthcare, Uppsala, Sweden). The procedure was done according to the manufacturer's instructions. The desalted samples were concentrated to approximately 60 µl using a vacuum centrifuge.

tRNA degradation

tRNA was degraded to nucleosides following Gehrke et al. (1982). Samples were heated at 100°C for 2 min then cooled on ice for approximately 10 min. RNA degradation was accomplished by the use of Nuclease P1 (US Biological, USA). The final sample volume was 23 µl containing 8 U Nuclease P1, 13 mM sodium acetate, pH 5.3, and 1.5 µM $ZnCl_2$. Incubation was at 37°C for 3 hours. Nucleotides were then dephosphorylated overnight at 37°C using 7.5 U bacterial alkaline phosphatase (Sigma P-4252) in 0.25 M Tris, pH 7.9. The reaction was stopped by adding methanol to 60% final concentration. After 30 min on ice the samples were centrifuged. Methanol and most water were removed from the supernatants by vacuum centrifugation, taking care not to take the samples to full dryness.

High-performance liquid chromatography (HPLC)

The method of Buck et al. (1983) was followed with modifications. A gradient from 0.01 mM KPO_4, pH 5.8 (buffer A) to acetonitrile-water (40%:60 %) (buffer B) was run at 1 ml per minute. The gradient was (% buffer B, minutes) : 0, 0; 0, 3; 5, 10; 25, 25; 50, 30; 75, 34; 75, 37; 100, 45; and 100, 48.

Studies of substrate specificity towards pre-tRNA and adenylates of a plant tRNA isopentenyltransferase

Yeast strains

S. cerevisiae strain MT8 (Dihanich et al., 1987) lacking the *MOD5* gene was used. Cultures were obtained from Dr. Gunvant Patil. The yeast strains were grown at 28°C on synthetic complete medium (Dihanich et al., 1987).

tRNA isolation from MT8

All steps were performed as described above.

Cultivation of *E. coli* transformed with his-tagged *AtIPT2*

The starter culture with 10 ml LB medium was inoculated with *E. coli* and incubated at 30°C with shaking at 170 rpm overnight. The overnight culture was added to Erlenmeyer flasks with 100 ml of LB medium (including 100 µl of chloramphenicol). The culture was further incubated at 25°C with shaking at 170 rpm. The growth was monitored by regular sampling and measuring absorbance at 600 nm. When the OD_{600} reached late log phase (approximately OD_{600}= 0.6 - 0.8), 1ml of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. The culture was further incubated for 4h before centrifugation whereafter the cells were stored at -70°C.

AtIPT2 purification

The protein was isolated under native conditions using Ni-NTA Spin Columns (QIAGEN). All steps were performed according to the Ni-NTA Spin Kit Handbook (Second edition, 2008). Protein was desalted by using TMD buffer (60 mM Tris-HCl, pH 7.5, at 24°C; 20 mM $MgCl_2$ and 2 mM DTT) before concentration measurement. Sample desalting was done using PD-10 Desalting Columns according to the manufacturer's instructions.

Enzyme assay

[3H]-DMAPP (American Radiochemicals, Saint Luis, MO, USA) was used as a substrate. The incubation mix contained 5 µl BSA; 5 µl [3H]- DMAPP; 5 µl TMD buffer 30 mM Tris-HCl pH 7.5 at 24°C; 10 mM $MgCl_2$ and 1 mM DTT; 5 µl tRNA and different amounts of enzyme, and adjusted with milliQ water to a final volume of 50 µl. The reaction was stopped after 8 minutes by adding 0.5 ml cold 10% TCA taking the samples to 0°C. The samples were collected on Whatman GF/C filters, washed with 10 ml of cold trichloroacetic acid and 10 ml of cold ethanol. Filters were left overnight to dry. The filters were counted in scintillation mixture.

Testing of the filtration procedure

Differing amounts of yeast tRNA (Sigma) was mixed with [32P]-labeled 15 basepair long DNA fragments. The samples were taken through the modificated filtration procedure (see description below) or washed in petri dishes as follows: two filters were placed in petri dishes with 10% cold TCA for 10 min. The step was repeated once more with freshly prepared petri dishes. Afterwards the filters were washed as described above with cold ethanol. Filters were left overnight to dry. The filters were counted in scintillation mixture.

To the samples, 0.5 ml of cold 10% TCA was added. The mixture was vortexed immediately after each addition. Tubes were then placed on ice for 10 minutes. Filters were wetted with a drop of 10% cold TCA (0.5 ml) before addition of the samples. The samples were pipetted to the filters and 0.5 ml of cold 10% TCA was added to the empty eppendorf tubes, vortexed and added to the filters. This eppendorf tube wash was repeated one more time. Afterwards the filters were washed according to standard procedures (see description above).

Results and Discussion

Functional complementation of yeast using moss tRNA-IPT genes

HPLC analysis of tRNA from transformed yeast

Four IPT homologues from moss were cloned by Dr. Gunvant Patil (data not shown). To test if they have the ability to modify tRNA, they were expressed in yeast MT8, a mutant strain lacking *MOD5*, the yeast IPT gene (Hopper et al., 1980). The loss results in an inability to form adenine compounds. The transformed yeast strains were grown at 28°C on synthetic complete medium lacking adenine (SC-ade medium)

(Dihanich et al., 1987). tRNA was prepared, degraded to nucleosides, and analyzed by HPLC.

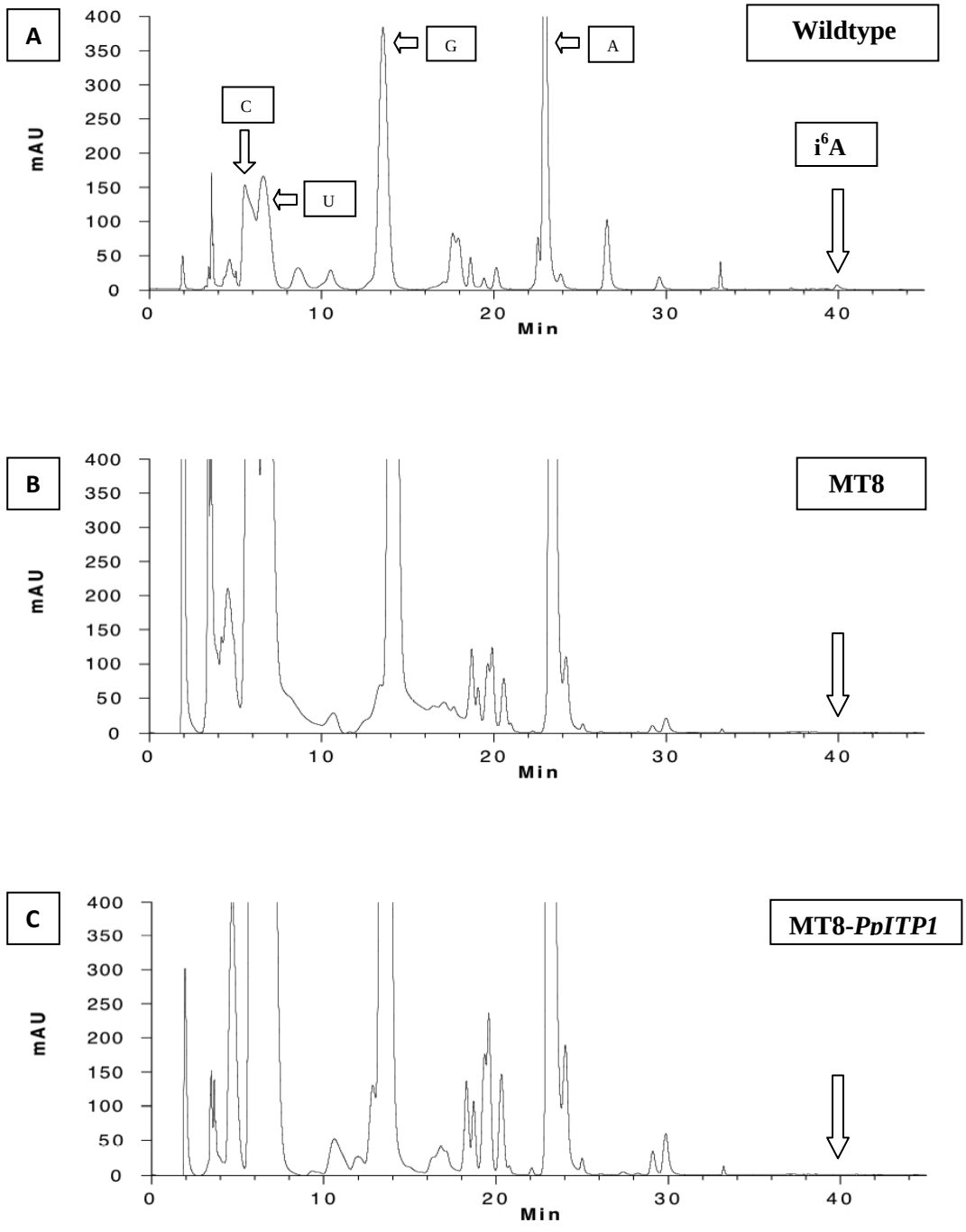
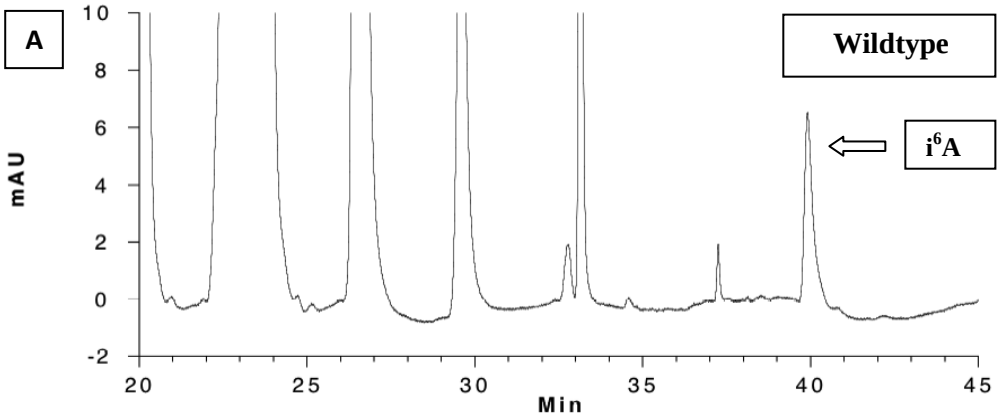
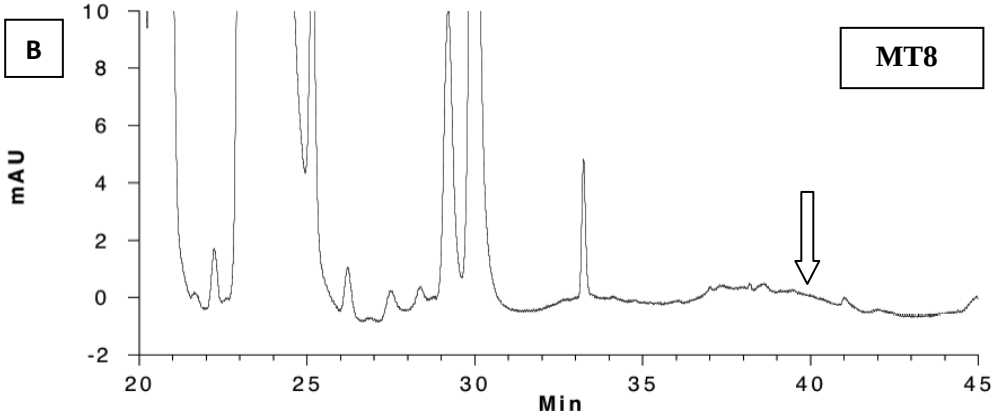
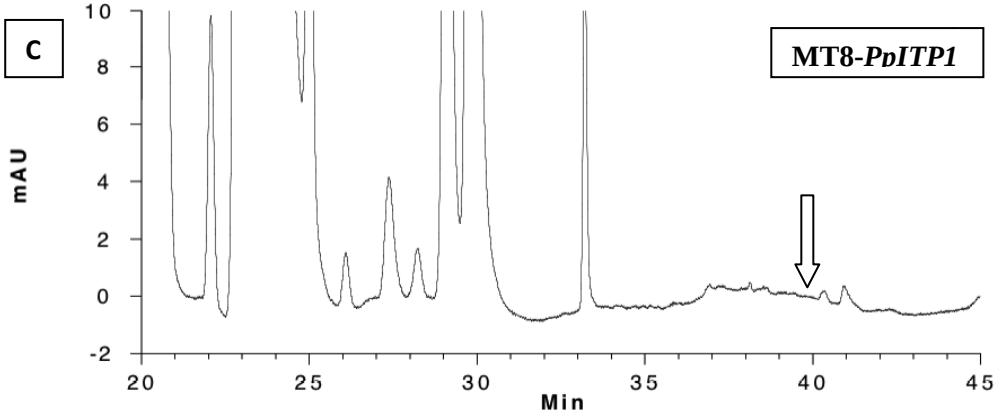


Fig. 3. HPLC chromatograms of total yeast tRNA nucleosides corresponding to 4 OD_{260} tRNA (wildtype) or 5 OD_{260} (MT8 and MT8-*PpITP1*) **A.** Wildtype yeast strain (obtained from Sigma) **; B.** MT8 with no transformation; **C.** MT8 transformed with *PpIPT1*. The large peak at 5-6 min (cytidine),7 min (uridine), 13-14 min (guanosine) and 23-24 min (adenosine). Arrows to the right shows the retention time for i^6A.


A
Wildtype
i6A
mAU
Min


B
MT8
mAU
Min


C
MT8-PpITP1
mAU
Min

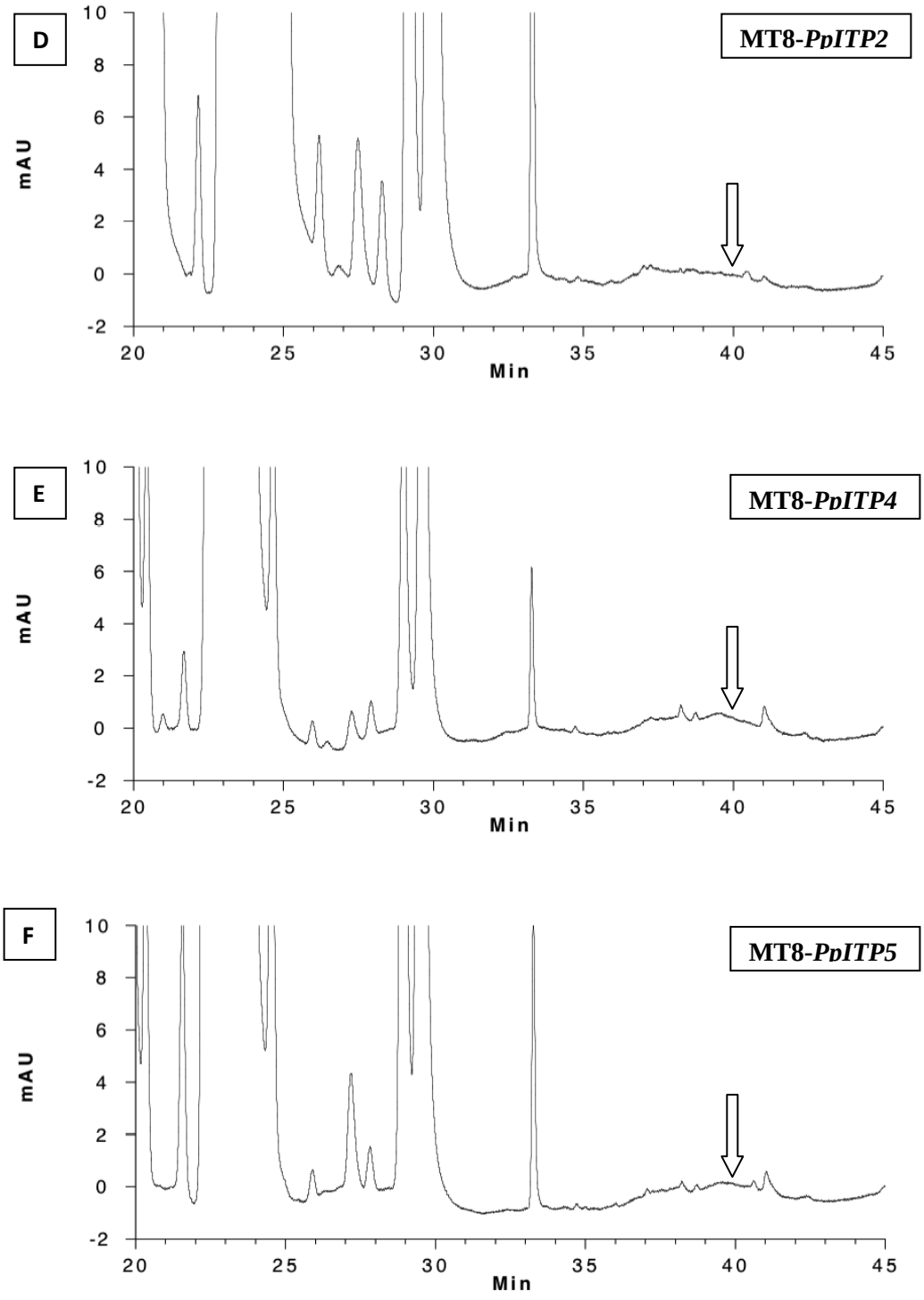


Fig.4. HPLC chromatograms of hydrophobic yeast tRNA nucleosides. 4 OD_{260} tRNA (wildtype) and 5 OD_{260} (MT8, MT8-*PpITP1*, MT8-*PpITP2*, MT8-*PpITP4*, MT8-*PpITP5*) tRNA was enzymatically digested and injected for analysis. Chromatograms are shown from 20 min, and include more hydrophobic nucleosides. The peak at 23-24 min shows adenosine. tRNA nucleosides from: **A.** Wildtype yeast strain (obtained from Sigma) ; **B.** MT8 with no transformation; **C.** MT8 transformed with *PpIPT1* **D.** MT8 transformed with *PpIPT2*; **E.** MT8 transformed with *PpIPT4*; **F.** MT8 transformed with *PpIPT5.* Arrows shows the retention time for i^6A.

Modified nucleosides in tRNA may have an essential role in cellular regulation. The modifications which occurs, varies between each other and can range from uncomplicated methylations to more advanced hypermodifications (Nishimura, 1979). Figs. 3 and 4 show chromatograms of the tRNA nucleoside composition of the yeast lines analyzed. Figure 3 shows the composition of nucleosides from yeast wildtype tRNA, untransformed MT8 and MT8-*PpITP1*. The scale is chosen to visualize the major nucleosides. Figure 3A shows four big peaks, for the major nucleosides. The big peak at 23-24 min corresponds to adenosine. This nucleotide is more hydrophobic and therefore elutes later than others. The next major peak at 13-14 min is guanine (fig. 3A). The third peak at 6-7 min represents uracil (fig. 3A). Cytosine is probably the smaller peak at 5 min (fig. 3A). The other smaller peaks are the modified nucleosides specific for tRNA (Buck and Ames, 1983). The differences in the sizes of these peaks can be explained by different growth conditions, and different levels of contamination from other forms of RNA remaining after purification.

RNA is not an easy material to isolate because of the size of the RNA molecules and the insignificant differences between different RNA types that provide challenges to find appropriate chromatographic conditions. RNA molecules affected by contamination precipitate or are irreversibly absorbed at different phases of chromatography. Another problem is cross-contamination of the peak of interest with other peaks e.g. due to large dead volume in the HPLC tubing between detector and fraction collector.

An internal standard would behave similarly to the sample, but would give a signal that could be distinguished from the sample . This would improve the precision of quantitative analysis. It would also correct for variability due to loss of RNA among samples.

The i^6A elutes at 40 minutes, and is barely seen in the overall analysis in Fig 3A. In Fig. 4 the scale of the chromatogram is enlarged to better visualize the hydrophobic area of the chromatogram and the less abundant modified nucleosides, like i^6A. Fig. 4A shows the tRNA nucleoside composition of a yeast wildtype line (Sigma) and Fig. 3B shows MT8, the mutant without yeast *MOD5*. The peak at 40 min in fig. 4A is i^6A. The mutant clearly lacks i^6A (Fig 4B). Figs 4C-F show the chromatograms from yeast cell lines transformed with the *P. patens* genes.

The successful complementation of the IPT deficiency in mutant MT8 by *P. patens* genes would result in the appearance of i^6A peak at 40 min. However, figs. 4 C-F shows this not to be the case.

Functional complementation of yeast MT8

To test if the moss IPT-Pfl61 constructs were present and could give the expected functional complementation, the transformed yeast lines were grown on different media lacking different amino acids (see table 1).

Table 1. Analysis of the yeast lines growth.

Type of yeast line	Medium type					
	YEPD*	SC*	SC-ade	SC-ura	SC-lys	SC-ade-ura
S. cerevisiae						
MT8	+	+	-	-	-	-
MT8+ Pfl61	+	+	-	+	-	-
P. patens:						
PpIPT1	+	+	+	+	+	+
PpIPT2	+	+	+	+	+	+
PpIPT4	+	+	+ + +	+	+ + +	+
PpIPT5	+	+	+ + +	+	+ + +	+

*SC - synthetic complete medium / *YEPD – Yeast Extract Peptone Dextrose medium

The MT8 strain cannot grow without uracil while Pfl61 contains the gene for uracil synthesis. Without Pfl61 MT8 will not be able to grow on a medium lacking uracil. The cell lines grow when uracil was omitted, which indicates that the plasmid is present in the yeast lines.

Yeast lines with *P. patens* genes (*PpITP1* and *PpITP2*) grew very poorly when no adenine or lysine was included. Interestingly, the other yeast lines with *P. patens* genes (*PpITP4* and *PpITP5*) grew very well.

Growth of functionally complemented yeast MT8

The relative effectiveness of complementation of yeast by *P. patens* genes was also quantified by measuring the growth rate. If the different IPT proteins have different relative ability to complement MT8 they would grow at different rates. The untransformed MT8 cultures were grown in SC medium with adenine. The other MT8 yeast lines transformed with alien genes were grown in SC medium without adenine. The reason for that was to avoid the loss of the plasmid in the transformed yeast lines and to study any effects of the introduced IPT genes on suppressing the nonsense mutation by tRNA SUP7. It has been shown that the tRNA SUP7 requires i^6A isopentylation to suppress the nonsense mutation (Laten et al., 1978). The growth was monitored by regular sampling and measuring absorbance at 600 nm and should reach the late log phase to continue with next step. MT8 with *PpIPT2* was grown by direct inoculation to SC-ade medium.

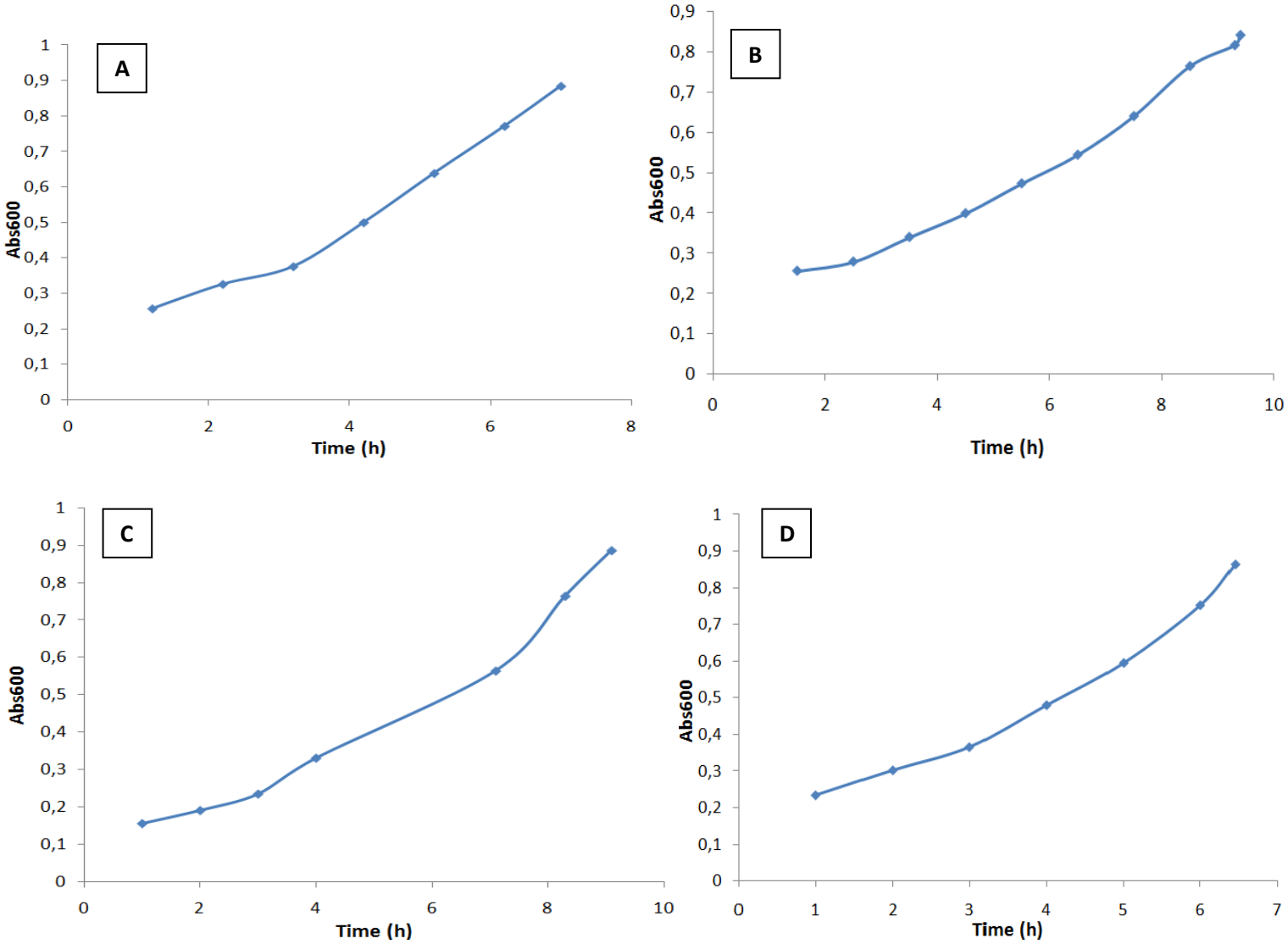


Fig. 5. Growth of *S. cerevisiae* MT8 strains. **A**. MT8 strain with no transformation; MT8 strain transformed with *Physcomitrella patens* isopentenyltransferase genes: *PpIPT1* (**B.** MT8-*PpIPT1*), *PpIPT4* (**C.** MT8-*PpIPT4*) and *PpIPT5* (**D.** MT8-*PpIPT5*). The cultures were grown at 28°C in the incubator shaker at 200 rpm to late log phase approximately (OD_{600}: 0.8 - 0.9). MT8 strain transformed with *P. patens* isopentenyltransferase genes *PpIPT2* (MT8-*PpIPT2*) was overnight cultures and therefore the growth curve is not presented. Mock control was not performed.

The doubling time (db) for the yeast cultures were calculated from Fig. 5. The highest value was found for MT8-*PpIPT1* (db = 5.5 h; fig.5B). The db for others cultures was more similar: MT8 (db = 4.3 h; fig.5A) MT8-*PpIPT4* (db = 4.0 h; fig.5C) MT8-*PpIPT5* (db = 3.4 h; fig.5D). The varying doubling times could be explained by the different capabilities of the moss genes for complementing MT8.

Discussion of functional complementation results

The results obtained were conflicting. The HPLC shows no presence of i^6A, yet at least two of the complemented yeasts, *PpITP4* and *PpITP5,* grew well without adenine and lysine, indicating that i^6A-tRNA must be present. MT8-*PpIPT1* did not grow well nor did it have i^6A. This is in contrast with results published by others (Yevdakova and Schwartzenberg, 2007). The short project time did not allow working out the many possible sources of discrepancies encountered. These could for instance be: 1) Loss of i^6A during tRNA processing before HPLC analysis. i^6A-tRNA is hydrophobic and may have been lost during purification. 2) Contamination of the salts and amino acids used to prepare media. The substances used for the medium may have been contaminated with adenine or other molecules that yeast can convert to adenine. 3) Contamination of the cultures with wildtype during growth in the functional complementation. Wild-type takes over during growth. 4) The cloned IPT genes did not contain the full coding sequences. Something went wrong in the construction of the plasmid. Investigations to address the sources of error include: 1) Test if there is contamination in salts and amino acids used in media by using another organism which cannot grow with adenine. 2) Test the transformed lines on different media lacking different amino acids to see if the functional complementation and construction of plasmid worked.

Setting up an assay to study substrate specificity towards pre-tRNA and adenylates of a plant tRNA isopentenyltransferase

The study aimed to determine the catalytic parameters like K_m and V_{max} of AtIPT2, for comparison with published values for other IPTs. In *E. coli,* IPT was found to be a far more abundant protein than expected based on the low levels of the catalytic product, i^6A-tRNA, that is needed for the protein translational machinery (Eastwood Leung et al., 1997). The reason was found to be a strong competitive inhibition by ATP with the DMAPP substrate. The high enzyme level is necessary to overcome this inhibition. I wanted to investigate if this property has been fixed in the enzyme evolved in higher organisms.

Even though tRNA-IPT is among the best studied of the tRNA-modifying enzymes, very little biochemical characterization has been done in general, and none for the plant enzymes. Only the *E. coli* enzyme has received a more thorough study (Eastwood Leung et al., 1997).

The assay uses pre-tRNA lacking the i^6A modification at position 37. For this study, tRNA from the MT8 yeast mutant lacking MOD5 (Dihanich et al., 1987), and thus lacking i^6A in tRNA, was purified and used. Histidine-tagged *A. thaliana* AtIPT2 was affinity purified by Gunvant Patil. [3H]-DMAPP was used as a side-chain donor. Samples were prepared containing different amounts of enzyme (0, 2, 5, 8 and 12 mg of AtIPT2). The reaction product was measured by incorporation of 3H into tRNA, which was precipitated using TCA and collected on glass fiber filters.

The first tests of the assay yielded no 3H activity on the filters. All steps of the method were then controlled. The tRNA batch prepared from MT8 was analyzed to ensure that it indeed lacked i^6A. Initial result from HPLC showed a debris peak obscuring the retention time of i^6A. Later analysis however confirmed the lack of i^6A

in this strain (Fig. 5B).

The filtration step was controlled to ensure that tRNA indeed was retained. For this step short strands of [32P]-labelled DNA was mixed with varying amounts of tRNA. In one control procedure additional filtration with TCA was used before the filters were washed according to standard procedures, to ensure that all the content was added to the filters. In the second procedure not only more TCA was used but also the time was extended. The results showed that the filtration was able to retain DNA of roughly the same size as tRNA, so this step very likely works reliably (data not shown). Aliquots of [3H]-DMAPP spotted on filters and then dried gave the expected cpm values, showing that the scintillation fluid is able to penetrate the filters.

Two factors were not tested because of time constraints: the integrity of the commercial DMAPP, and if the catalytic activity of the his-tagged IPT had indeed survived the affinity purification procedure.

In yeast and *E. coli*, tRNA substrate specificity was found to be determined solely by the sequence of the anticodon arm (Eastwood Leung et al., 1997; Soderberg and Poulter, 2000). The tRNA sequences of *A. thaliana* present in the Genbank and all possible moss tRNA genes (binding to codons starting with a U) have been extracted using tRNA-Scan (G. Patil, unpublished). This could possibly lead to insights regarding the co-evolution of tRNA and a tRNA-modifying enzyme. A search for possible sRNA interactions was initiated. The tRNA-IPT protein has many RNA-interacting regions. The enzyme could conceivably use alternative RNA substrates, possibly destined to be cytokinin precursors. Alternatively the catalytic activity could be modified by competition with the pre-tRNA substrate, allowing access for a substrate like ATP. sRNAs can be searched for in *A. thaliana* and *P. patens* genomic databases using BLAST based on similarity with known tRNA substrates. A preliminary scan of published sRNA sequences in the the microRNA database (http://www.mirbase.org/) was done but did not identify any promising homologous tRNA candidates (data not shown). Time did not permit an attempt to use column-bound AtIPT2 to affinity purify sRNAs from cell extracts.

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STUDIER AV tRNA-ISOPENTENYLTRANSFERASERS ROLL I BILDANDET AV CYTOKININER
Grzegorz Furman
Institutionen för växtbiologi och skogsgenetik
Uppsala Biocentrum
Fakulteten för naturresurser och lantbruksvetenskap
Sveriges lantbruksuniversitet (SLU)
Uppsala