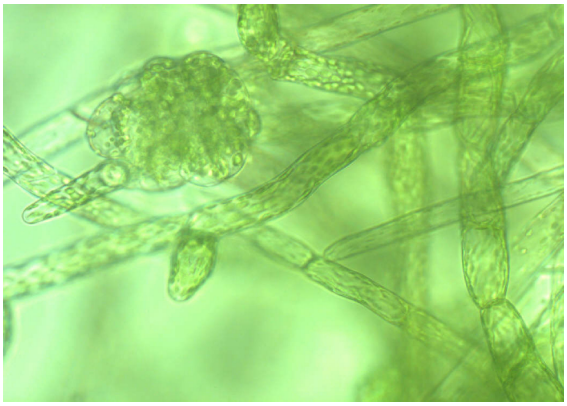
Molecular and biochemical evidence for tRNA-dependent cytokinin biosynthesis in the ancient land plant *Physcomitrella patens* (Hedw.) B.S.G.



Dissertation

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Pure science, the search for knowledge
without knowing where it will lead,
is part and parcel of what will make
the world a better place for all mankind.

Carolyn Shoemaker
1998, Science

Content

Abbreviations

A^{37}	adenine in position 37 of tRNA molecule
aa	amino acid
Acc. No.	accession numbers
AHK	histidine kinase of *Arabidopsis thaliana*
AtIPT2, 9	tRNA-*IPT* genes of *Arabidopsis thaliana*
BLAST	basic local alignment search tools
bp	base pair
cDNA	copy DNA
CRE	cytokinin receptor, e.g. CRE1/AHK4 of *Arabidopsis thaliana*
cZ	*cis*-zeatin *(base)*
CKX	cytokinin oxidase/dehydrogenase
DEPC	diethylpyrocarbonat
ddH_2O	*aqua bidest*
DMAPP	dimethylallyl diphosphate
dw	dry weight
DZ	dihydrozeatin
EST	expressed sequence tag
gDNA	genome DNA
fw	fresh weight
HMBDP	4-hydroxy-3-methyl-2butenyldiphosphate
iP	N^6-Δ^2-isopentenyladenine *(base)*
IPT	isopentenyltransferase
NCBI	National Center for Biotechnology Information
NJ-tree	neighbourhood joining tree
ORF	open reading frame
OD	optical density
PpIPT1	tRNA-*IPT* gene of *Physcomitrella patens*
PpACT3	actin biosynthesis gene of *Physcomitrella patens*
RT-PCR	reverse transcription - polymerase chain reaction
-R	riboside *(nucleoside),* e.g. cZR
-RMP	monophopsphate (*nucleotide),* e.g. cZRMP
-(R)OG	(riboside)-O-glucoside
-(R)NG	(riboside)-N-glucoside
ScMOD5	tRNA-*IPT* gene of *Saccharomyces cerevisiae*
tZ	*trans*-zeatin *(base)*
ZOG	zeatin-O-glucosyltransferase
ß-Glc	*ß*-glucosidase

Summary

Cytokinins are of critical importance to numerous developmental processes in plants. There have been described two cytokinin biosynthetic pathways, each one using a different type of isopentenyltransferases (IPTs) as a key enzyme. In the first pathway, adenylate-IPTs (EC 2.5.1.27) prenylate adenylic nucleotides to cytokinin nucleotides thus catalysing the direct *de novo* biosynthesis of free cytokinins. In the second pathway, tRNA-IPTs (EC 2.5.1.8) catalyse cytokinin formation by isopentenylation of tRNA, the degradation of which liberates cytokinin nucleotides. Seed plants have been shown to possess both forms of IPTs.

In the thesis I report on the *in silico* based identification and on the functional characterisation of an IPT encoding gene (*PpIPT1*) from the bryophyte *Physcomitrella patens* (Hedw.) B.S.G. Analysis of the *PpIPT1* amino acid sequence revealed high similarities to tRNA-IPTs of other plants. No adenylate-IPT genes were found in the sequenced *Physcomitrella* transcriptome/genome. *PpIPT1* functionally complemented a defective tRNA-*IPT* gene of *Saccharomyces cerevisiae* (*ScMOD5*) in the strain MT-8. Dephosphorylated tRNA hydrolysates from *PpIPT1*-transformed MT-8 showed cytokinin activity in a moss bioassay and the presence of isopentenyladenosine in HPLC analysis, in contrast to those prepared from untransformed MT-8. A comparison of pro- and eukaryotic homologues revealed two classes of tRNA-IPTs; PpIPT1 belongs to a prokaryotic-type with predicted chloroplast targeting.

Physcomitrella is known as an especially useful model system for the research of cytokinin biosynthesis due to its cytokinin overproducing mutants. I have characterised a temperature sensitive *ove* mutant, *ove*ST25, for changes in its cytokinin content during the thermal induction. Cytokinins were determined in tissue and culture medium as well as in tRNA-hydrolysates by combined liquid chromatography-mass spectrometry (LC-MS).

Not depending on temperature conditions c*is*-zeatin riboside-*O*-glucoside (cZROG) was found to be the predominant form in the tissue of *ove*ST25 and wild type; in culture medium c*is*-zeatin-*O*-glucoside (cZOG) predominated in both genotypes. Thermoinduction in *ove*ST25 caused a drastic increase of extracellular N^6-(Δ^2)-isopentenyladenine (iP) and c*is*-zeatin-riboside-*O*-glucoside (cZROG), 10fold and 4fold respectively. In the wild type no significant changes were measured.

In a comparative analysis of cytokinin content in whole cultures and in tRNA-hydrolysates cZ-type cytokinins were found to be predominant in both fractions (99% and 91%, respectively, in wild type of *Physcomitrella*). The iP-type represented the second dominant group with ~1% in whole culture- and >8% in tRNA-cytokinins. The resemblance of

cytokinin distribution in tRNA and whole cultures of *Physcomitrella* suggests a tRNA origin of cytokinins in *Physcomitrella*.

RT-PCR-based expression studies with the tRNA-IPT gene *PpIPT1* in the *ove*ST25 mutant revealed enhanced transcription levels at the inducing temperature of 25ºC compared to non-inducing conditions (15ºC). A transgenic wild type line with cytokinin deficiency due to cytokinin oxidase/dehydrogenase overexpression (tCKX7) exhibited also high *PpIPT1* expression levels indicating that cytokinin deficiency might upregulate tRNA-mediated cytokinin biosynthesis.

Presented results indicate a potential role of *PpIPT1* for cytokinin biosynthesis in *Physcomitrella* and point out the relevance of the tRNA-mediated pathway for cytokinin production in moss.

1. Introduction

1.1 Cytokinins

Cytokinins are adenine derivatives with distinct substitutions attached to the N^6 position of the adenine ring. They occur as bound forms in the tRNA of most organisms of domains Eucarya and Bacteria and represent one of the numerous modifications of tRNA molecules (Persson et al., 1994). Cytokinin nucleotides are localised in tRNAs recognising UNN codons at the adenine in position 37 (A^{37}) 3´-adjacent to the anticodon and have functions related to tRNA binding to the mRNA-ribosome-complex during translation (Fig. 1). It was demonstrated for *Escherichia coli* that the isopentenylation of tRNA-nucleotide 37 (iPA^{37}) affects the codon-anticodon affinity (Konevega et al. 2006).

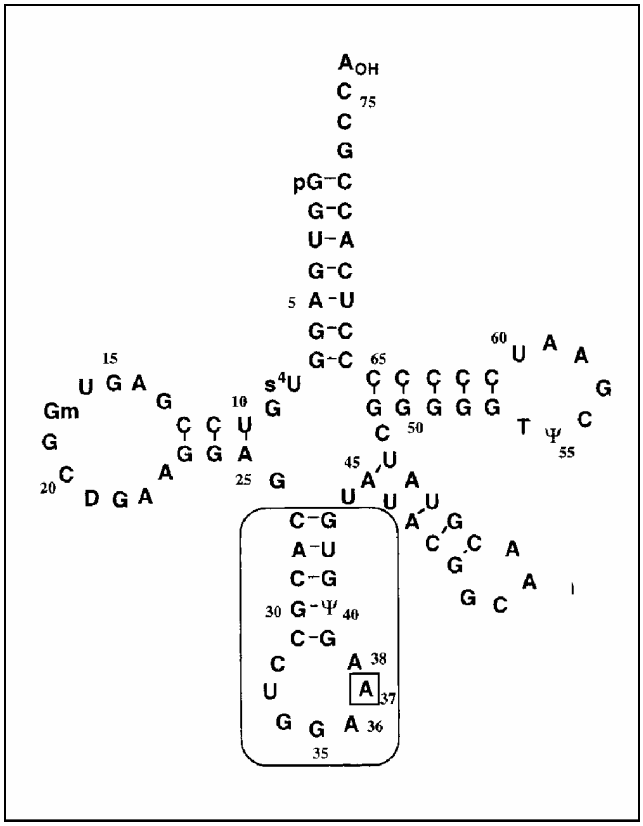

Fig. 1 Cloverleaf structure of *E.coli* $tRNA^{Ser}$ (GGA) with designation of nucleotide modifications (figure from Motorin et al. (1997)). Anticodon stem and loop containing A^{37} residue are boxed. Isopentenylation of A^{37} residue (iPA^{37}) is essential in increasing the efficiency and fidelity of the anticodon-codon interaction during translation (Konevega et al. 2006).

Plants along with the bound cytokinins possess also significant amounts of free cytokinins. In contrast to bound cytokinins, free forms act as plant hormones affecting diverse biological processes (Werner et al. 2001; Kieber 2002). Since the discovery of these

phytohormones as inducers of plant cell division (Miller et al. 1955) and differentiation (Skoog and Miller 1957), they have been recognised as central regulators of plant development (Sakakibara 2006). Cytokinins also increase nutrient sink strength, delay senescence, stimulate outgrowth from lateral buds, and inhibit cell elongation (Mok 1994).

The most abundant class of cytokinins is of isoprenoid-type: N^6-Δ^2-isopentenyladenine (iP) carries an unmodified isopentenyl side chain, whereas *trans*-zeatin (tZ) and *cis*-zeatin (cZ) carry hydroxylated side chains (Fig. 2).

Several plant species have been shown to contain adenine derivatives with aromatic substitutiens (Strnad 1997). The structure and conformation of the side chain are critical to the activity of the respective cytokinins. One of the most abundant cytokinins in higher plants, tZ, displays a high cytokinin activity, while its *cis* isomer, cZ, possesses a significantly lower activity (for review see Haberer and Kieber 2002).

Cytokinins exist in three interconvertible forms: free base, riboside (in which a ribose is attached to the N^9 of the purine ring) and nucleotide forms (in which the ribose moiety contains a 5´-phosphate group). Free cytokinins are readily converted to their respective nucleoside and nucleotide forms, which have lower levels of biological activity.

Via glucosilation of purine moiety, cytokinins are transformed in inactive *N*-glucosides of Z- and iP- cytokinins, or in storage *O*-glucosides of Z- type when glucosilation occurres at isoprenoid side chain (Mok and Mok 2001) (Fig. 2).

In general, a plant contains numerous species of cytokinin molecules modified in different ways. The distribution of the various cytokinins may differ significantly between plant species. For *Physcomitrella* a total of 20 different isoprenoid and aromatic cytokinins have been described (Schwartzenberg et al., manuscript submitted).

Concerning cytokinin signaling, side chain variations in isoprenoid cytokinins are highly significant for the diversification of the hormones function. Z- and iP- types were shown to differ in their affinity to different types of receptors (sensor histidine kinases). In binding assays with *Arabidopsis* receptors tZ was shown to bind with high affinity to both the AHK3 and CRE1/AHK4 receptors. iP and iPR however bound approximately 10-fold stronger to CRE1/AHK4 than to AHK3 (Romanov et al. 2006). Similar data were demonstrated for cytokinin receptors of *Zea mais* (Yonekura-Sakakibara et al. 2004). During qualitative studies on the basis of the bacterial promoter activation test tZ was more active in case of ZmHK2 (a homologue of AHK3). In contrast, ZmHK1-expressing bacteria strongly responded to iP (a homologue of CRE1/AHK4).

Recently, two CRE1/AHK4 -homologues, PpCRE1 and PpCRE2, were identified in *Physcomitrella* (Trouiller et al. 2004). Both receptors were shown to have partially distinct roles in cytokinin signalling and to be functionally redundant for cytokinin-induced bud formation.

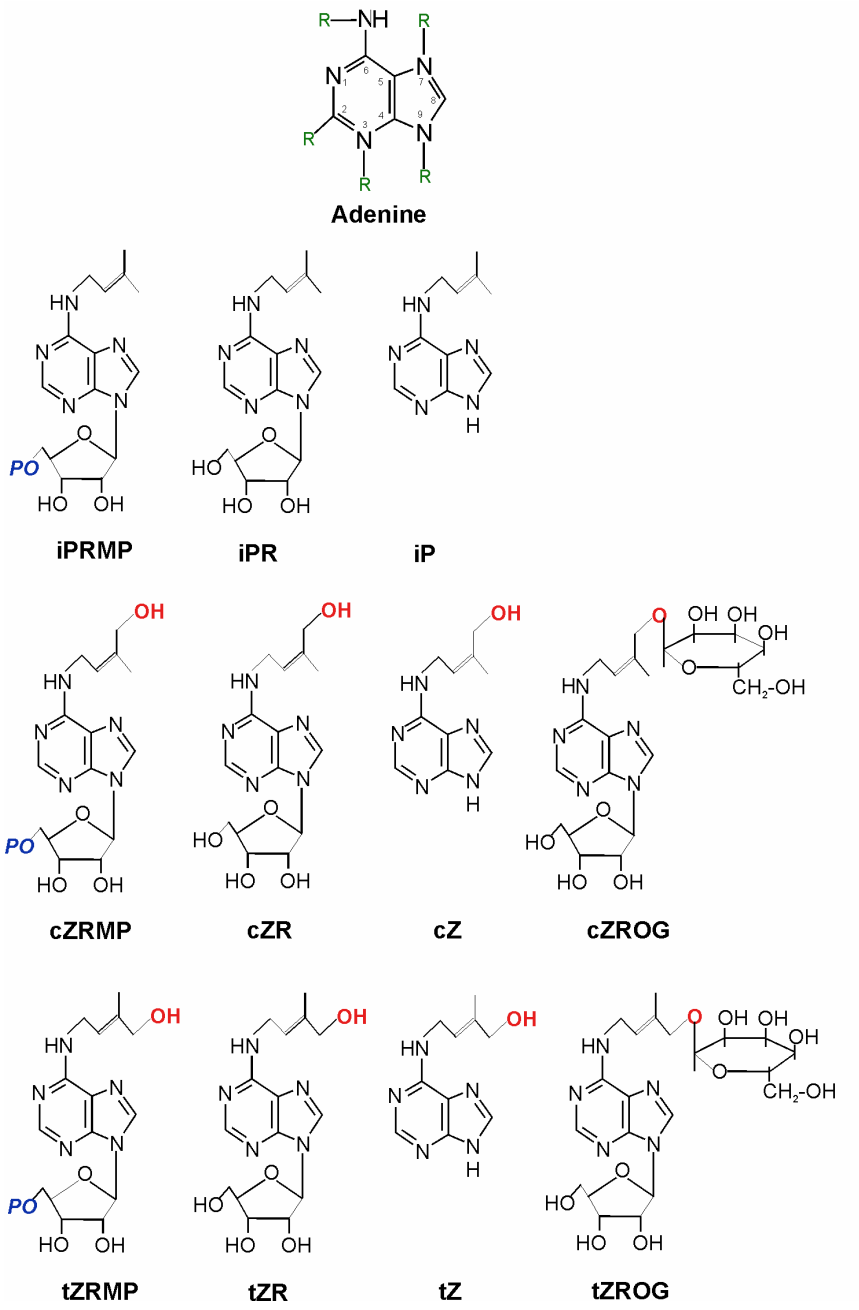

Fig. 2 Structural formulas of natural occuring isoprenoid-cytokinins, which are important in the current investigation. Adenine derivatives: (iP) N^6-Δ^2-isopentenyladenine, (cZ) *cis*-zeatin and (tZ) *trans*-zeatin; their (-R) riboside, (-RMP) nucleotide, (-OG) *O*-glucoside forms.

1.2 Cytokinin biosynthesis

Central enzymes of cytokinin biosynthesis are isopentenyltransferases (IPTs), which transfer an isopentenyl group, e.g. dimethylallyl diphosphate (DMAPP), to the N^6 atom of free or bound adenosine nucleotides (Hwang and Sakakibara 2006). Based on the nucleotide substrates, there have been described two cytokinin biosynthetic pathways.

(a) The direct *de novo* biosynthesis of free cytokinins, where adenylic nucleotides are prenylated to cytokinin nucleotides by adenylate- isopentenyltransferases (IPTs) (EC 2.5.1.27) (Fig. 3a). In this pathway, known as AMP/ADP/ATP-IPT pathway, free iP- or Z-type cytokinins are directly synthesised depending on whether the substrate is DMAPP or the hydroxylated side chain donor 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP), respectively (Kakimoto 2001, 2003; Takei et al. 2001; Sakakibara et al. 2005).

(b) Alternatively cytokinins can be liberated from tRNAs, which contain cytokinins as hypermodified nucleotides. The post-transcriptional prenylation of the N^6 at A^{37} in the UNN recognising tRNAs is catalysed by tRNA-IPTs (EC 2.5.1.8) (Fig. 3b).

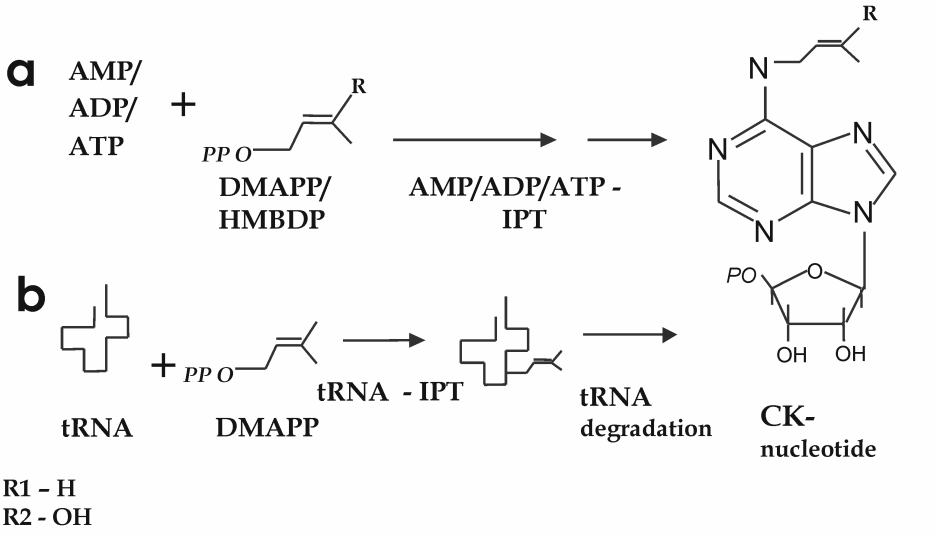

Fig. 3 Simplified scheme of cytokinin biosynthetic pathways. (a) Direct synthesis of free cytokinins by adenylate- isopentenyltransferases (adenylate-IPTs). (b) Prenylation of adenine by tRNA-IPTs in position 37 of tRNA-species recognising codons starting with U and release of cytokinin nucleotides by tRNA degradation

The generation of specific cytokinin forms was clarified due to discovery of nine biosynthetic genes of *Arabidopsis thaliana* (Kakimoto 2001, 2003; Takei et al. 2001) and the analyses of the corresponding mutants (Miyawaki et al. 2004, 2006).

Endogenous plant adenylate *IPT* genes were first identified *in silico* by analysis of *Arabidopsis thaliana* databases using bacterial AMP:IPT sequences as queries. *E. coli* expressing *AtIPT1, 3, 4, 5, 6, 7* and *8* were shown to secrete iP and tZ into the culture medium (Kakimoto 2001; Takei et al. 2001). *AtIPT4* overexpressed in *Arabidopsis* calli caused shoot regeneration even in the absence of exogenous cytokinin (Kakimoto 2001). AtIPT1 and AtIPT4 were shown to prefer adenosine di- and triphosphates to AMP and were designated DMAPP:ATP/ADP-IPTs. The substrate preference of ATP and ADP was also demonstrated for the *Oryza sativa* enzymes OsIPT1 and OsIPT3, which were recently

characterised among 8 other rice adenylate IPTs by Sakamoto et al. (2006). Taken together these data suggest that ATP and ADP are the preferred substrates for seed plant adenylate IPTs.

Each member of *Arabidopsis* ATP/ADP-IPT gene family has a unique tissue- and stage specific expression pattern, determined via fusion of *GUS* to the *IPT* regulatory sequences (Kasahara et al., 2004; Miyawaki et al., 2004). Expression of *AtIPT3, 5* and *7* is relatively high in the vegetative organs. *AtIPT8* is expressed exclusively in reproductive organs, with highest level in immature seeds; *AtIPT4* - primarily in immature seeds, and *AtIPT1* - in ovules and vegetative organs. The effect of cytokinins on IPT gene expression was examined for *AtIPT1, 3, 5* and *7*, and a negative regulation was revealed for all these genes (Miyawaki et al., 2004).

In seed plants both the adenylate- and the tRNA-IPT mediated pathway contribute to cytokinin biosynthesis (Maaß and Klämbt 1981; Kakimoto 2001, 2003). Miyawaki et al. (2006) have demonstrated for *Arabidopsis* that seven adenylate-*IPT* genes are responsible for the biosynthesis of the bulk of iP- and tZ- type cytokinins whereas two tRNA-IPT genes are responsible for cZ- and iP- type cytokinins.

Mutations in tRNA-IPT have a crucial influence on the translation precision and lead to pleiotropic phenotypes in microorganisms (for review see Taller 1994). Microbial tRNA-IPT encoding genes were identified in *MiaA* from *E. coli* (Rosenbaum and Gefter 1972), *A. tumefaciens* (Gray et al.1992) and in *MOD5* from *Saccharomyces cerevisiae* (Martin and Hopper 1982; Dihanich et al. 1987).

The isopentenyl (i^6)-side chain is known to derive from the mevalonate pathway (MVA) or from the methylerythritol phosphate pathway (MEP). Depending on whether DMAPP or HMBDP is used as a side chain donor either iP- or cZ- hypermodification of tRNA occurs (for review see Persson et al. 1994, Kasahara et al. 2004). In prokaryotes, tRNA is a common source of free cytokinins (Gray et al. 1996; Koenig et al. 2002).

In the early 1980´s the tRNA pathway (Fig. 3b) was investigated as a potential source of cytokinins in plants by Maaß and Klämbt (1981). The authors demonstrated in *Phaseolus vulgaris* that cytokinins can originate from the turnover of tRNA. The first plant tRNA-*IPT* genes, *AtIPT2* and *AtIPT9,* were identified by Kakimoto (2001) and Takei et al. (2001). *AtIPT2* was functionally described by Golovko et al. (2002) using the complementation based on a defective mutant allele of *MOD5* in *S. cerevisiae*. For both, *AtIPT2* and *AtIPT9,* an ubiquitous expression in *Arabidopsis* tissue was shown by Miyawaki et al. (2004). Later, for *Oryza sativa* two genes, *OsIPT9* and *OsIPT10,* were also suggested to encode tRNA-IPTs (Sakamoto et al. 2006).

Recently, from work of Miyawaki et al. (2006) it has become evident that AtIPT2 and ATIPT9 are required for the formation of cZ- and iP- type cytokinins, thus showing that tRNA-mediated pathway is essential for the biosynthesis of cZ-cytokinins in *Arabidospsis*.

Although both tRNA- and adenylate-IPT pathways contribute to cytokinin biosynthesis in plants (Maaß and Klämbt 1981; Kakimoto 2001, 2003; Miyawaki et al. 2006), the tRNA pathway was considered to be insufficient to account for the significant source of cytokinins in seed plants (Barnes et al.1980).

Generally it can be stated that the tRNA-mediated cytokinin biosynthesis is not yet well described for plants (see Hwang and Sakakibara 2006).

1.3 Moss as a useful model system for cytokinin research

The bryophytes and flowering plants diverged in land plant evolution >400 million years ago (in the early Silur). Morphology and life cycle of mosses differ significantly from those of seed plants (Reski, 1998). In contrast to higher plants, the gametophyte (haploid) generation dominates in the life cycle of mosses over the sporophyte (diploid) generation. The tissues of gametophytic leafy shoots are much simpler than sporophytic shoots of flowering plants. Moss sporophytes differentiate from the zygote and are epiphytic to leafy shoots.

Mosses, due to their simple developmental differentiation and due to their sensitivity to growth substances, have been used as model systems for numerous works regarding hormonal action and homeostasis (for reviews see: Cove 2005, Schwartzenberg 2006). During the last years large genomic resources have been established for *Physcomitrella* (Nishiyama et al. 2003, Decker et al. 2006) and underline the suitability and the advantages of this moss species with respect to evolutional studies.

Cytokinins in mosses, unlike in higher plants, affect plant development at a single-cell level. They induce differentiation of the 2-dimensional filamentous protonema cells into 3-dimensional structures, the so called buds (Brandes and Kende 1968). Hahn and Bopp (1968) established a bioassay based on *Funaria hygrometrica* cultures, where the number of induced buds was used to estimate the concentration in exogenously supplied cytokinin samples.

The moss *Physomitrella patens* has several advantages especially concerning studies of cytokinin metabolism and biosynthesis as this plant provides unique *ove* mutants, which were shown to have drastically increased cytokinin amounts exceeding that of wild type plants up to 100fold (Wang et al. 1981b). In seed plants mutants with such extreme cytokinin overproduction are unknown, e.g. cytokinin level in the *Arabidopsis* mutant *amp1* is only about 5fold above wild type (Chaudhury et al. 1993). Cytokinin overproduction mutants of *Physcomitrella* were first selected by Ashton et al. (1979) on the basis of bud overproducing

phenotype. In feeding studies using radiolabelled adenine, it was demonstrated, that *ove* mutants incorporate more radioactivity into the cytokinin fraction than the wild type (Wang et al. 1984).

Among *ove* mutants some show temperature sensitivity: at 15°C these mutants produce wild type-like gametophores, at 25°C abnormal bud formation occurs, which results in callus-like structures. A temperature sensitive bud overproducing phenotype was first described by Futers et al. (1986) for the mutant *ove*409. Most of *ove* mutations, including *ove*409, were shown to be recessive and are divided into three complementation groups (Featherstone et al. 1990). Futers et al. (1986) characterised *ove*409 in comparison with a mutant *ove*A78 (both belong to the same complementation group) and wild type. The authors summarised that *ove* allele need not necessarily code for a temperature sensitive gene product. Already at 15°C both *ove* mutants produced more cytokinin than wild type. At 25°C all three strains showed increased cytokinin levels compared with 15°C, due to a general effect of temperature on cytokinin production. Cytokinin levels in wild type were low so that normal development continued at both temperatures while those in *ove*A78 were high enough at both temperatures to cause increased production and abnormal development of plant. In case of *ove*409 the cytokinin production increased high enough only at 25°C to provoke elevated production of abnormal buds. These studies showed that changes in mutants' phenotype correlated with the higher production of N^6-(Δ^2-isopentenyl)adenine (iP). Schulz et al. (2001) studied another temperature sensitive mutant, *ove*ST25, and also demonstrated a strong increase of iP and N^6-(Δ^2isopentenyl)adenosine (iPR) concentrations in the culture medium. These characteristics make temperature-sensitive *ove* mutants particularly useful for studies of cytokinin biosynthesis and homeostasis.

What causes the high amount of cytokinins in *ove* mutants - changes in cytokinin biosynthesis, interconversion or degradation? Previous studies have revealed that *ove* mutants have an enhanced cytokinin biosynthesis and are not deficient in cytokinin breakdown (reviewed by Schwartzenberg 2006). Schulz et al. (2001) addressed the question of the metabolic mechanisms underlying the *ove* phenotype and carried out *in vivo* labelling with tritiated-iPR and *in vitro* determinations of cytokinin oxidase/dehydrogenase activity. Analysis of *Physcomitrella ove* mutants *ove*A78, *ove*A201, *ove*B300, and *ove*ST25 showed that all genotypes were able to degrade cytokinins. The cytokinin conversion of the riboside (iPR) to the base (iP) was notably stronger in all *ove* mutants. Therefore, it was concluded that cytokinin overproduction was not caused by deficiency in cytokinin breakdown. This puts forward changes in the early steps of cytokinin biosynthesis. As *ove* mutants have been described as loss of function mutants (Featherstone et al. 1994) it is assumed that the *ove* mutation concerns negative regulators of early cytokinin biosynthetic steps.

1.4 Aims of research

So far no complex investigations concerning cytokinin biosynthesis in evolutionary early plants were published. The presented research work was aimed to the comprehension of cytokinin biosynthesis in the model plant *Physcomitrella patens,* with respect to genetics and evolution of cytokinin physiology.

The general question to answer was: *Via* which pathway(s) cytokinins are biosynthesised in *Physcomitrella*?

Using the existing genomic/transcriptomic resources for this plant in combination with molecular-biological and biochemic-physiological approaches the following themes were addressed:

1. Identification of genes involved in cytokinin biosynthesis in *Physcomitrella,* and their functional characterisation.

2. Characterisation of cytokinin biosynthesis in wild type and a conditional cytokinin overproducing mutant, *ove*ST25 on the level of cytokinin production and expression of biosynthetic genes.

3. Comparative analyses of cytokinin biosynthesis gene(s), its (their) proteins and catalytic products of *Physcomitrella* with those of the primitive organisms, such as bacteria, cyanobacteria and yeasts and of the higher plants with respect to evolution of cytokinin biosynthesis.

2 Materials and methods

2.1 Source of chemicals, enzymes and molecular biological kits

Chemicals (p.a. quality), enzymes and molecular biological kits were purchased at Amersham Biosciences (Freiburg, D), AppliChem (Darmstadt, D), Biozym (Oldenburg, D), Duchefa (Haarlem, NL), Fluka (Neu-Ulm, D), Invitrogen (Karlsruhe, D), Genecraft (Münster, D), Merck (Darmstadt, D), MBI Fermentas (St. Leon-Roth, D), MWG Biotech AG (Ebersberg, D), Roth (Karlsruhe, D), Roche Diagnostics (Mannheim, D), Serva (Heidelberg, D), Sigma Aldrich (Taufkirchen, D), Stratagene (Heidelberg, D), Qiagen (Hilden, D).

2.2 Media and reagents

Media and reagents were prepared using water, which was purified to quality *aqua bidest via* Milli-pore Milli-Q Plus (Millipore, Billerica, USA). As a rule, solutions were sterilised by 20 min autoclaving at 125°C and $2 \cdot 10^5$ Pa.

If not indicated further in the text, standard receipts for molecular biological methods were applied according to Sambrook et al. (1989).

2.3 Plant material

Wild type of *Physcomitrella patens* (Hedw) B.S.G was originally collected from Grandsden Wood, Huntingdonshire, UK (1968) and provided by D. Cove (Univ. of Leeds, UK).

Cytokinin overproducing mutant *ove*ST25 was obtained by UV-mutagenesis of thiamine auxotrophic wild type *thiA1*d strain and provided by E. Russo and A. Hofmann (MPI of Molecular Genetics, Berlin, D). At 15°C of cultivation *ove*ST25 produces normal leafy shoots like wild type; at 25°C a thermoinduced cytokinin overproduction occurs with strongly increased iP concentrations in the medium causing the formation of many abnormal buds (Schulz et al. 2001, Schwartzenberg 2006).

A transgenic *Physcomitrella* strain tCKX7 with enhanced cytokinin degradation was provided by M. Fernandez Nunez (Biocentre Klein Flottbek, University of Hamburg, D). This strain was obtained by overexpression of the cytokinin oxidase/dehydrogenase (CKX) gene *AtCKX2* (*Arabidopsis thaliana*) in wild type of *Physcomitrella*. Increased CKX activity caused

a significant reduction of extracellular iP and iPR (Schwartzenberg et al. 2007, manuscript submitted).

2.4 Plant culture conditions

All *Physcomitrella* strains were grown in liquid culture in a medium described by Wang et al (1980): $Ca(NO_3)_2$ 0.359 mM, $FeSO_4$ 0.035 mM, $MgSO_4$ 1.01 mM, KH_2PO_4 1.84 mM, KNO_3 10 mM.

The medium was supplemented with Hoagland´s trace elements (1 ml) and vitamins: p-aminobenzoic acid (1.8 µM), nicotine acid (8 µM) and thiamine/HCl (1.5 µM). The pH was adjusted to 6.5 (KOH).

Liquid culture medium (400 ml) was inoculated with about 300 mg of protonema filaments that had been freshly cut up with an ultra-turrax blender (IKA, Staufen, Germany) to filaments of 10 to 20 cells each. Culture flasks (1000 ml, Schott, Mainz) closed with cotton were aerated with water saturated, sterile air (ca. 600 ml/min). Cultures were grown at 15°C (maintainance) or 25°C, as given in results, under white light (Philips TLM) at 100 µmol m^{-2} s^{-1} (400-700 nm) and a light-dark cycle of 16 : 8 h.

For the micro-bioassay for cytokinin activity (the determination of budding frequency) *Physcomitrella* protonema was grown on solid Knop-agar medium (Hahn and Bopp 1968): KH_2PO_4 250 mg/l, $MgSO_4$ x 7 H_20 250 mg/l, KCl 250 mg/l and $Ca(NO_3)_2$ x $4H_2O$ 1g/l, Fe-EDTA 12.5 mg/l, agar 1% (w/v), pH 6.4 (KOH).

2.5 *In silico* analyses

Sequence search and comparison with BLAST (tBLASTn, evalue cutoff 0.0001) were done in *COSMOSS* (http://www.cosmoss.org/bm/BLAST; Rensing et al. 2005), *PHYSCO*base (http://moss.nibb.ac.jp/blast/blast.htm; Nishiyama et al. 2003) and NCBI (http://www.ncbi.nlm.nih.gov/blast/mmtrace.shtml). Multiple sequence alignments and drawing the phylogeny inferences using “neighbourhood joining” method (a NJ-tree) were obtained with CLUSTAL W algorithm (http://clustalw. genome.ad.jp/). Predicted targeting sites for a potential signal peptide and a chloroplast transit peptide localisation of proteins were done in MultiLoc (http://www-bs.informatik.uni-tuebingen.de/ Services/MultiLoc; Hoeglund et al. 2006). Search for putative cleavage sites of leader sequences was carried out with TargetP and ChloroP (http://www.cbs.dtu.dk/services/).

2.6 Reconstruction of *Physcomitrella* gene *PpIPT1*

A *Physcomitrella* cDNA library in pBSIISK+ (Schwartzenberg et al. 1998) was used as a template to establish the full-length sequence *PpIPT1* using polymerase chain reaction (PCR)- based techniques. Oligonucleotide primers were designed on BJ163403 and BJ173373 EST sequences (see Fig. 4). All reactions were performed on a Mastercycler 5330plus (Eppendorf, Hamburg).

A standard RCR-regime used:

95℃ for 5 min, following 30 cycles with: 94℃ for 1 min, annealing with appropriate temperature (specific for each primer pairs) for 1 min, 72℃ for 1 min 30 sec. A final incubation at 72℃ for 10 min ended the PCR.

A standard PCR-mix (of 25 µl end volume) contained:

2.5 µl of 10× reaction bufer (Fermentas, St. Leon-Rot);
0.25 µl of dNTPmix, 25 mM each dNTP (Fermentas);
2 ×0.5 µl of primers (forward and reverse), 50 pmol/µl each (MWG Biotech, Ebersberg);
template DNA: 10 pg -1ng of pDNA (or 10 ng - 1 µg of gDNA);
1U of high fidelity PCR enzyme mix (Fermentas);
PCR water (Fermentas).

To identify an unknown region linking *Physcomitrella* ESTs, BJ173373 and BJ163403, the mehtod of nested PCR was used allowing the reduction of the contaminations in PCR-product due to possible mispriming. Two sets of primers were used in two successive runs of polymerase chain reaction; the second set of primers was intended to amplify a secondary target within the first run product.

The following primer pair with the annealing temperature of 57℃ was used for the first PCR:

pr-137 5'-GGCCGCGCCTTCCTTGA-3' (reverse) ;
pr-144 5'-GGATTCCACACCATCTAATTGA-3' (forward).

1 µl from the reaction mix of the obtained PCR-product was used for the next PCR-run using a second pair of primers, located between the first ones (annealing temperature of 57℃):

pr-135 5'- AGCTAGCACCTTCGGTCGCAG-3' (reverse);
pr-145 5'-CTGGAAAGGTAGGAG CTCCG -3 (forward) (see Fig. 4).

Missing 5' and 3' ends of the *PpIPT1* were identified by inverse PCR as described by Huang (2002). Two opposite direction primers, pr-151 and pr-152 specific to the known EST-

sequences, were designed to anneal to the region of unknown sequence in *PpIPT1* and its flanking vector sequences (see Fig. 4):
pr-151 5'-CGACCTCTATGTAACGTTGCT-3' (forward, from 3'-end);
pr-152 5'-CAAACGCTCTTACGAAGTTGTTCAAGGAG-3' (reverse, from 5'-end).

The PCR was performed in 30 cycles, at the annealing temperature of 61°C and with an elongation time of 2 min. The recovered ~3,800 bp-PCR product was sequenced and compared *in silico* with the vector sequence of pBS II SK+ (2,961 bp) and the ESTs belonging to *PpIPT1* (1,453 bp) using the BLASTn program of NCBI (http://www.ncbi.nlm.nih.gov/blast/ bl2seq/wblast2.cgi).

Sequencing was commercially performed by DNA-Cloning Service, www.DNA-Cloning-Service.de, Hamburg).

The entire open reading frame (ORF) region in the compiled sequence of *PpIPT1* was identified using the ORF-finder program of NCBI (http://www.ncbi.nlm.nih.gov/ gorf/gorf.html).

The gemomic sequence of *PpIPT1* (4,702 bp) and its intron/exon architecture was established *in silico* by alignment of *Physcomitrella* genome sequence selected from *PHYSCO*base (http://moss.nibb.ac.jp/blast/blast.htm; Nishiyama et al. 2003) with *PpIPT1* cDNA sequence in the BLASTn program of NCBI (http://www.ncbi.nlm.nih.gov/blast/ bl2seq/wblast2.cgi).

2.7 Complementation of *mod-5-1* mutation in *S. cerevisiae* strain MT-8 by *PpIPT1*

2.7.1 Cloning the *PpIPT1* gene into the yeast expression vector pFL61

To study the function of *PpIPT1* in yeast, its ORF was cloned into the yeast expression vector pFL61. pFL61 (5,425 bp) carrying a *Not*I restriction site, the URA3 selective marker encoding orotidine-5'-monophosphate decarboxylase and the yeast phosphoglycerate kinase promoter (Minet et al. 1992) was a gift of M. Minet (CNRS, Gif s. Yvette, France).

Using 10 ng DNA of *Physcomitrella* cDNA library in pBSIISK+ (Schwartzenberg et al. 1998) as a template, the *PpIPT1* ORF was amplified with specific primers pr-230/pr-232 (s. Fig. 4) containing each a *NotI* restriction site (annealing temperature of 61°C):
pr-230 5'-*CGGCCGC*TGAAGTGATATGGTGA-3' (forward);
pr-232 5'-*CGGCCGC*AACGTTACATAGAGGTC-3' (reverse).

The resulting PCR-product was recovered from PCR mix using the Invisorb®Spin PCRapid KIT for purification of PCR-fragments (Invitek GmbH, Berlin, D) and digested with the *Not*I restriction endonuclease (Fermentas) for further ligation with vector DNA, which was previously linearised with *Not*I.

Cohesive termini of *PpIPT1* and pFL61 were ligated (in molar ratio 5:1, respectively) by T4 DNA ligase (Fermentas) according to the protocol (Fermentas). Ligation product was inserted in pDNA of *E.coli* for amplification. (For the protocols of *E. coli* transformation and plasmid DNA preparation see 2.8 and 2.9, respectively).

2.7.2 Transformation of *S. cerevisiae* strain MT-8

S. cerevisiae strains used were MT-8 and ALB8 (Benko et al. 2000), both a gift of A. Hopper and M. Whitney (Pensylvania State University, USA). Yeasts were mantained at 28℃ on YPD medium (Sherman 1991): 10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, 20 g/l bacto-agar.

Yeast MT-8 strain was transformed with (a) pFL61-*PpIPT1,* (b) the empty vector pFL61, as a control, and (c) pFL61-*Sho* (Zubko et al., 2002), as an additional (negative) control for *mod5*- complementation (for details see 3.1.3 Results). The construct pFL61-*Sho* was a gift from E. Zubko and P. Meyer (LIBA, University of Leeds, UK).

The employed method of rapid yeast transformation was based on lithium acetate/PEG treatment and carried out as described by Rine (2006): Yeast cells were inoculated with 10 ml of YPD medium and grown overnight at 30℃ to an OD_{600} of about 1. The overnight culture was centrifuged at 2,000 rpm for 5 min. The pellet was resuspended in 1 ml of sterile ddH_2O and centrifuged for 5 sec at low speed. After removing the supernatant, the pellet was resuspended in 200 µl 1X TEL solution (1 mM EDTA, 0.1 M lithium acetate, 10 mM Tris, pH 7.5) and incubated at 30℃ for 20 min with shaking. A 100 µl aliquot of the cell suspension was transferred to a second tube. A 10 µl volume of herring sperm DNA (10 mg/ml) was added as carrier to each tube. Five hundred ng of transformation DNA was added to one of the tubes. The second tube was used as a negative control. After 20 min incubation (30℃) 0.7 ml of PEG/TEL buffer (1 ml 5 TEL, 4 ml 50% (w/v) PEG 4,000) was added to each tube and mixed by pipetting. The mixtures were incubated for 20 min (30℃). The pellets collected by centrifuging for 10 seconds at low speed were resuspended with 1 ml of sterile ddH_2O and centrifuged again for 10 sec. The final pellets were resuspended in 100 µl of sterile ddH_2O. The obtained cell suspensions were plated out on selective media as indicated below.

2.7.3 Selection and growth of transformed yeast lines

The used selective media were based on synthetic complete (SC) medium (Sherman 1991): 6.7 g/l of bacto-yeast nitrogen base, 20 g/l dextrose, 20 g/l bacto-agar, 20 mg/l adenine sulfate, 20 mg/l uracil, 20 mg/l L-tryptophan, 20 mg/l L-histidine-HCl, 20 mg/l L-arginine-HCl, 20 mg/l L-methionine, 30 mg/l L-tyrosine, 30 mg/l L-leucine, 30 mg/l L-isoleucine, 30 mg/l L-lysine, 50 mg/l L-phenylalanine, 100 mg/l L-glutamic acid, 100 mg/l L-asparatic acid, 150 mg/l L-valine, 200 mg/l L-threonine, 400 mg/l L-serine.

MT-8 transformants, carrying either pFL61-*PpIPT1* or pFL61-*PhSho* constructs orthe empty vector (as a transformation control), were selected on medium lacking uracil (SC –ura). Only transformed cells were able to grow on SC –ura due to their uracil auxotrophy based on the function of *URA3* of pFL61. During the following 5 days of incubation at 30°C the plates were sealed with Parafilm to awoid dryness.

Randomly chosen colonies were used for PCR-screening.

For complementation of *mod5-1,* transgenic yeast lines along with control lines were tested for growth on medium without adenine (SC –ade) as indicated under results.

Additionally, all lines were grown on a rich medium YPD (s. 2.7.2) for the observation of colouration, which is indirectly influenced by tRNA-IPT activity as indicated under results.

2.7.4 Plasmid DNA preparation from yeast culture

In order to verify whether the transformed yeast lines carried the right DNA constructs, plasmid DNA preparations were carried out.

To obtain a yeast culture for plasmid DNA preparation, a liquid SC –ura medium was inoculated with the preselected yeast cells to grow at 30°C with shaking for 36 h.

The plasmid DNA was prepared using a modified method described by Robzyk and Kassir (1992): The cell pellet collected by spinning down 10 ml of yeast culture was resuspended in 100 µl of STET bufer (8% sucrose, 50 mM Tris pH8, 50 mM EDTA, 5% Triton X-100). Approximately 200 mg of steril 0.50 mm glass beads were added prior to vortexing for 5 min. Another 100 µl of STET was added, and the sample was vortexed. The mix was incubated for 3 min in a boiling water bath, cooled briefly on ice and spinned in a microfuge for 10 min at 6,000 g. 200 µl of supernatant was transferred into a new tube. 100 µl of 7.5 M NH_4OAc was added and incubated at -20°C for 1 h. The mix was centrifuged at 13,000 g for 15 min, and 200 µl of supernatant was collected. 400 µl of ice cold ethanol was added, and the mix was spinned at 13,000 g for 15 min. The obtained pDNA- pellet was

rinsed with 70% ethanol and resuspended in 20 µl of sterile ddH_2O. pDNA was stored at -20°C prior to further use.

2.7.5 PCR- screening of transformed yeast lines

The plasmid DNA (2.7.4) of transformed yeast lines was checked by PCR. PCR was performed as indicated in 2.6 using the Taq DNA Polymerase (Genecraft, Münster). The pDNA from yeast colonies harbouring the pFL61-*PpIPT1* construct showed a 1,245 bp PCR-fragment, wich was amplified with pr-135 (specific to the *PpIPT1*-insert) and pr-183 (specific to region of pFL61-vector 5´-flanking the insert) at the annealing temperature of 56°C :

pr-183 5'-CAAGGGGTGGTTTAGTTTAGTAGA-3' (from vector);

pr-135 5'-AGCTAGCACCTTCGGTCGCAG-3' (from insert)

2.8 Transformation of *E.coli* cells *via* electroporation

E.coli strain XL1 blue (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44) (Stratagene, La Jolla, USA) was used to harbour or multiply DNAs or ligation products.

For transformation of *E.coli* with foreign DNA the following media (Sambrook et al. 1989) were applied:

2YT (10 g/l yeast extract, 16 g/l trypton, 5 g/l NaCl), pH 7.0;

LB (5 g/l yeast extract, 10 g/l trypton, 10 g/l NaCl), pH 7.6;

LB-agar (5 g/l yeast extract, 10 g/l trypton, 10 g/l NaCl, 7.5 g/l agar), pH 7.6;

SOC (10 g/l yeast extract, 5 g/l trypton, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 20 mM $MgSO_4$, 10 mM $MgCl_2$).

The pH of the media was ajusted using NaOH.

The equipment used:

electroporator 2510 (Eppendorf, Hamburg);

1 mm electroporation cuvettes (Biozym, Hamburg).

2.8.1 Preparation of *E.coli* competent cells

E.coli competent cells were prepared as described by Chuang (1995) with some modifications: *E.coli* pre-culture (LB) was grown overnight at 37°C (shaking). 1 ml of the fresh pre-culture was grown in 300 ml of 2YT at 18°C (shaking) to mid-log phase at an optical density of OD_{600} 0.4 - 0.5 and placed on ice for 10 min. The following washing

procedure using 500 ml of ice cold MilliQ water and centrifugation at 4°C, 5,000 g for 15 min (2K15 centrifuge, Sigma, Taufkirchen, Germany) was repeated four times. Finally the bacterial pellet was resuspended in 1 ml of 7% DMSO.

The cell suspension was aliquoted (50 µl) and quick-froozen in liquid nitrogen to store at -80°C prior use.

2.8.2 Electroporation

Before electroporation 5-10 ng of plasmid DNA or ligation product were dissolved in 5 µl of MilliQ water. The solution was gently mixed with 50 µl of *E.coli* competent cells (thawed in ice-cold water) and pipeted into a pre-cooled electroporation cuvette.

Electroporation was performed at a charging voltage setting of 1400 V during 2 - 5 ms depending on the salt concentration of the DNA-solutions. The cells were immediately washed out from the cuvette with 2 ×150 µl SOC at room temperature and incubated at 37°C for 1h under shaking.

Then the transformation mix was plated on LB-plates containing appropriate selective antibiotic(s) to overnight growth at 37°C.

2.9 Plasmid DNA preparation

For preparation of plasmid DNA in a scale of up to 20 µg (mini-preparation) 3 ml of LB medium (s. 2.8) containing appropriate selective antibiotic(s) were inoculated with the preselected *E.coli* cells (s. 2.8.2). The bacterial culture was grown overnight at 37°C under shaking. Then bacteria were sedimented at 6,000 g for 5 min (Biofuge pico, Heraeus, Hanau). The obtained bacterial pellet was used for pDNA preparation using QIAprep Spin Miniprep Kit (Qiagen, Hilden) following the instructions of the manufacturer.

For maxi-preparation of plasmid DNA, a bacterial pre-culture in 3 ml of LB medium (s. 2.8) containing appropriate selective antibiotic(s) was grown for 6 h at 37°C under shaking. Then the pre-culture was transferred into 250 ml of 2YT medium (2.8) containing the same selective antibiotic(s) to grow overnight at 37°C under shaking. Bacteria were sedimented at 6,000 g for 5 min (2K15 centrifuge, Sigma, Taufkirchen). The bacterial pellet was used for pDNA preparation using Plasmid Maxi Kit (Qiagen, Hilden).

Obtained plasmid DNA was resuspended in ddH_2O (the volume was dependent on the Kit- protocol recommendations) and stored at -20°C prior use.

2.10 Kryopreservation of bacterial and yeast strains

The kryo-stocks were prepared to preserve bacterial (or yeast) strains harbouring the plasmid DNA. 1 ml of fresh bacterial (or yeast) culture was added to a 2 ml tube (Eppendorf, Hamburg) containing 1 ml sterile glycerol solution (65% (v/v)) glycerol, 0.1 M $MgSO_4$, 0.025 M TrisCl pH8.0) and the suspension was briefly and gently mixed. The mix was immediately frozen in liquid nitrogen.

Kryopreserved samples were stored at -80°C.

2.11 Determination of cytokinins from cultures of *Physcomitrella*

2.11.1 Preparation of tissue and culture medium samples for cytokinin measurements

Tissue and culture medium (see 2.4) from *Physcomitrella* cultures were separated by nylon mesh filtration (mesh width 0.2 mm). Excess of culture medium was further removed from tissue samples by vacuum filtration. Tissue samples were weighed and frozen in liquid nitrogen.

Cytokinins from culture medium samples were concentrated and pre-purified by solid phase extraction using Sep Pak, C18-cartridge (Waters, Eschborn). Cartridges were conditioned according to the recommendations of the manufacturer. After loading, the C18-cartridges were rinsed with water and eluted with 3 ml methanol. The methanol fractions were filtered (0,2 µl FP 030/3; Schleicher&Schuell, Dassel, Germany) into small glass flasks. Samples were stored at -20°C until LC-MS analysis.

2.11.2 Cytokinin extraction

Cytokinins from tissue as well as from culture medium samples were extracted and purified by V. Motyka and P. Dobrev according to Dobrev and Kaminek (2002) at the Institute of Experimental Botany, Prague, Czech Republic.

Frozen plant material (equiv. 0.4-1.2 g FW and 50-200 ml for tissue and media, respectively) was homogenised in liquid nitrogen and extracted overnight with 10 ml methanol/water/formic acid (15:4:1, v/v/v, pH ~ 2.5, -20°C). For analyses of endogenous cytokinins, 50 pmol of each of following twelve deuterium labelled standards were added: [$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z7G, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPA, [$^2H_6$]iP7G,

[$^2H_6$]iP9G, [$^2H_5$]DHZ, [$^2H_5$]DHZR (products of Apex Organics, Honigton, UK) were added**.** Derivatives of cZ derivatives were determined on the basis of retention times and MS spectra of corresponding *trans*-isomers. The extracts were purified using Sep-Pak® Plus tC18 cartridges (Waters, Milford, MA, USA) and Oasis® MCX 6cc mixed mode (cation exchange and reverse-phase) columns (150 mg, Waters, USA). After washing with 5 mL of 1M formic acid and 5 ml of methanol cytokinin nucleotides were eluted with 5 mL 0.35 M NH_4OH and cytokinin bases, ribosides and glucosides with 5 mL 0.35 M NH_4OH in 60% (v/v) methanol. Separated cytokinin nucleotides were dephosphorylated by incubation with calf-intestine alkaline phosphatase (Sigma) and determined as corresponding nucleosides.

2.11.3 LC-MS determination of cytokinins

LC-MS analysis of purified cytokinin samples (2.11.1 and 2.11.2) was performed by J. Malbek and V. Motyka at the Institute of Experimental Botany, Prague, Czech Republic, as described in Lexa et al. (2003).

A Rheos 2000 HPLC quaternary gradient pump (Flux Instruments, Switzerland) and HTS PAL autosampler (CTC Analytics, Switzerland) coupled to an ion-trap mass spectrometer LCQ (Finnigan, USA) equipped with an electrospray interface were used. The mass spectrometer was operated in the positive ion, full-scan MS/MS mode. Quantification was carried out using a multilevel calibration graph with deuterated cytokinins as internal standards. The levels of 27 different cytokinin derivatives were measured. Cytokinin nucleotides were quantified as their corresponding ribosides after alkaline phosphatase treatment. The detection limit was calculated for each compound as 3.3 σ/S, where σ is the standard deviation of the response and S the slope of the calibration curve. Three independent experiments were done. Each sample was injected at least twice.

2.12 Determination of tRNA- derived cytokinins

2.12.1 Extraction of tRNA from the *S. cerevisiae* strains ALB8, MT-8 and MT-8-*PpIPT1*.

tRNA was selectively extracted with a modified method based on yeast cell wall lysis in hot (65°C) phenol (Domdey et al. 1989):

The following reagents were used:

0.05 M sodium acetate (pH 5.3)/ 0.001 M EDTA;
10% w/v SDS;
phenol equilibrated with 0.05 M sodium acetate (pH 5.3) (AppliChem, Darmstadt);
phenol-chloroform (1:1) (Merck, Darmstadt);
ethanol absolute (Roth, Karlsruhe).

Yeast cells were grown overnight in 200-250 ml of YPD (see 2.7.2) medium at 30°C under shaking and harvested by centrifugation at 2,000 g for 5 min at 4°C. The cell pellet was washed twice in ice-cold sterile distilled water and then resuspended in 10 ml of ice-cold solution of 0.05 M sodium acetate (pH 5.3) containing 0.001 M EDTA.

The cell suspension was vortexed vigorously for 3 min in the cold and an equal volume of hot (65°C) phenol was added. This mix was vortexed for 4 min and then rapidly chilled on ice until phenol crystals appeared. To separate the phases the mixture was centrifuged at 4,000 g, and the tRNA containing aqueous phase was decanted to a clean, sterile centrifuge tube. These steps were repeated again.

The aqueous phase was then extracted with a half volume of phenol/chloroform for 5 min at room temperature and transferred to a clean sterile centrifuge tube. Sodium acetate solution (5 M) was added to the aqueous phase containing tRNA to result in a final concentration of sodium acetate of 0.3 M. Then 2.5 volumes of absolute ethanol were added to precipitate the RNA.

The extracted tRNA was resuspended in a small volume of diethylpyrocarbonat (DEPC)- treated water (2 ml) and kept at -80ºC.

2.12.2 Extraction of tRNA from *Physcomitella* wild type and *ove*ST25

Plant tRNA was selectively extracted from the 10 g of fresh tissue with a modified method based on phenol/*m*-cresol treatment of homogenised plant tissue (Maas and Klämbt 1981).

The moss tissue was dried, divided into samples of 10 g (fw), quickly frozen in liquid nitrogen and homogenised by grinding with mortar and pestle using liquid nitrogen. Ten mM Tris-HCl (pH 7.5) / 100 mM NaCl buffer was added to the homogenate to make a 20 ml volume. The sample was vortexed for 5 min.

An equal volume of phenol (pH 8, equilibrated by Tris-HCl and stabilized by 8-hydroxyquinoline (Sigma Aldrich, Taufkirchen) mixed with *m*-cresol (per 100 ml phenol 12.4 ml of *m*-cresol was taken) was added to the samples and vortexed properly for 5 min. After centrifugation of the mixture for 10 min at 1,500 rpm a 20% K-acetate solution (0.1 volume of the mixture) and absolute ethanol (2.5 volumes of the mixture) were added to the aqueous

phase, mixed and incubated over night at -18℃. Then the mixture was centrifuged for 30 min at 12,000 rpm (4℃). The precipitate was collected and resuspended in 3M Na-acetate (pH 6.0) by shaking over night (4℃) followed by centrifugation for 45 min at 12,000 rpm (4℃). The supernatant was diluted with the DEPC- treated water (1:1) and 2 volumes of absolute ethanol was added. After 30 min incubation on ice, tRNA was precipitated by centrifugation for 30 min at 12,000 rpm (4℃) and the pellet was resuspended in a small volume of DEPC- treated water (2 ml) and kept at -80℃ before further purification.

2.12.3 DEAE cellulose chromatography of tRNA

Purification of tRNA was achieved by chromatography on DEAE cellulose columns according to Buck et al. (1983). The DEAE-cellulose (Serva, Heidelberg) was saturated and equilibrated in a 2×5 cm column with Tris-Mg buffer: 0.1 M Tris-HCl, 0.01M $MgCl_2$ (pH 7.5). The same buffer was added (1-2 ml) to the tRNA- sample (2.12.1 or 2.12.2) solution to result in an end volume of 10 ml. The tRNA sample was then applyed to a column of DEAE-cellulose. 20 ml of the Tris-Mg buffer were applyed and allowed to flow through the column. To elute tRNA 70ml of Tris-Mg buffer containing 0.2 M NaCl (pH 7.5) was applyed.

tRNA was precipitated from the eluting buffer with 0.7 volume of isopropanol followed by incubation at -20℃ over night. Then the mixture was centrifuged at 15,000 g at 4℃ for 45 min. The tRNA- pellet was washed with 70% ethanol and finally precipitated by centrifugation at maximal speed at 4℃ for 30 min. The pellet was dried and dissolved in DEPC-treated H_2O.

Purified tRNA samples were adjusted to an optical density (OD_{260}) of 5.0 and checked by separation using gel electrophoresis (2% agarose / TAE). Samples were stored at –80℃ prior to further treatments.

2.12.4 Preparation of tRNA samples for cytokinin measurements

For alkaline hydrolysis aliquots of purified tRNA (5 units of tRNA corresponding to 1 ml of a solution of OD_{260}= 5) (2.12.3) were incubated in 0.5 M KOH (37℃) for 15 h followed by neutralisation with $HClO_4$ (Maaß and Klämbt 1981, modified) and adjustment of pH to 7 using Tris-HCl.

The hydrolysates were separated from the precipitating $KClO_4$ by centrifugation at 10,000 g for 3 min and treated with 15 units alkaline phosphatase (CIAP) (MBI Fermentas, St. Leon-Roth) for 15 h at 37℃. CIAP protein was precipitatied in 80% ethanol (final concentration) by incubation for 1 h and centrifuged at 15,000 g (4℃) for 45 min.

The supernatant containing the tRNA- riboside mixture was evaporated to dryness by rotary film evaporation (rotary evaporator of Labo Rota SE 320, Resona Technics, Gossau, Germany) and the residue was dissolved either in 1 ml of 10% methanol (for further HPLC-analysis) or sterile ddH_2O (for determination of cytokinin bioactivity on moss tissue).

2.12.5 HPLC determination of cytokinins from tRNA- samples

Samples of dephosphorilated tRNA-hydrolysates were prepared and purified as described above (2.12.4). Cytokinin nucleotides were determined as their corresponding ribosides in the dephosphorylated tRNA hydrolysates.

Prior chromatography hydrolysates were purified from protein traces as followed: Per 100 µl of the digest 20 mg of wet packed silicic acid (100 mesh, equilibrated in hydrolysis buffer) was added. The sample was mixed gently on ice for 15 min. After centrifugation of the mixture the supernatant was collected and mixed 1:1 with 10% methanol in water (v/v). To remove traces of silicic acid prior to HPLC the mixture was centrifuged again and the supernatant was collected and injected for HPLC.

Ribosides were separated by a Kontron-BioTEK HPLC system equipped with a diode array detector (DAD 540+, BioTEK, Bad Friedrichshall, Germany) for UV-spectroscopic analysis of effluents. Separation was performed by a gradient ranging from 10% methanol in water (v/v) to 100% methanol at a flow rate of 0.8 ml min^{-1}. Chromatograms and UV spectra were analysed using the Kroma 2000 software package (Version 1.83, BioTek) (see Schwartzenberg et al. 2003).

Three independent experiments were done. Each sample was injected at least twice.

2.12.6 UPLC-MS/MS determination of cytokinins from tRNA

Samples of dephosphorilated tRNA-hydrolysates were prepared as described above. Cytokinin nucleotides were determined as their corresponding ribosides in the dephosphorylated tRNA hydrolysates.

The following UPLC-MS/MS procedure used for cytokinin analysis was a modification of the method described by Faiss et al. (1997) and performed by O. Novak and M. Strnad at the Institute of Experimental Botany, Palacký University, Olomouc, Czech Republic).

Deuterium-labelled CK internal standards (Olchemim Ltd., Czech Republic) were added, each at 5 pmol per sample to check the recovery during purification and to validate the determination. The standards were [$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_5$]tZOG, [$^2H_5$]tZROG,

[$^{2}H_5$]tZRMP, [$^{2}H_3$]DHZ, [$^{2}H_3$]DHZR, [$^{2}H_3$]DHZ9G, [$^{2}H_3$]DHZOG, [$^{2}H_3$]DHZROG, [$^{2}H_3$]DHZRMP, [$^{2}H_6$]iP, [$^{2}H_6$]iPR, [$^{2}H_6$]iP9G, [$^{2}H_6$]iPRMP, [$^{2}H_7$]BA, [$^{2}H_7$]BAR,[$^{2}H_7$]BA9G, [$^{2}H_7$]BARMP, [$^{15}N_4$]mT, and [$^{15}N_4$]oT. All topolins were analysed using internal deuterium standards for [$^{15}N_4$]mT and [$^{15}N_4$]oT as no other labelled standards were available. After 3 h extraction, the homogenate was centrifuged (15,000 g at 4°C) and the pellets were re-extracted. The combined supernatants were concentrated to approximately 1.0 ml by rotary evaporation under vacuum at 35°C. The samples were diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5). The extracts were purified using a combined (diethylamino)ethyl (DEAE)-Sephadex (Sigma-Aldrich, St. Louis, MA, USA) (1.0 x 5.0 cm)-octadecylsilica (0.5 x 1.5 cm) column and immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against cytokinins (Faiss et al., 1997). This resulted in three fractions: (1) the free bases and 9-glycosides (fraction B), (2) a nucleotide fraction (NT) and (3) an *O*-glucoside fraction (OG). The metabolic eluates from the IAC columns were evaporated to dryness and dissolved in 20 µl of the mobile phase used for quantitative analysis.

The cytokinin fractions were analysed by ultra-performance liquid chromatography (UPLC) (Acquity UPLCTM; Waters, Milford, MA, USA) linked to a Quattro microTM API (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray interface. The purified samples were dissolved in 15 µl MeOH/H2O (30/70) and 10 µl of each sample was injected onto a C18 reversed-phase column (Acquity UPLCTM is based on a combination of high pressure and small bridged ethylsiloxane/silica hybrid particles; BEH Shield RP18; 1.7 µm; 2.1 x 150 mm; Waters). The column was eluted with a linear gradient of 15mM ammonium formate (pH 4.0, A) and methanol (B), with retention times for the monitored compounds ranging from 2.50 to 6.50 min. The binary gradient (0 min, 10% B; 0-8 min, 50% B) was applied with a flow-rate of 0.25 ml/min and a column temperature of 40°C. Quantification was obtained by multiple reaction monitoring of [M+H]+ and the appropriate product ion. For selective MRM experiments, optimal conditions were as follows: capillary voltage 0.6 kV, source/desolvation gas temperature 100/350 °C, cone/desolvation gas 2.0/550 l/h, LM/HM resolution 12.5, ion energy 1 0.3 V, ion energy 2 1.5 V, entrance 2.0 V, exit 2.0 V, multiplier 650 eV. The dwell time, cone voltage, and collision energy in collision cell corresponding to exact diagnostic transition were optimized for each cytokinin. On the basis of retention time stability, the chromatographic run was split into eight retention windows. The dwell time of each MRM channel has been calculated to obtain 16 scan points per peak during which time the inter channel delay was 0.1 sec. In MRM mode, the limit of detection (LOD) for most of cytokinins was below 5.0 fmol and the linear range was at least five orders of magnitude.

2.13 Moss bioassay with cytokinins derived from yeast tRNA

Five samples of undifferentiated protonemal tissue of *Physcomitrella* wild type were prepared as follows: 500 µl aliquots taken from a 7-day-liquid culture were placed onto a 1 mm layer of Knop-agar medium on microscopic cover-glasses (400 mm^2) as a support. The excess of liquid was evaporated under sterile conditions and the weight resulting from tissue and agar medium was determined.

Riboside mixtures obtained after dephosphorylation of tRNA- hydrolysates from the yeast lines MT-8–*PpIPT1*, MT-8 and ALB8 were taken up in 100 µl of sterile ddH_2O and applied to the tissue. As a positive control iPR was used at a final concentration of approximately 1.75×10^{-8} M and as a negative control sterile ddH_2O was applied.

After 6 days of incubation the bud formation was recorded by microscopic observation using an inverse microscope.

2.14 RT-PCR analysis of *PpIPT1* expression

Total RNA from 10 days old cultures of wild type, tCKX7, *ove*ST25 grown at 25°C or *ove*ST25 cultured at 15ºC were prepared using the plant RNA Kit (AnalytikJena Group INNUSCREEN, Berlin, Germany). From the resulting RNA for each genotype 4 µg aliquots were treated with RNase-free DNase (Fermentas, St. Leon-Rot).

For synthesis of first strand cDNA the RevertAid™ M-MuLV RevertAidTM Reverse Transcriptase from Moloney Murine Leukemia Virus (M-MuLV RT) (Fermentas GmbH, St. Leon-Rot, Germany) was used. The enzyme possesses an RNA-dependent and DNA-dependent polymerase activity and a ribonuclease H activity specific to RNA in RNA-DNA hybrids, which provide the accurate synthesis of a single cDNA strand from a single RNA template.

To prepare a reaction, 4 µg of DNase- treated total RNA was mixed with 1µl of random nonamer primers (final concentration of 2,5 µM) (Sigma-Aldrich, Steinheim) and DEPC-treated water for a volume of 11 µl, incubated at 70°C for 5 min and chilled on ice; then the following was added in the order indicated: 4 µl of 5× reaction buffer, 1 µl of dNTP mix (final concentration of 2,5 mM each) (Fermentas) and DEPC- treated water for a final volume of 19 µl; the mix was first incubated at 25°C for 5 min, then 1 µl (200 U) of M-MuLV RT was finally added to perform the reaction. The reaction mix (end volume of 20 µl) was incubated at 25°C for 10 min, and then at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and chilled on ice. The synthesized cDNA (5 µl of reaction mix) was directly used for PCR.

As a control for eventual DNA contaminations an additional reaction mixture for each RNA template was prepared without final addition of M-MuLV.

The reverse transcription products were amplified in *real time* PCR reaction with the SYBR® green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich Chemie GmbH, Steinheim). The SYBR green I fluorescent dye binds selectively to double-stranded DNA. Detection of the DNA can then be monitored by measuring the increased fluorescence throughout the PCR cycles. At room temperature, the Taq DNA polymerase remains inactive due to JumpStart Taq antibody binding. The elevated temperature of the first denaturation cycle is sufficient to disrupt the complex, restoring full enzyme activity. By preventing nonspecific product formation, this polymerase results in more accurate C_T values and an improved standard curve for sample quantitation.

PCR ReadyMix (12.5 µl) was added to cDNA templates (5 µl), specific primers and PCR-water (Sigma-Aldrich) for a final reaction volume of 25 µl.

For RT-PCR of *PpIPT1* the following primers were used:

pr-99 5'-AAGTGGTGAGCGATGTCCTG-3';

pr-98 5'-AGCAACGTTACATAGAGGTC-3' (see Fig. 4),

(the annealing temperature of 55°C).

As a control reaction for constitutive expression a RT-PCR of *PpACT3* (an actin encoding gene (AY382283)) was performed using following primers:

pr-214 5'-CGGAGAGGAAGTACAGTGTGTGGA-3';

pr-215 5'-ACCAGCCGTTAGAATTGAGCCCAG-3' (Nakamura et al. 2005),

(the annealing temperature of 57°C).

For *real time* monitoring of amplification, which was performed in the standard PCR-regime (see 2.6), an iCycler iQ (Bio-Rad Laboratiories GmbH, München) was used.

PCR-data processing was done using the iCycler iQ Optical System Software (version 3.0a). Additionally PCR-products were separated by 1.5% agarose TAE gel - electrophoresis.

3 Results

3.1 Identification and characterisation of a tRNA-IPT gene from *Physcomitrella*

3.1.1 *In silico* identification of *Physcomitrella* ESTs with homologies to IPTs

To identify putative cytokinin biosynthetic genes from *Physcomitrella*, corresponding EST-data bases were screened using 30 plant adenylate- and tRNA-IPTs as queries in tBLASTn (s. 2.5). The search revealed only two ESTs, BJ163403 and BJ173373, for all queries. These ESTs showed highest homology to AtIPT9 from *Arabidopsis* and OsIPT9 and OsIPT10 from *Oriza sativa* (> 50%- identity on aa level, Tab. 1). All three mentioned proteins were previously annotated as tRNA-IPTs (Kakimoto 2001, Sakamoto et al. 2006).

Astonishingly, there were no ESTs found with significant homology to adenylate-IPTs in the existing *Physcomitrella* databases (http://www.mossgenome.
org/links.php).

3.1.2 Reconstruction of the *Physcomitrella* IPT gene, *PpIPT1*

From a phagemid cDNA library a 520 bp fragment was amplified by PCR using the primers pr-137 and pr-144, designed on the basis of the sequences of the ESTs BJ173373 and BJ163403, showing that both ESTs represent only one gene, named *PpIPT1*.

The sequence of the *PpIPT1* cDNA was established by sequencing the 520 bp PCR fragment containing a 196 bp fragment joining the two ESTs. The missing 5´- and 3´ -ends were recovered by inverse PCR with the primers pr-151 and pr-152 using cDNA library as template (s. 2.6) (Fig. 4). The entire cDNA (Acc. No. EF512463) comprised 2,080 bp (Fig. 5).

A continuous ORF of 1,674 bp as well as 322 bp 5´- and 84 bp 3´- untranslated regions were identified. The ORF encodes a predicted gene product of 557 amino acids with a molecular mass of 61,995 Da. For cloning and functional analysis the entire ORF of *PpIPT1* was amplified by PCR using the primers pr-230 and pr-232.

Genomic locus of *PpIPT1,* as withdrawn from the *Physcomitrella* genomic database *PHYSCO*base (http://moss.nibb.ac.jp/blast/blast.htm; Nishiyama et al. 2003), comprises 4,702 bp containing 10 introns (between start und stop codons) (Fig. 6).

Tab. 1 Screening of Physcomitrella transcriptome for putative IPTs. Results of tBLASTn search using 30 plant IPTs as queries (http://www.ncbi.nlm.nih.gov/) for screening in *COSMOSS* (http://www.cosmoss.org/bm/BLAST). The two detected Physcomitrella ESTs correspond to one gene, named *PpIPT1* showing the highest homology to the tRNA-IPTs *AtIPT9, OsIPT9* and *OSIPT10*

Query			**Hit**			
Plant IPT	**Accesion number**	**Plant species**	***Physcomitrella* ESTs**	**Score, bits**	**Identities, %**	**Positives, %**
AtIPT1	BAB59040	*Arabidopsis thaliana*	BJ163403	91	37	63
AtIPT2*	AB062609	"	BJ163403	85	42	65
AtIPT3	BAB59043	"	BJ163403	97	43	66
AtIPT4	BAB59044	"	BJ163403	70	40	61
AtIPT5	BAB59041	"	BJ163403	94	41	67
AtIPT6	BAB59045	"	BJ163403	62	41	58
AtIPT7	BAB59046	"	BJ163403	80	39	64
AtIPT8	BAB59047	"	BJ163403	74	38	61
AtIPT9*	AB062615	"	BJ163403 BJ173373	**152** **153**	**68** **41**	**79** **61**
OsIPT1	AB239797	*Oryza sativa*	BJ163403	77	35	54
OsIPT2	AB239798	"	BJ163403	72	40	67
OsIPT3	AB239799	"	BJ163403	90	38	54
OsIPT4	AB239800	"	BJ163403	82	34	53
OsIPT5	AB239801	"	BJ163403	88	36	56
OsIPT6 OsIPT7	AB239803 AB239805	" "	BJ163403 BJ163403	80 69	38 32	62 54
OsIPT8	AB239805	"	BJ163403	72	35	57
OsIPT9*	AB239806	"	BJ163403	**104**	**50**	**70**
OsIPT10*	AB239807	"	BJ163403 BJ173373	**156** **157**	**65** **44**	**78** **62**
BrIPT1	BAE43826	*Brassica rapa*	BJ163403	90	39	64
BrIPT3	BAE43827	"	BJ163403	76	40	58
BrIPT5	BAE43828	"	BJ163403	83	43	62
BrIPT7	BAE43829	"	BJ163403	80	44	61
IPT	BAE75936	*Pisum sativum*	BJ163403	90	40	65
IPT	BAE75937	"	BJ163403	85	37	61
IPT	BAE75938	"	BJ163403	92	42	64
IPT	BAE75939	"	BJ163403	83	36	60
Sho	AF346892	*Petunia hybrida*	BJ163403	79	30	58
IPT	AAT28191	*Glycine max*	BJ163403	84	33	59
IPT	AAS94327	*Humulus lupulus*	BJ163403	81	31	56

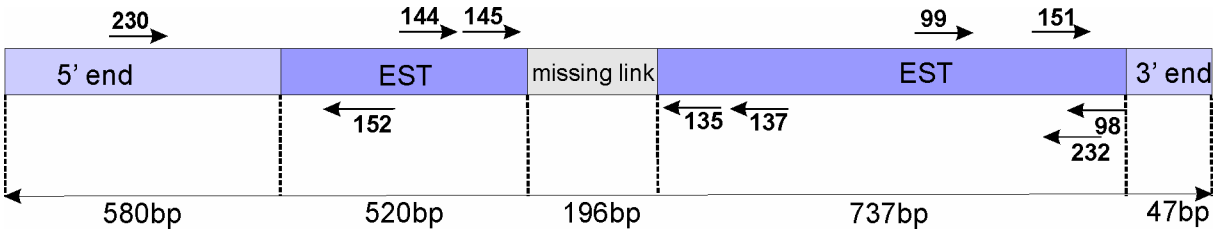

Fig. 4 Reconstruction of entire cDNA of *PpIPT1.* A fragment joining the ESTs (see Tab. 1) and the missing 5´- and 3´ -ends was recovered by PCR-based techniques with the primers pr-144/pr-137 and pr-151/pr-152; respectively, using *Physcomitrella* cDNA library as a template.

Fig. 5 The reconstructed nucleotide- und amino acid sequences of *PpIPT1* (Acc. No. EF512463).

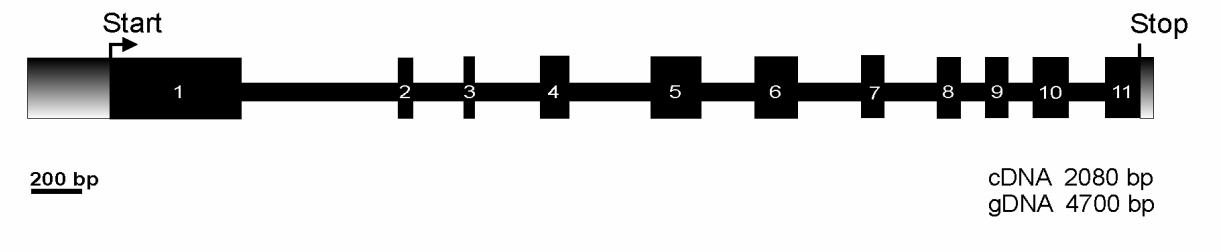

Fig. 6 Structure of *PpIPT1* gDNA (4,702 bp) with designation of ORF (2,080 bp). Exon/intron arrangement of gDNA was established *in silico* by alignment of *Physcomitrella* genome sequence from *PHYSCO*base (http://moss.nibb.ac.jp/blast/blast.htm; Nishiyama et al. 2003) with *PpIPT1* cDNA sequence in program BLASTn for two sequences of NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). ORF of *PpIPT1* was identified using the program ORF-finder of NCBI (http://www.ncbi.nlm.nih.gov/ gorf/gorf.html).

3.1.3 *PpIPT1* complements *mod5-1* mutation of a yeast tRNA- IPT gene *ScMOD5*

To test if *PpIPT1* encodes for a functional tRNA-specific IPT, as predicted by homology analysis (Tab. 1), a complementation assay with the yeast mutant MT-8 carrying an inactivated tRNA IPT gene (Sc*MOD5*) was performed (s. 2.7).

The *S. cerevisiae* strain MT-8 harbours three sets of genetic modifications (Gillman et al. 1991). First, there are UAA nonsense (point) mutations in the adenine biosynthetic gene *ADE2* (in addition to the *LYS1* and *CAN1 genes*). In order to grow MT-8 requires media containing adenine. Second, the strain contains a gene encoding the suppressor tRNA *SUP7.* The tRNA *SUP7* can insert a tyrosine at UAA, thereby suppressing the above mentioned point mutations and restoring metabolic independence. However, tRNA *SUP7* was shown to require isopentenylation (i^6A modification) to be able to suppress the UAA nonsense mutations (Laten et al. 1978). Third, the *MOD5* locus encoding the single yeast tRNA-*IPT* is disrupted through gene replacement. This prevents the i^6A modification of cytoplasmic and mitochondrial tRNAs, including the nuclear encoded tRNA *SUP7*, which renders *SUP7* unable to suppress the above mentioned nonsense mutations.

As a result of the non-functional *mod5-1* locus, MT-8 cells are not able to grow on media lacking adenine, and they accumulate a red pigment when grown on rich media. Complementation of *mod5-1* by a functional tRNA-IPT restores the adenine independence of the cells and reduces the accumulation of red pigment (Golovko et al. 2002).

In the complementation test we used ALB8, a *ScMOD5* line of *S. cerevisiae,* as a positive control. ALB8 was created on the basis of the MT-8 strain through integration of a construct containing a functional *ScMOD5* at the *ura3-1* locus of strain MT-8 (Gillman et al. 1991). By this targeted restoration of IPT functionality the auxotrophy of the strain for uracil was obtained. As a consequence of the functional *IPT* gene ALB8 keeps a white colour when growing on rich media (YPD), whereas MT-8 accumulates a red pigment.

To carry out the complementation assay the ORF of *Physcomitrella PpIPT1* was amplified using specific pimers (pr-230 and pr-232) (Fig. 4), and cloned at the *Not*I-site into the yeast expression vector pFL61 (s. 2.7.1) carrying an *URA3* selective marker (Minet et al. 1992) (Fig. 7).

The yeast line MT-8 was transformed with 3 constructs (1) pFL61-*PpIPT1*, (2) with the empty expression vector pFL61 as a negative control and (3) with the construct pFL61-*Sho*, carrying an adenylate *IPT* gene from *Petunia hybrida, Sho* (Zubko et al. 2002), as an additional (negative) control for *MOD5*- complementation. Transformed yeast lines were selected on SC media lacking uracil (s. 2.7.2 - 2.7.5).

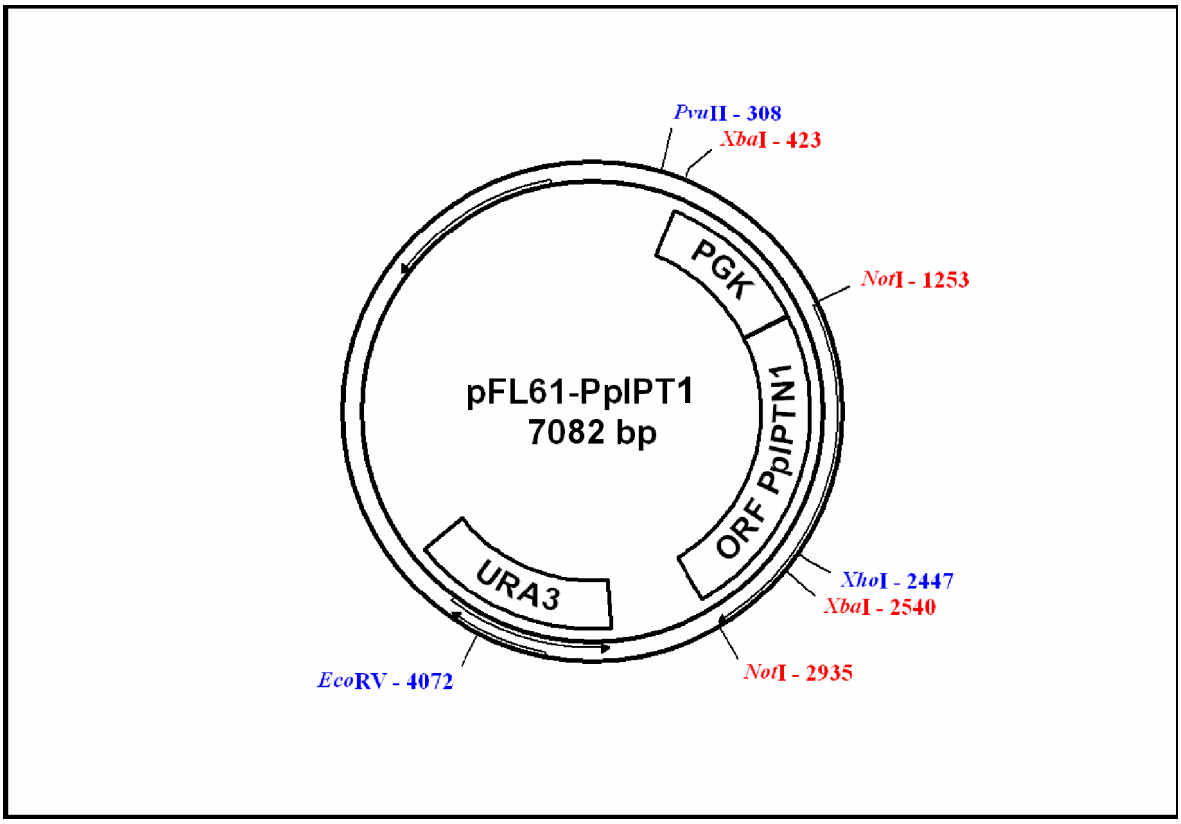

Fig. 7 Scheme of the constuct containing the isopentenyltransferase gene *PpIPT1* from *Physcomitrella* in the yeast expression vector pFL61 for functional complementation of *Sacharomyces cerevisiae* tRNA-*IPT* mutant in order to confirm the function of *PpIPT1*. PGK - phosphoglycerate-promotor; *URA3* – a selection marker for yeasts transformation (s. Minet et al. 1992).

The growth of the different strains and transformants on different media is presented in Fig. 8: When offering SC medium lacking uracil but containing adenine, the recombinant MT-8 lines exhibit growth, whereas the non-recombinant lines MT-8 and AlLB8 failed to grow (Fig. 8 a). On a medium containing uracil but lacking adenine, ALB8 expectedly showed growth as it contains a functional *MOD5* gene (Fig. 8 b).

From the growth of MT-8 containing pFL61-*PpIPT1* it can be deduced that *PpIPT1* complements the defect of the *mod5-2* allele and thus encodes for a functional tRNA- IPT (Fig. 8 b). The control transformant MT-8- pFL61-*PhSho* was unable to grow in the absence of adenine (Fig. 8 b). All strains grew well on both complete media, SC- or YPD (Fig. 8 c,d).

The complementation of the IPT deficiency in MT-8 by pFL61-*PpIPT1* also had phenotypical consequences with regard to the pigment accumulation on YPD purple colour, the MT-8 carrying pFL61-*pPIPT1* had only a slight pigment accumulation (Fig. 8 d). Apparently the IPT activity mediated by pFL61-*PpIPT1* seemed less strong than in the pigment-free ALB8 expressing the yeast gene.

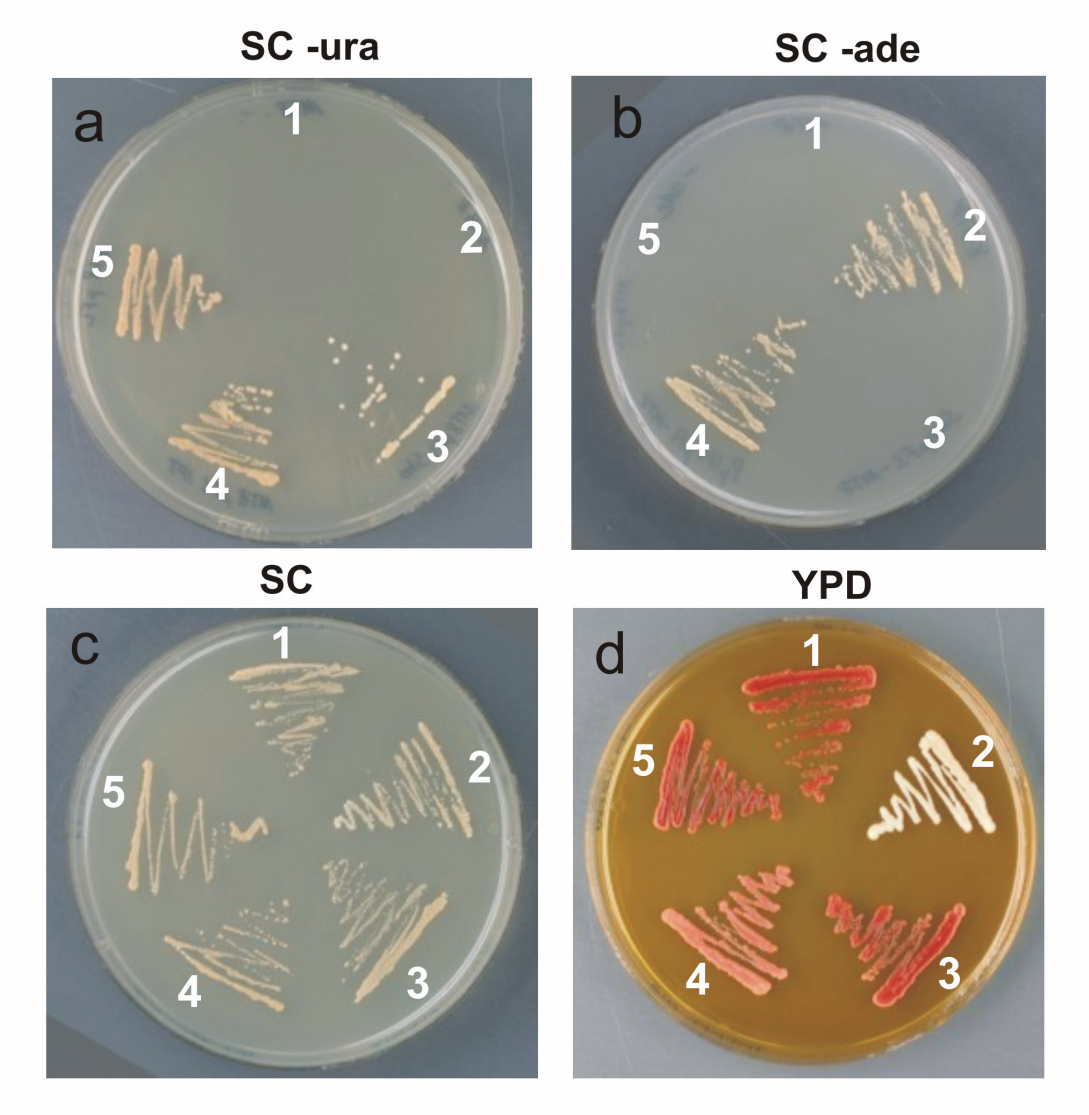

Fig. 8 a-d Yeast *mod5-1* complementation assay demonstrating tRNA-IPT function of PpIPT1. Yeast lines (1) MT-8, (2) ALB8, (3) MT-8-pFL61-*Sho*, (4) MT-8-pFL61-*PpIPT1* and (5) MT-8-pFL61 were streaked out on selective SC media either lacking uracil (a) or adenine (b) or on the control media complete SC (c) and YPD (d). Line ALB8 expresses the yeast tRNA-*IPT* gene (Sc*MOD5*). Line MT-8 has a disrupted locus of tRNA-*IPT* (*mod5-1*). When grown on YPD, ALB8 remains white, while MT-8 accumulates a red pigment. MT-8 was transformed with the plasmid containing the Physcomitrella *PpIPT1* cDNA, thus reducing the pigmentation. As a control MT-8 was transformed with pFL61 without insert and with *Sho* (adenylate-*IPT* gene from *Petunia hybrida*); in both cases pigmentation was not affected.

3.1.4 Cytokinin in tRNA from *PpIPT1* transformed MT-8 yeast line corresponds to iPR

As *PpIPT1* expressed in MT-8, was able to restore the strain´s growth on medium lacking adenine, it was essential to verify, whether this was indeed mediated by an N^6-isopentenylation of tRNA resulting in the formation of cytokinin nucleotides. Therefore tRNA was extracted, purified by DEAE-cellulose chromatography and hydrolysed to nucleotides

(2.12.1, 2.12.3). The nucleotide mixture was treated by alkaline phosphatase and the resulting ribosides were analysed by reverse phase HPLC for the presence of cytokinins (2.12.4 - 2.12.5) (Fig. 9).

The tRNA samples prepared from the *MOD5* expressing line ALB8 revealed a peak at the elution time of isopentenyladenosine (iPR) at 27 min (Fig. 9 a). The IPT deficient mutant MT-8 lacked an iPR signal in its tRNA hydrolysate.

The expression of the *Physcomitrella* gene *PpIPT1* in MT-8 resulted in the appearance of a significant iPR signal. The concentration of iPR measured in 2.5 OD_{260} units ml^{-1} of MT-8-*PpIPT1*- tRNA was estimated to be ~10^{-8} M and thereby ca. 13-fold lower than in ALB8 tRNA.

The UV spectra recorded by diode array detector demonstrated that the iPR co-eluting peaks from tRNAs of ALB8 and MT-8 PFL61-*PpIPT1* had the same absorption characteristics as the iPR standard with a maximum at 269 nm (Fig. 9 b).

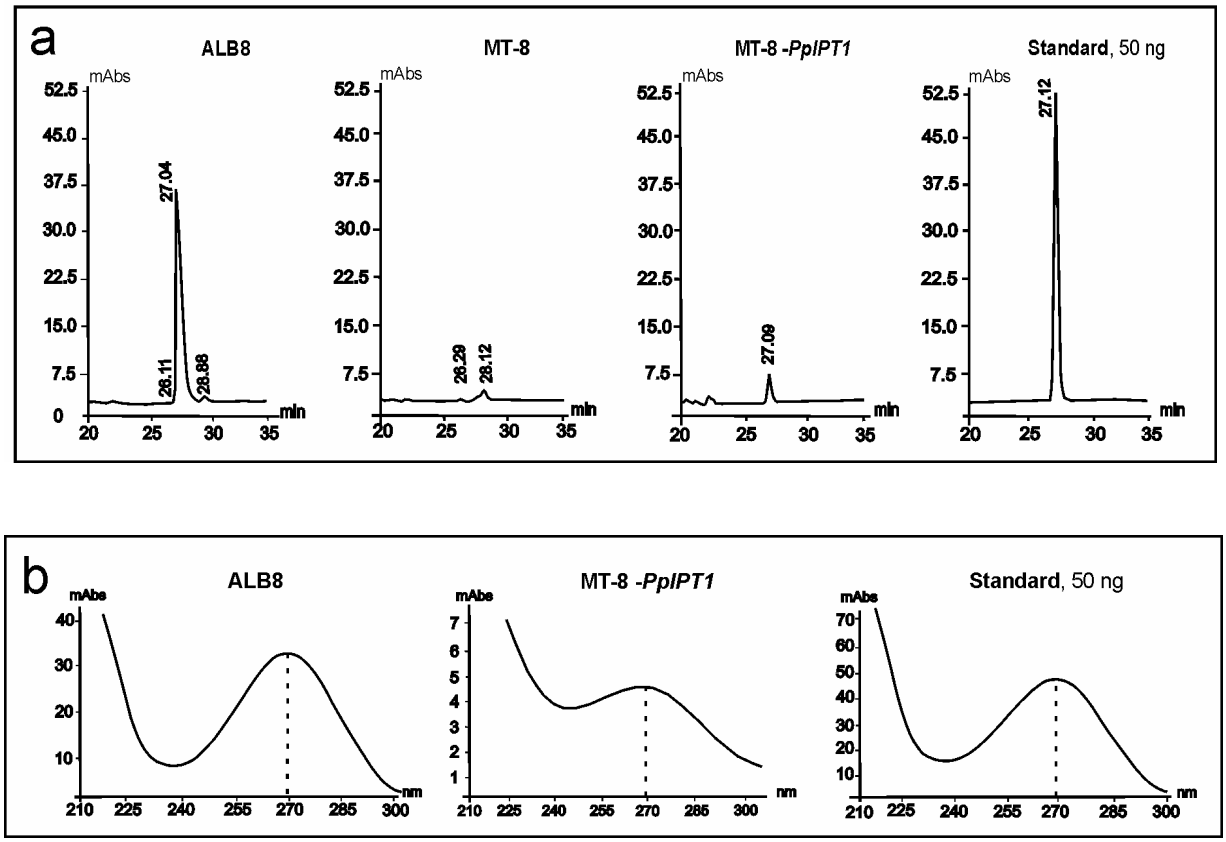

Fig. 9 HPLC analyses for isopentenyladenosine in yeast tRNA. (a) Chromatograms of hydrolysed and dephosphorylated tRNA (2.5 OD_{260} units) from *S. cerevisiae* lines: ALB8 carrying the functional yeast *IPT* gene *ScMOD5*; MT-8 having a disrupted *ScMOD5* locus*;* MT-8 expressing the *Physcomitrella PpIPT1* cDNA (MT-8 –*PpIPT1*) and iPR standard (50ng). The iPR corresponding peak (27 min) is present in ALB8 and MT-8 –*PpIPT1* with different intensities and absent in MT-8 sample. (b) UV spectra of iPR peaks from ALB8 and MT-8-*PpIPT1* samples exhibit maximum at 269 nm and correspond to iPR standard.

3.1.5 Cytokinin derived from tRNA of MT-8–*PpIPT1* demonstrates biological activity in moss bioassay

In order to unequivocally prove that *PpIPT1*-mediated modifications in the yeast tRNA result in biologically active cytokinin, a micro-bioassay on *Physcomitrella* protonema was performed (s. 2.13).

Exogenous application of cytokinins induces buds on moss protonema, which further develop to gametophores (Hahn and Bopp 1968, Reski and Abel 1985). Riboside mixtures obtained after dephosphorylation of yeast tRNA-hydrolysates were administered to small amounts of undifferentiated protonema of Physcomitrella wild type. After 6 days, bud formation was recorded by microscopic observation. On protonema treated with ribosides derived from tRNA samples of MT-8–*PpIPT1* and ALB8 75 and 110 buds/400 mm^2 were induced, respectively (Fig. 10 I and II). No bud development was observed on protonema treated with ribosides derived from MT-8-tRNA or after application of H_2O (Fig. 10 IV and V). As a positive control iPR was used at a concentration of 1.75×10^{-8} M for which 90 buds/400 mm^2 were counted (Fig. 10 III).

Thus, the modifications in the yeast tRNA, caused by *PpIPT1* expression, resulted in biologically active cytokinin and the chromatographic analysis indicates that *PpIPT1* activity catalyses the formation of iPR-nucleotides in the yeast background.

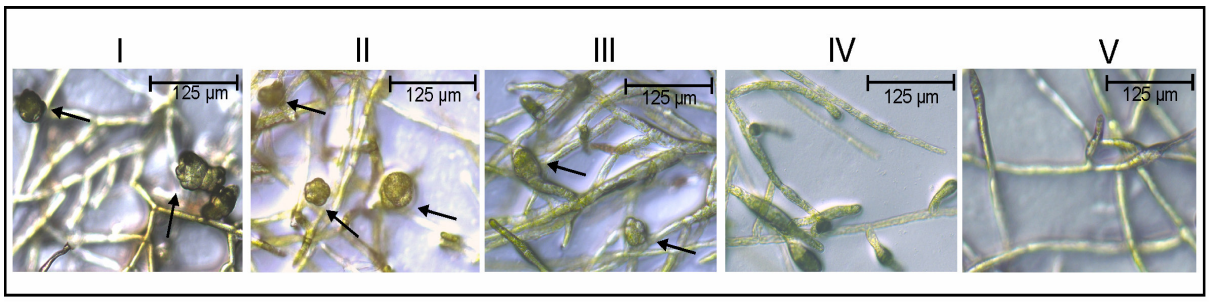

Fig. 10 Cytokinin bioassay based on bud formation on *Physcomitrella* wild type protonema; nucleosides of tRNA samples (~2,5 A_{260} units) from MT-8-*PpIPT1* (I) and ALB8 (II) were applied in comparison with iPR reference, 1.75×10^{-8} M (III); no bud induction occurred when nucleosides from tRNA samples of non-transformed MT-8 (IV) or water (V) were administered. Microscopic photos were made 6 days after treatment.

3.1.6 Protein properties of PpIPT1 - comparison with other eukaryotic and prokaryotic tRNA-IPTs

The predicted amino acid sequence of PpIPT1 showed strong homology with eukaryotic and prokaryotic tRNA-IPTs: 49% positives (identical plus similar aa) with AtIPT2, 63% with AtIPT9 (both *A. thaliana*); 53% with OsIPT9, 69% with OsIPT10 (both *O. sativa*); 49% with ScMOD5 (*S. cerevisiae)*; 45% with EcMiaA (*E. coli*); 47% with AgtMiaA *(A. tumefaciens*), 45% with SsIPT1 and 46% with SsIPT1 (both of cyanobacterium *Synechococcus sp*.) (s. 2.5).

All of the 10 tRNA-IPTs analysed in this study share a ATP/GTP binding motif at their N- termini, which typically consists of a glycine-rich sequence followed by a conserved lysine and a serine or threonine [G or A]-x(4)-G-K-[S or T] (Hirst and Sternberg 1993). In PpIPT1 this P-loop motif locates at the position 155-162 (Fig. 11). The PpIPT1 sequence is the longest among the analysed tRNA-IPTs, the predicted gene product having a molecular weight of 61 kDa. All other eukaryotic tRNA-IPTs have predicted molecular masses below 53 kDa.

The eukaryotic tRNA-IPTs are characterised by a C-terminal extension of ca. 100 aa resulting in total sequences ≥ 417 aa whereas the longest prokaryotic protein is predicted to consist of only 316 aa (Fig. 11). Compared to the eukaryotic proteins, the prokaryotic tRNA-IPTs are shorter because of lack of C-terminal extension. In addition PpIPT1 has a N-terminal extension of 97 aa, when compared with AtIPT9 (Fig. 11).

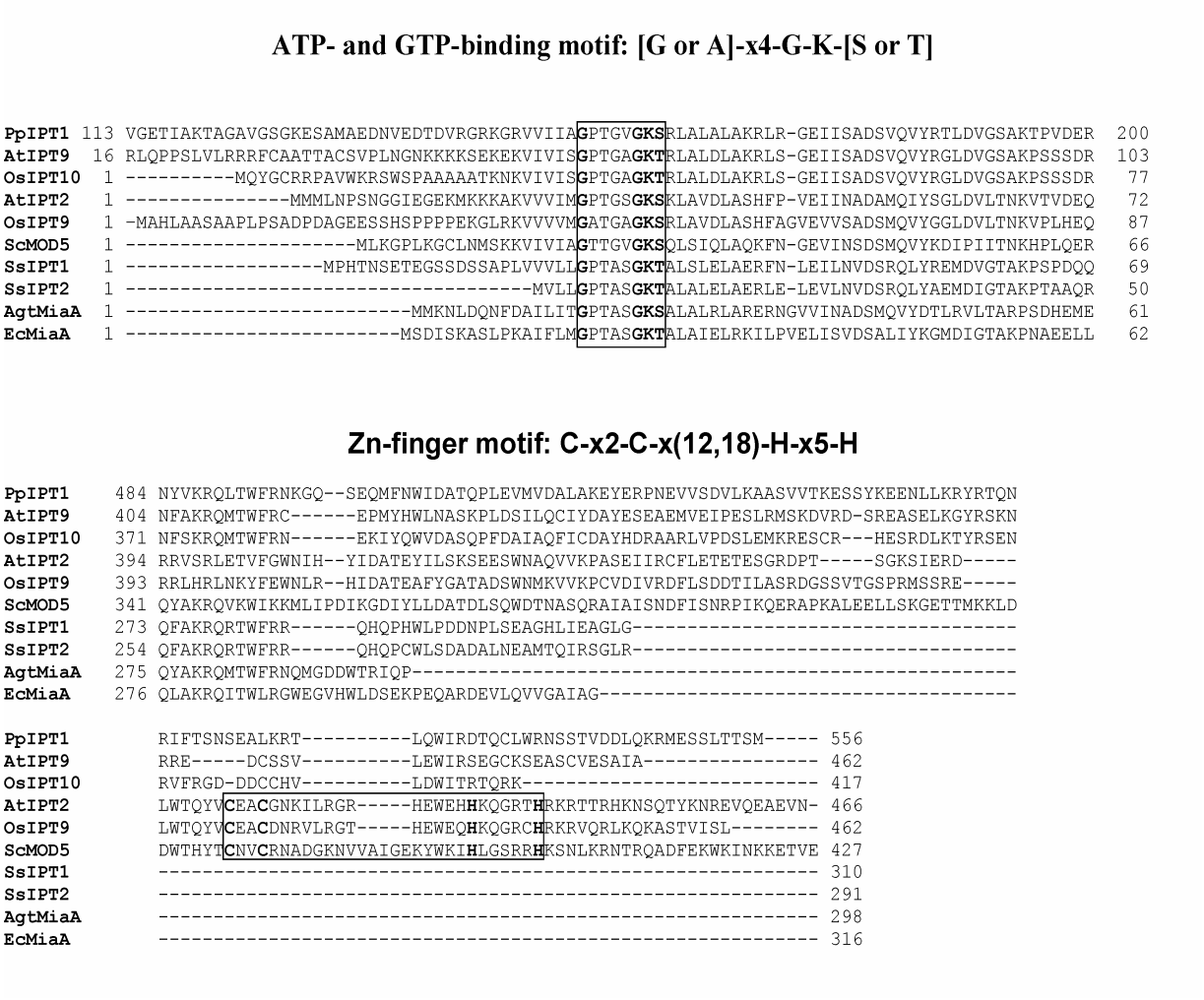

Fig. 11 Conserved motifs in PpIPT1 (EF512463) aa sequence and other pro- and eukaryotic tRNA-IPTs, accession numbers of proteins and genes (in brackets): AtIPT2 - BAB59042.1 (AB062609) *A. thaliana;* AtIPT9 - BAB59048 (AB062615) *A. thaliana;* OsIPT9 - BAE47452.1 (AB239806) *O. sativa;* OsIPT10 - BAE47453.1 (AB239807) *O. sativa*; ScMOD5 – AAA34785 (M15991) *S. cereviseae*; SsIPT1 - ZP_01125424 (NZ_AAOK01000008.1:11474..12406) *Synechococcus sp*. WH 7805; SsIPT2 - ZP_01079100 (AANP01000001.1:242844..243719), *Synechococcus sp.* RS9917; AgtMiaA - AAA22091.1 (M83532) *A. tumefaciens*; EcMiaA - AAA24174.1 (M63655) *E. coli*. Above - distribution of ATP/GTP-binding motif (P-loop); below – distribution of C2H2 Zn-finger-like motif (present only in AtIPT2, OsIPT9 and ScMOD5).

3.2 Evidence for tRNA-dependent cytokinin biosynthesis in *Physcomitrella* by comparative studies of wild type and mutant *ove*ST25

3.2.1 Temperature-induced bud-formation in *ove*ST25

To study morphological changes in relation to cytokinin overproduction the temperature-sensitive *ove*ST25 mutant and wild type were grown first for 7 days at the non-inducing temperature of 15°C and then for another 7 days at the inducing temperature of 25ºC. The appearance of buds in *ove*ST25 was first observed on the 4th day after its transfer to 25°C. Three days later the formed buds showed malformed callus-like structures, typical for the *ove* phenotype (Fig. 12). The wild type control did not phenotypicaly respond to the temperature increase.

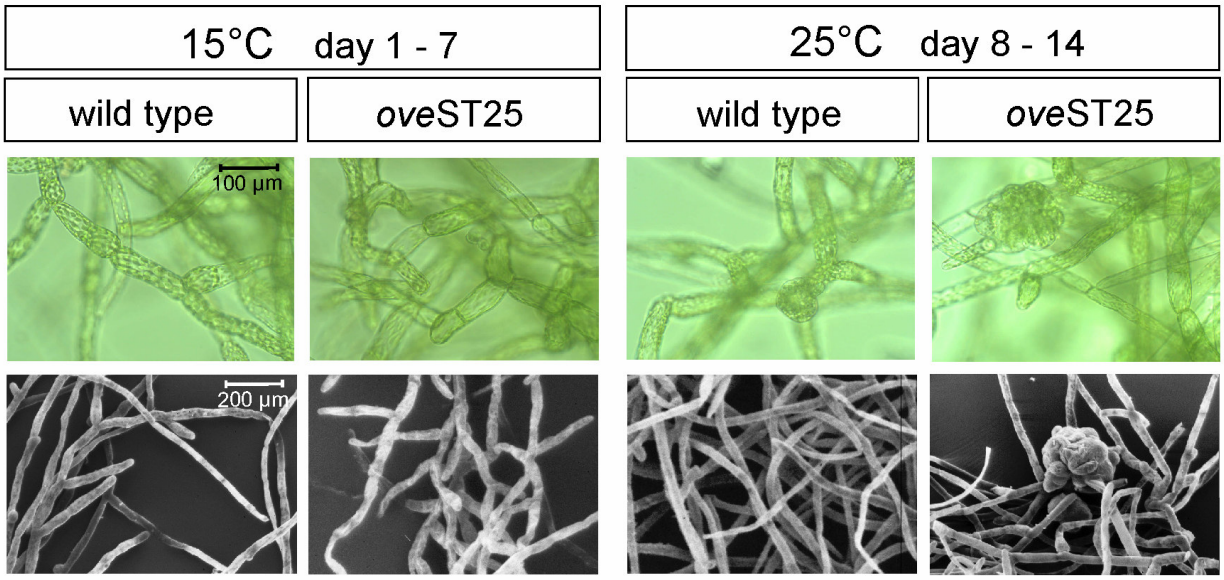

Fig. 12 Light microscopy (top) and scanning electron micrographs (bottom) demonstrating temperature induced bud formation causing the *ove* phenotype *ove*ST25 in comparison to wild type. Strains were cultivated in liquid culture for 7 days at **15°C**, then they were transferred to **25°C** (for other 7 days).

3.2.2 Cytokinin content in *Physcomitrella* and kinetics of temperature induced cytokinin overproduction in *ove*ST25

Cytokinin production by wild type and *ove*ST25 was studied during the time course of thermoinduction by means of HPLC-MS (s. 2.11). The culture samples of both *Physcomitrella* genotypes (s. 2.11.1) were analysed by J. Malbek and V. Motyka at the Institute of Experimental Botany, Prague, Czech Republic (s. 2.11.2 – 2.11.3). Eleven different isoprenoid cytokinins belonging to iP-, cZ-, tZ- and dihydro-zeatin (DHZ)- types, were found in *Physcomitrella* cultures (Tab. 2, Tab. 3).

Cytokinin content

In the tissue the cZ-type cytokinins: cZ and the *O*-glycosides cZROG and cZOG as well as the nucleotide cZRMP were the prevailing forms (s. Tab. 2). The concentration maximum of detected cytokinins reached 1180 pmol/g fw (cZROG). The tZ-type cytokinins were mostly under detection limit. For the dihydrozeatin-type forms (DHZ) only minor concentations of riboside (DHZR) and nucleotide (DHZRMP), ranging from 0.32 to 7.30 pmol/g dw, were detected. The iP-type cytokinins iP-, iPR- and iPRMP- were determined in concentrations between 0.45 and 9.2 pmol/g fw. No significant amounts of their *N*-glucosides could be found.

As mosses release significant amounts of cytokinins into the extracellular space, cytokinins were also analysed in the culture medium (s. Tab. 3). The amounts of the predominant *O*-glycosides of cZ-type cytokinins were measured ca. 0.30 pmol/ml. No extracellular DHZ-type cytokinins were found, but traces of the tZ-type (tZR) were detected in concentration of 0.01 pmol/ml. For the iP-type cytokinins, the iP- and iPR- form were found in concentrations of 0.130 pmol/ml and 0.014 pmol/ml, respectively. Cytokinin nucleotides were detected but not quantified in the culture medium.

The level of cytokinin amounts in mutant *ove*ST25 was generally higher, than those in wild type (Tab. 2, Tab. 3).

Tab. 2 Endogenous cytokinins in tissue of *Physcomitrella* wild type and *ove*ST25 in pmol/g fresh weight. Cultures were suspended in fresh medium at day 0 and cultured at 15℃ for 5 days, on day 5 cultivation temperature was raised to 25℃. The given data correspond to day 4 (15℃) and day 8 (25℃). Data were obtained by LC-MS analysis (s. 2.11.2-2.11.3), which was carried out at the Institute of Exp. Botany (Prague, Czech Republic) by J. Malbek and V. Motyka. (nd - not determined, udl - under detection limit)

	Genotype	iP	iPR	iPRMP	cZ	cZR	cZRMP	cZOG	cZROG	ZR	DHZR	DHZRMP
15℃	**wild type**	0.45	0.65	5.0	0.60	udl	90	60	512	udl	udl	0.32
	***ove*ST25**	2.60	0.13	8.8	1.30	udl	460	420	1002	udl	2.20	0.57
25℃	**wild type**	1.35	0.75	3.7	1.15	udl	145	89	581	udl	0.51	0.27
	***ove*ST25**	3.65	0.16	9.2	2.20	udl	420	404	1180	udl	7.30	1.00

Tab. 3 Endogenous cytokinins in extracellular fraction (culture medium) in pmol/ml. Results correspond to same experiment presented in Tab. 2, for further information s. Tab. 2.

	Genotype	iP	iPR	iPRMP	cZ	cZR	cZRMP	cZOG	cZROG	tZR	DHZR	DHZRMP
15℃	**wild type**	0.015	0.007	nd	0.007	0.003	nd	0.19	0.05	0.01	udl	nd
	***ove*ST25**	0.012	0.005	nd	0.006	0.003	nd	0.22	0.05	0.01	udl	nd
25℃	**wild type**	0.015	0.006	nd	0.014	0.005	nd	0.22	0.08	0.01	udl	nd
	***ove*ST25**	0.130	0.010	nd	0.020	0.013	nd	0.30	0.25	0.01	udl	nd

Temperature-mediated induction of cytokinin overproduction

Temperature increase from 15℃ to 25℃ caused cytokinin raise in both genotypes of *Physcomitrella,* with a more distinct tendency in the mutant *ove*ST25. When comparing the changes in cytokinin amounts in tissue (Tab. 2) and culture medium (Tab. 3), the increase of cytokinins levels was found to be more pronounced in the extracellular fraction.

In *ove*ST25 extracellular concentrations of the predominant *O*-glucoside forms of cZR and cZ increased significantly at 25°C compared to 15°C, 5fold and 1.5fold, respectively. Also for the minor extracellular compounds cZ and cZR, the concentrations raised significantly (3fold and 4fold, respectively). The most distinct temperature-mediated increase in *ove* mutant was found for extracellular iP (11fold). The concentration of its riboside iPR increased only 1.5fold (Tab. 3).

In contrast, in the wild type control the cytokinin concentrations raised not significantly during temperature increase. No increase of extracellular iP-type compounds on thermo-induction was detected. However, in case of cZ-type cytokinins the 2fold increase of cZ (from 0.007 to 0.014 pmol/ml), as well as slight increase of cZR and cZROG were measured (Tab. 3).

The kinetics of temperature-mediated cytokinin overproduction during the transition from 15℃ to 25℃ is depicted in Fig.13. In the extracellular space the cytokinin species: iP, iPR, cZROG, cZR, cZ, were found to be affected already 36h after temperature increase and accumulated in *ove* mutant.

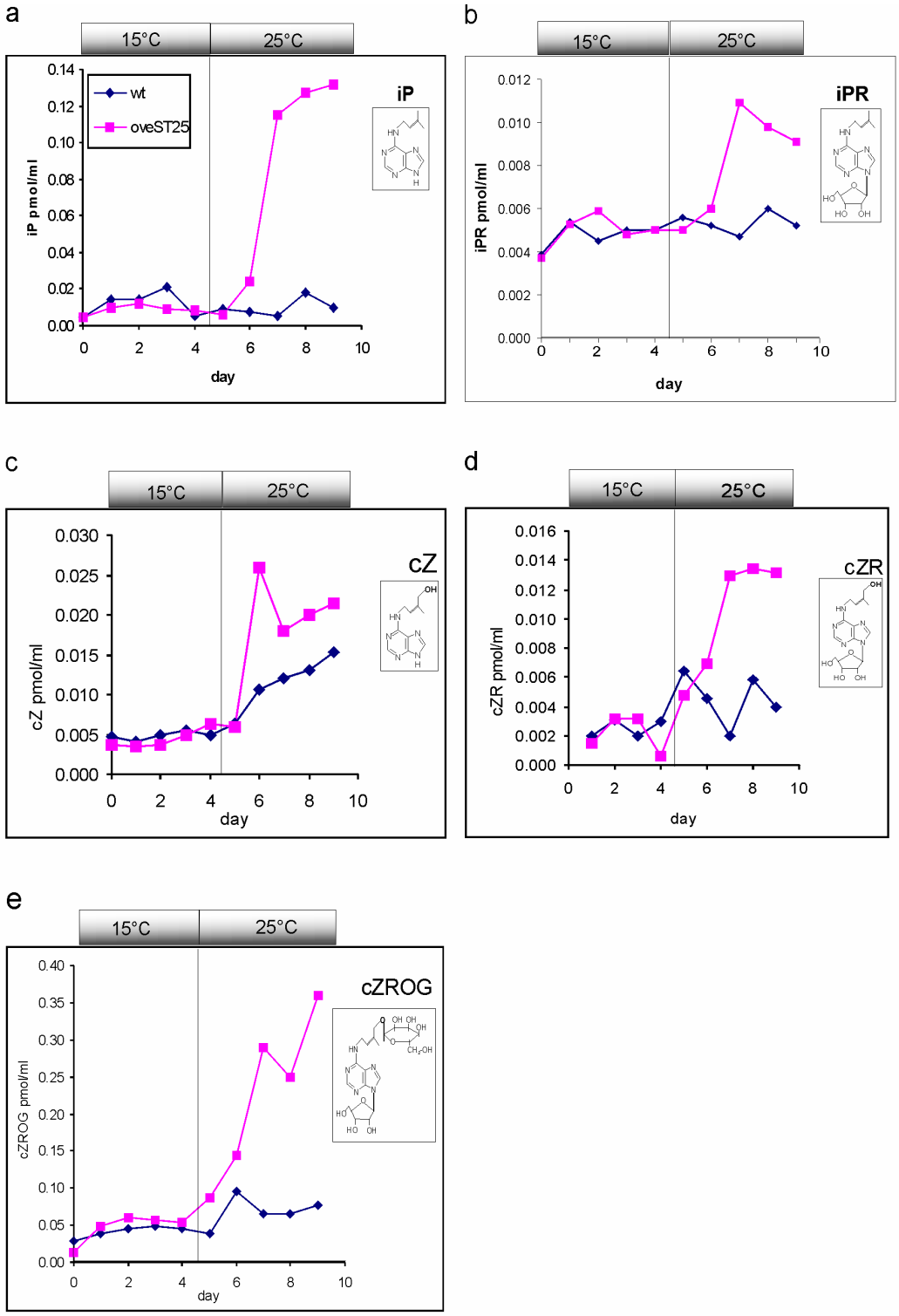

Fig. 13 Thermal dependence of <u>extracellular</u> cytokinin concentrations in temperature sensitive mutant *ove*ST25 and wild type during thermoinduction (liquid culture). On the 4th day the temperature was raised from 15℃ to 25℃ (for further information see Tab.2). The LC-MS analysis revealed the cytokinins iP, iPR, cZ, cZR, cZROG to be affected already 36h after temperature increase and to accumulate in culture medium of the *ove* mutant (as given in Tab. 3).

3.2.3 cZR and iPR are major cytokinins in *Physcomitrella* tRNA

As the major cytokinin forms in *Physcomitrella*, cZ and iP, were shown to be also major cytokinin constituents of tRNA in higher plants (Vreman et al. 1978, Miyawaki et al. 2006) as well as in primitive plants (Letham und Palni 1983, reviewed by Persson et al. 1994), an analysis of cytokinins in tRNA from *Physcomitrella* was added. Futhermore it should be checked whether the cytokinin composition in tRNA changes during temperature- mediated cytokinin overproduction.

The ribosides obtained from dephosphorylated tRNA-hydrolysates of *ove*ST25 and wild type (s. 2.12.2 – 2.12.4) were analysed by reverse phase HPLC in our laboratory (s. 2.12.5). In addition the complete spectrum of tRNA-bound cytokinins was determined by UPLC-MS/MS analysis (s. 2.12.6) in cooperation with O. Novák and M. Strnad (Palacký University & Institute of Experimental Botany, Olomouc, Czech Republic).

The tRNA samples (2.5 OD_{260} units ml^{-1}) prepared from *ove*ST25 and wild type tissue revealed peaks at the elution time of cZR and iPR at 17 and 27 min, respectively (Fig. 14). UV spectra recorded by diode array detector demonstrated that these iPR- and cZR- co-eluting peaks had the same absorption characteristics than the corresponding reference compounds with an absorbtion maximum at 269 nm (data not shown). It was therefore concluded that in *Physcomitrella* tRNA contains nucleotides of cZR and iPR as major forms.

In order to monitor whether further cytokinin forms were present in tRNA-samples an UPLC-MS/MS analysis of tRNA hydrolysates was carried out. From total of 40 measurable cytokinins 3 ribosides were detected: cZR, tZR and iPR (Tab. 4).

The UPLC-MS analysis confirmed that the dominant cytokinins in tRNA from both, wild type and *ove*ST25, were cZR and iPR (Tab. 4). The total amount of cytokinins in 1 µg of tRNA was lower in wild type-sample. tRNA from *ove*ST25 contained twice higher values for iPR, as well as more cZR compared to wild type tRNA cytokinins.

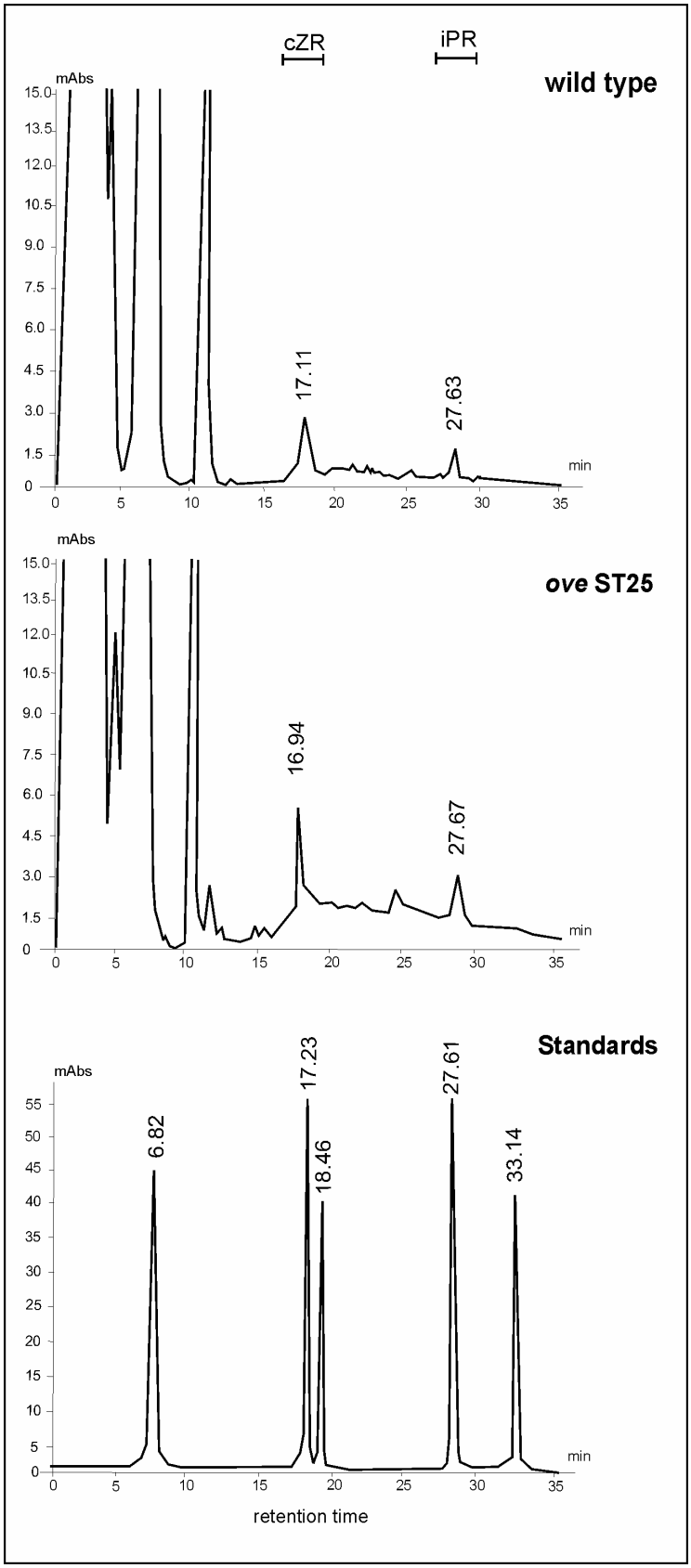

Fig. 14 HPLC profiles (s. 2.12.2 – 2.12.5) of hydrolised and dephosphorylated tRNA-samples (2.5 OD_{260} units) from *Physcomitrella* tissue revealing cZR and iPR cytokinin species in wild type as well as *ove*ST25. cZ and iPR eluted at 17min and 27min, respectively. Standards: Ado (6.82 min), tZR (16.13 min), cZR (17.16 min), iPA (27.32 min), BAPR (33.14 min)

Tab. 4 Cytokinin concentrations in 1µg of tRNA hydrolysate from *Physcomitrella* wild type and *ove*ST25 (pmol/µg) (s. 2.12.6). Both genotypes were cultured at 25°C. UPLC-MS/MS analysis was carried out by O. Novák and M. Strnad at Palacký University & Institute of Experimental Botany (Olomouc, Czech Republic). Nucleotides were determined as their corresponding ribosides in dephosphorylated tRNA hydrolysates.

	cZRMP	tZRMP	iPRMP	total sum
wild type	2.2164	0.0091	0.2221	2.4
***ove*ST25**	2.2830	0.0071	0.5287	2.8

3.2.4 Enhanced transcription of *Physcomitrella* tRNA-IPT gene *PpIPT1* in *ove*ST25

In order to address the question of the contribution of the tRNA-mediated pathway to cytokinin biosyntheis in *Physcomitrella* an expression analysis of the identified tRNA-IPT gene *PpIPT1* (EF512463) was carried out for wild type as well as for the temperature-inducible mutant *ove*ST25 (s. 2.14). In addition a wild type transgenic strain tCKX7 (25°C), characterised by cytokinin deficiency due to enhanced cytokinin oxidase/dehydrogenase activity (Schwartzenberg et al. 2007, a manuscript is submitted) was included as a control into this experiment.

For *ove*ST25 cultured at the inducing temperature of 25°C, a low Ct value of 18, compared to 23 in the wild type, pointed a high expression level of *PpIPT1*. Under non inducing conditions the expression of *PpIPT1* in the *ove* mutant was low as indicated by a high Ct value of 22 (Fig. 15).

When comparing the *PpIPT1* expression level in all three analysed genotypes under the same conditions, *ove*ST25 mutant and cytokinin oxidase/dehydrogenase overexpressing strain tCKX7 displayed a significantly higher expression level of *PpIPT1* with a Ct number of 18, than wild type (Ct value of 22) (Fig. 15).

As a constitutive control an actin biosynthetic gene *(PpACT3*, AY382283*)* was used. Its RT-products were amplified independently from genotypes and/or culture conditions with a Ct of 17.

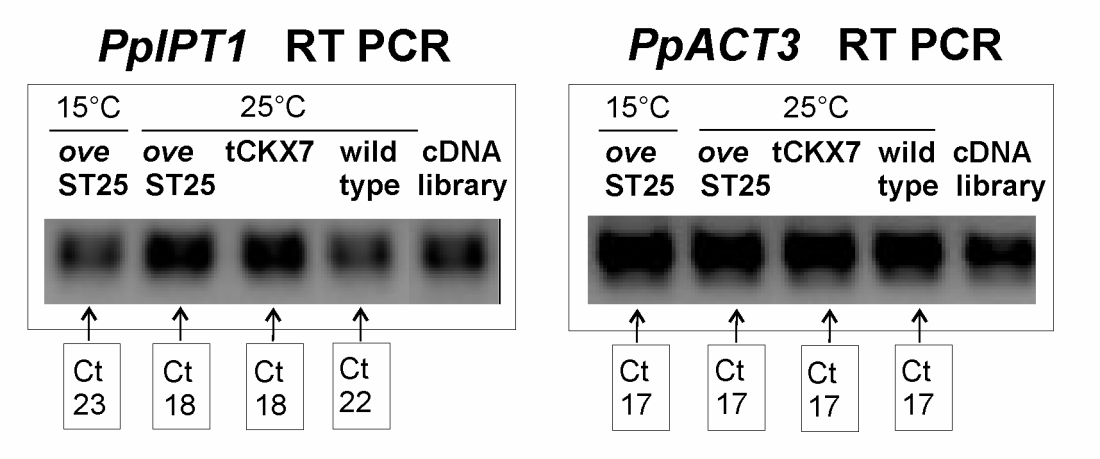

Fig. 15 RT-PCR analysis of *PpIPT1* (EF512463) expression in temperature-sensitive *ove*ST25 at 15°C and 25°C compared to wild type and transgenic line with enhanced cytokinin degradation, tCKX7, (both cultivated at 25°C). Equal amounts of RNA template were used. One representative experiment out of four is shown. Agarose gel electrophoresis separation (1.5%) after 30 PCR cycles: on the left graph a PCR specific to *PpIPT1* (240 bp fragment with pr-99/98); on the right graph a PCR specific to *PpACT3* (AY382283), 350 bp fragment with pr-214/215); as a PCR positive control is *Physcomitrella* cDNA phagemid library. The threshold cycles (Ct values) of semiquantitative *real time* PCR are given below. Standard deviation of Ct values did not exceed 0.4.

Summarising, *PpIPT1* is stronger expressed in both the cytokinin overproducing mutant *ove*ST25 and the tCKX7 line with enhanced cytokinin degradation of the wild type. With respect to tRNA-mediated cytokinin biosynthesis the studies of tRNA-IPT gene expression are consistent with the assumption of an increased tRNA-dependent cytokinin biosynthesis in *ove*ST25.

4 Discussion

The aim of the research was to characterise cytokinin biosynthesis in the moss *Physcomitrella patens* with respect to the two known biosynthetic pathways: the tRNA-mediated pathway and the direct synthesis of free cytokinins.

Using publicly available *Physcomitrella* genome/transcriptome databases and i*n silico* approaches for searching cytokinin biosynthetic genes, a putative IPT-candidate (*PpIPT1)* was identified.

As tRNA-IPT genes and adenylate IPT genes share considerable homologies, the function of the selected gene product had to be unequivocally demonstrated. The functional description of *PpIPT1* gene of *Physcomitrella* was achieved by a complementation assay, i.e. *via* restoring the tRNA-IPT activity in the tRNA-IPT- defective mutant MT-8 of *Saccharomyces cerevisiae.*

The question was asked, how the gene architecture and the protein properties of *PpIPT1* are related to IPT homologues from bacteria, cyanobacteria, yeasts and higher plants.

Cytokinin overproducing mutants *Physcomitrella*, such as the conditional mutant *ove*ST25, are useful tools to study plant cytokinin biosynthesis. If *PpIPT1* is relevant for cytokinin biosynthesis, its gene expression should be altered in oveST25. Semiquantitative RT-PCR analyses were carried out to clarify this question.

In addition the cytokinin profile of *Physcomitrella* wild type and *ove*ST25 was established under inducing and non-inducing conditions. It was asked whether the distribution of cytokinins contains already information on the preferred pathway of cytokinin biosynthesis in *Physcomitrella*. With respect to the tRNA biosynthetic pathway, the cytokinin composition of whole culture extracts was compared with the one of purified *Physcomitrella* tRNA.

In the following chapter, the experimental data obtained in the various approaches are evaluated and discussed in detail. The second part of the discussion focuses on the question, whether in *Physcomitrella* cytokinin biosynthesis takes mainly the tRNA-IPT or the adenylate-IPT pathway.

4.1 Identification and functional characterisation of a putative cytokinin biosynthetic gene from *Physcomitrella*

4.1.1 *Physcomitrella* genome/transcriptome screening for *IPT*-genes revealed tRNA-IPT candidates

The publicly available EST-databases for *Physcomitrella*, *PHYSCO*base and *COSMOSS* (Nishiyama et al. 2003, Rensing et al. 2005) cover almost the complete *Physcomitrella* transcriptome.

By screening these databases with sequences of 30 different plant IPTs, including adenylate- and tRNA-IPTs, I detected only two ESTs representing one gene, which was named *PpIPT1* (Tab. 1). Further genome-wide screens in which *PpIPT1* itself served as a query, also gave no indication of additional IPTs. Therefore it was deduced that *PpIPT1* is an important candidate for adenine isopentenylation or cytokinin biosynthesis in *Physcomitrella*.

Besides the gene *PpIPT1*, a partial sequence of a second *IPT* gene, named *PpIPT2,* was found by searching in non-publicly available databases by S. Rensing and R. Reski (Univ. of Freiburg, Germany). The screening revealed an EST representing a partial gene sequence, which showed a strong similarity to tRNA-IPTs (Rensing, Reski, Schwartzenberg and Yevdakova, unpublished). Experiments aiming at the reconstruction and functional characterisation of *PpIPT2* are underway.

Computational analyses of the whole *Physcomitrella* genome (http://www.mossgenome.org/links.php) revealed no hints for the existence of adenylate-IPTs (Tab. 1) raising the question of the pathway of cytokinin biosynthesis in this evolutionary old land plant. However, the possibility that functional adenylate- IPTs sharing little or no homology to known IPTs exist in the *Physcomitrella* genome cannot be completely ruled out.

The absence of adenylate-*IPT*s puts forward that tRNA-*IPT*s might be involved in cytokinin biosynthesis in *Physcomitrella* , where *PpIPT1* could play an important role.

4.1.2 Functional characterisation of PpIPT1 as a tRNA-IPT

BLAST analyses revealed that *PpIPT1* (and also *PpIPT2,* not shown) share significant homologies with tRNA-IPTs (Tab. 1). This homology led to a functional complementation assay using the yeast mutant strain MT-8, which has already been used for the characterisation tRNA-*IPT* genes (Golovko et al. 2000, 2002). I first achieved confirmation that the yeast complementation assay was able to differentiate between adenylate- and tRNA-IPTs as the control plasmid pFL61-Sho containing an adenylate-IPT from *Petunia*

hybrida could not reconstitute adenine autotrophy in MT-8 (Fig. 8 b). The finding that MT-8 transformed with pFL-*PpIPT1* grows on adenine- free medium demonstrates that isopentenylation of tRNA is restored and characterises PpIPT1 as a functional tRNA-IPT enzyme.

The HPLC analysis of dephosphorylated tRNA hydrolysates derived from MT-8-*PpIPT1* confirmed the presence of the cytokinin riboside iPR. In addition to the co-elution with iPR standard, the UV spectra support that indeed iPR was formed by a functional *PpIPT1* gene product (Fig. 9 a,b). Further, biological evidence for PpIPT1 generating active cytokinins was obtained after the application of tRNA-derived ribosides to undifferentiated *Physcomitrella* protonema (Fig. 10). Bud formation on moss protonema has been used in bioassays since the works of Bopp and Brandes (1964) and Hahn and Bopp (1968). Although regarded as less active than free bases, cytokinin ribosides have been reported to be bud inducing cytokinins in moss (Kende and Whitaker 1974). It should also be considered that *Physcomitrella* protonema is capable of effectively transforming iPR to iP (Schulz et al. 2001, Schwartzenberg et al. 2003) and thus can convert the tRNA derived cytokinin riboside to the more active base.

While riboside samples derived from MT-8 tRNA where unable to induce buds, those from MT-8-*PpIPT1* tRNA as well as from ALB8 tRNA showed significant formation of buds (Fig. 10). The concentration of iPR estimated in the MT-8-*PpIPT1* tRNA sample was ~10^{-8} M and hereby 75 buds per 400 mm^2 were induced; the number of buds induced by an iPR standard of comparable concentration was in the same order (not shown). I therefore presume that the iPR in the tRNA samples was responsible for the bud induction.

4.1.3 *Physcomitrella* cytokinin biosynthetic gene *PpIPT1* possesses features of homologues from evolutionary early organisms

In silico analyses of gene structure and protein properties of the identified *Physcomitrella* PpIPT1 in comparison with tRNA-IPTs of other eukaryotic as well as prokaryotic organisms revealed several characteristics, which are essential to demonstrate similarities and differences reflecting evolutional development of tRNA-IPTs.

PpIPT1 shares protein properties of prokaryotic tRNA-IPTs

(a) Sequence homology reveals two groups of tRNA-IPTs

The analysis of homologies on amino acid sequence level showed that *Physcomitrella* PpIPT1 forms one group of tRNA-IPTs together with AtIPT9 of *Arabidopsis* and OsIPT10

from *Oryza sativa*. NJ-tree shows, that members of this group (I) are more closely related to the prokaryotic tRNA-IPTs SsIPT1, SsIPT2, EcMiaA and AgtMiaA from *Synechococcus*, *Escherichia coli* and *Agrobacterium*, respectively, than to eukaryotic tRNA-IPTs (II) (Fig. 16). Group II includes the plant enzymes ATIPT2 and OsIPT9 and the yeast enzyme ScMOD5.

Sakamoto et al. (2006) used the term prokaryotic-type tRNA-IPTs for the genes OsIPT10 and AtIPT9. Although no functional analysis for the plant *PpIPT1*-homologues genes *AtIPT9* and *OsIPT10* has been published, aa sequence similarities indicate that they code for tRNA-IPTs. Moreover, AtIPT9 (together with AtIPT2) has been described as putative tRNA-IPTs by Kakimoto (2001) and Takei et al. (2001) and recently characterised to be absolutely required for the synthesis of cZ- cytokinins (Miyawaki et al. 2006) .

(b) tRNA-IPTs of both groups, I and II, share an ATP/GTP P-loop binding motif

All analysed tRNA-IPTs including PpIPT1 have in their N-termini the ATP/GTP P-loop binding motif (Fig. 11 and Fig. 16). Leung et al. (1997) have found a competitive inhibition of MiaA activity by ADP and ATP as well as by other nucleotide di- and tri-phosphates. The study revealed a competition on the level of DMAPP binding and the authors presume that it occurs at the P-loop site. Thus tRNA-IPTs might be regulated in their activity by nucleotide di- and tri-phosphates. The P-loop motif is also a structural feature of adenylate-IPTs such as the agrobacterial Ti plasmid encoded IPT.

(c) Group I, including PpIPT1, is characterised by absence of Zn-finger-like motif

Another well known conserved region in tRNA-IPTs is the Zn-finger-like motif C–x2–C–x(12,18)–H–x5–H (Miller et al. 1985, Krishna et al. 2003), which was previously described to occur in single copy in ScMOD5, at position 376-404, as well as in AtIPT2, at 419-441, (Golovko et al. 2002). ScMOD5 has been shown to prenylate cytoplasmic and mitochondrial tRNA in yeast (Dihanich et al. 1987). AtIPT2 has been functionally described as tRNA-IPT by Glovko et al. (2002).

In the comparative analysis of 10 tRNA-IPTs the Zn-finger-like motif was also found in OsIPT9, at position 423-440, belonging to group II along with ScMOD5 and AtIPT2 (Fig. 11). Thus the Zn-finger-like motif, which is present in only one copy, appears to be a typical conserved feature in tRNA-IPTs of the group II.

Despite their C-terminal extension, all eukaryotic tRNA-IPTs belonging to group I, including PpIPT1, lack this motif. This motif is also absent in the 4 other tRNA-IPTs representing group I (Fig. 11 and Fig. 16).

The function of Zn-finger-like motif in tRNA IPTs was discussed in connection with protein-protein interactions and nuclear localisation (Golovko et al. 2000). It seems unlikely that this motif has an essential function for the binding of the tRNA substrate as enzymes of group I also show functional tRNA prenylation.

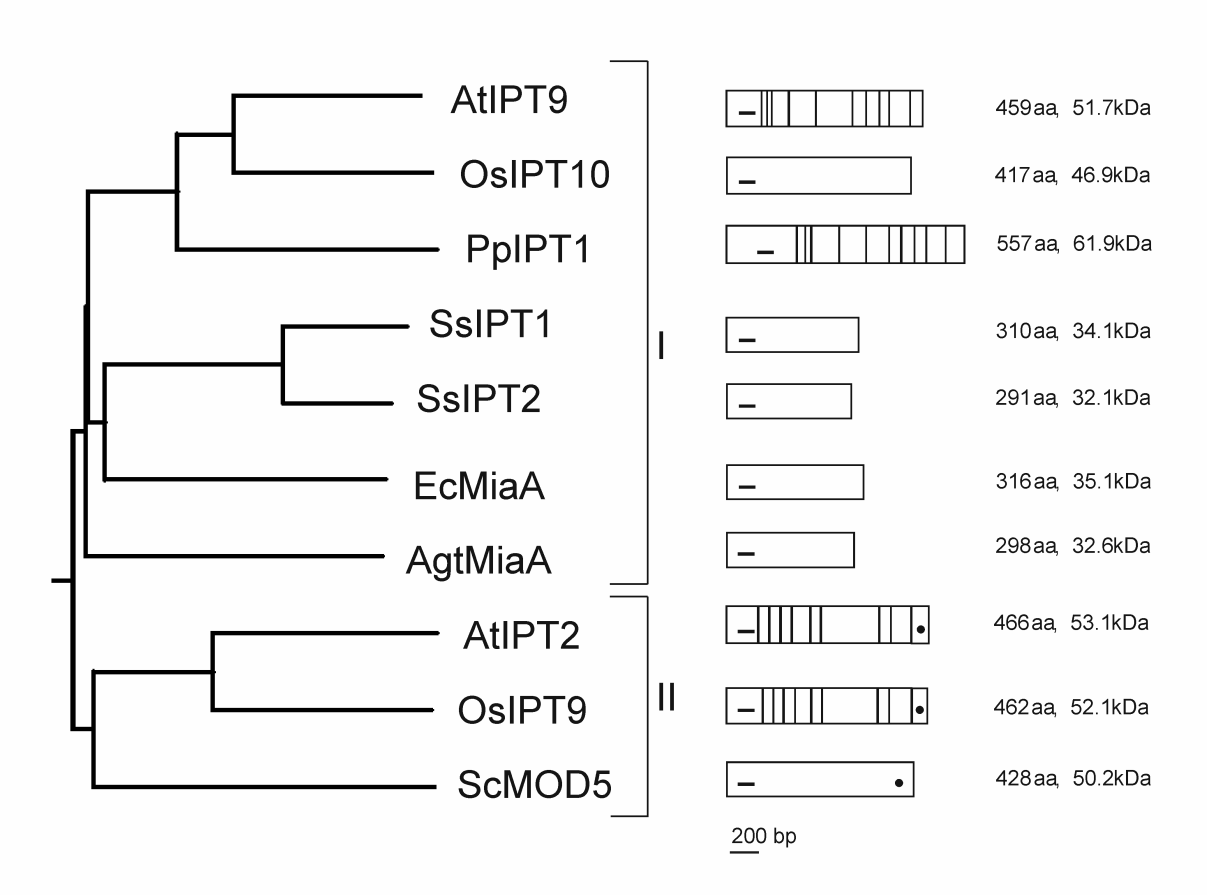

Fig. 16 NJ tree depicting the homologies of 10 pro- and eukaryotic tRNA- isopentenyltransferases on aa level (ClustalW). Two main groups (I and II) are distinguished. Location of encoded conserved motifs as well as of introns are indicated in boxes symbolising coding sequences (see Fig. 11 for gene bank acc. no.). Bars in boxes represent location of ATP/GTP binding motif (group I and II); closed circles indicate zinc-finger like motif (group II).

(d) Predicted chloroplast targeting of the prokaryotic-type plant tRNA-IPTs from group I

Employing *in silico* analyses, a strong probability for chloroplast targeting was predicted for AtIPT9, OsIPT10 and PpIPT1 by MultiLoc (Tab. 5).

However, this predicted localisation is restricted to members of group I (see Fig. 16). For PpIPT1 and OsIPT10, besides chloroplast, a possible peroxisomal targeting was estimated. Using computational analysis by ChloroP we were so far unable to determine a

putative cleavage site for a potential chloroplast transit peptide. This could be due to the fact that the mentioned program is not yet sufficiently adapted for bryophyte protein analysis.

Tab. 5 Predicted cellular targeting location of eukariotic IPTs of group I (see Fig. 16). Probability was determined in chloroplasts (chl), mitochondria (mit), nucleus (nuc), cytoplasm (cyt), peroxisoma (per), Golgi app. (gol) by MultiLoc SVMaac analysis (release8/2006) http://www-bs.informatik.uni-tuebingen.de/ Services/MultiLoc.

IPT	chl	mit/chl	nuc/cyt	nuc	per
AtIPT9	0.99	0.93	0.95	0.50	-
OsIPT10	0.53	0.99	0.88	0.62	0.64
PpIPT1	0.97	-	0.73	-	0.90

For the members of group II, no chloroplast but preferentially cytosolic, nuclear and mitochondrial localisation can be anticipated according to MultiLoc analyses (Tab. 6). Kasahara et al. (2004) demonstrated by *GFP*- fusion in Arabidopsis, that AtIPT2 is located indeed in the cytosol.

Tab. 6 Predicted cellular targeting location of eukariotic IPTs of group II, for further information see Tab. 5.

IPT	cyt	nuc	mit	nuc/cyt	gol
AtIPT2	0.69	0.97	-	-	0.93
OsIPT9	0.67	0.69	0.76	0.55	-
ScMOD5	0.77	0.66	0.82	-	-

For the single copy yeast gene *MOD5* it was demonstrated that it is responsible for the isopentenylation of both cytosolic and mitochondrial tRNA (Martin and Hopper 1982, Dihanich et al. 1987). Translation initiates at codon 1 as well as at codon 12 of the open reading frame hereby generating two distinct proteins, of which the long isoform is translocated into the mitochondria (Gillmann et al. 1991).

In chloroplasts the presence of tRNA-IPT activity seems essential as the sequencing of the *Physcomitrella* plastid genome has revealed the presence of 31 tRNA genes (Sugiura et al. 2003). Among these, there are 8 encoding for tRNAs recognizing an UNN codon. In order to assure a proper anticodon-codon binding (see Miller et al. 1976) these tRNAs require an N^6-modification at A^{37}. Actually cytokinins were detected in chloroplast tRNA isolated from *Euglena gracilis* and *Spinacea oleracea* (Taller 1994). It seems likely that in *Physcomitrella*

PpIPT1 contributes to plastidic tRNA modification and thus might be an essential factor for correct protein biosynthesis in chloroplasts. With respect to the results of MultiLoc analysis, it seems possible that PpIPT1 is also responsible for the prenylation of tRNAs in cytoplasm and nucleus.

Intron-exon architecture of intron- carrying eukaryotic tRNA-IPT genes differs between groups I and II

By analysis of gene architecture of eukaryotic tRNA-IPT genes, *OsIPT10*, a member of the group I, was found to lack introns. However, *PpIPT1* and *AtIPT9* have typical eukaryotic intron-exon architecture. Both genes have 10 introns. Whereas, the intron-carrying members of the group II have only 9 introns (Fig. 16). This points out that the intron-exon architecture among the intron-carrying *tRNA-IPTs* of each group seems to be partly conserved.

4.2 Molecular and biochemical characterisation of cytokinin biosynthesis in *Physcomitrella* wild type and *ove*ST25 mutant

4.2.1 Expression levels of tRNA-*IPT genes*

In order to clarify the role of the gene *PpIPT1* in cytokinin biosynthesis, its expression was studied in the temperature-sensitive mutant *ove*ST25. Temperature increase from 15° to 25°C and subsequent LC-MS- based cytokinin quantification confirmed the cytokinin overproduction in this *ove* mutant (Tab. 2, Tab. 3, Fig. 13). By semi-quantitative RT-PCR (3.2.4) it was found that this overproduction of cytokinins was correlated with an increased expression of *PpIPT1*. The measured difference of 5 Ct units between *ove*ST25 cultured at 15°C and 25°C indicated an extremely high transcriptional level of *PpIPT1* under inducing conditions.

Interestingly, *PpIPT1* expression in the wild type-derived cytokinin oxidase/dehydrogenase overexpressing transformant tCKX7 with enhanced cytokinin degradation was on same high level as in *ove*ST25 cultured at 25°C. It can therefore be presumed that also a CKX-mediated cytokinin deficiency can lead to an enhanced expression of genes involved in tRNA-mediated cytokinin biosynthesis. In this view the upregulation of *PpIPT1* expression in tCKX7 is regarded as a counter reaction to cytokinin deficiency as a part of cytokinin homeostatic regulation.

In *Arabidopsis* the tRNA- dependent cytokinin biosynthetic pathway was also shown to be upregulated in triple and quadruple mutants for the adenylate IPTs AtIPT1, 3, 5, and 7. In

such mutants with extreme low amounts of tZ-type cytokinins the amount of cZ- cytokinins was shown to increase significantly (Miyawaki et al. 2006).

Concerning the second gene *PpIPT2* (4.1.1) its expression was, in contrast to *PpIPT1*, found to be very weak in *Physcomitrella* wild type, and so far it was not possible to reconstitute its full length sequence when using a phagemid cDNA library of *Physcomitrella* wild type. However, in *ove* mutants RT-PCR experiments revealed *PpIPT2* to be expressed at measurable levels (data not shown).

Taken together, RT-PCR results indicate that the tRNA-IPT gene *PpIPT1* (and presumably also *PpIPT2)* might play a role in cytokinin overproduction in the mutant *oveST25.*

4.2.2 Cytokinin content in tRNA- and whole culture- extracts of *Physcomitrella* wild type and *ove* mutant

Resemblance in the distribution profiles of tRNA- and whole culture- cytokinins

The functional characterisation and expression studies of the tRNA-IPT gene *PpIPT1* pointed out the possible implication of tRNA for the biosynthesis of cytokinin in *Physcomitrella*. If tRNA is a source for free cytokinins, similarities in the distribution profile of the various cytokinin species should become evident when comparing cytokinin contents in tRNA with those of whole culture extracts.

To realise this approach, the moss tRNA was purified, hydrolised and dephosphorylated. The cytokinin nucleotides were determined as their corresponding ribosides by HPLC and by UPLC-MS/MS (3.2.3).

Analysis of hydrolysates of tRNA from *Physcomitrella* wild type and *ove*ST25 mutant revealed the cytokinins cZRMP, iPRMP and tZRMP, presented in order of decreasing amounts (Fig. 14, Tab. 4). While both, cZRMP and iPRMP were abundant, the *trans*-isomer of zeatin tZRMP was only found in traces.

The amount of cytokinins in the tRNA-pool of *Physcomitrella* is generally low: ~4pmol of cytokinins were detected per 400 pmol of tRNA (assuming that 1µg of tRNA equals about 400 pmol). The low content of cytokinins is probably mainly due to the fact, that only tRNAs recognising UNN codons contain cytokinins (Edmonds et al. 1991).

To determine the cytokinin composition in whole cultures of wild type and the *ove*ST25 mutant, a HPLC-MS method was applied (3.2.2). It was important to analyse and consider

cytokinins from both, tissue *and* culture medium, as mosses release significant amounts of hormones into the extracellular space during liquid culture.

In the tissue, from 27 cytokinins analysed, eleven different forms belonging to the cZ-, tZ-, DHZ- and iP- types were detected (Tab. 2). In the culture medium, the same cytokinins as those found in tissue occured, with the exception of DHZ- type forms, which were entirely lacking (Tab. 3).

When calculating the total cytokinin production of a whole culture (cytokinins in tissue plus those in medium) the dominant cytokinins were found to be of cZ- type group, represented by cZROG, cZPMP, cZOG and cZ (Tab. 2, Tab. 3).

The relative distribution of cytokinins determined for the tRNA- and whole culture- pool of *Physcomitrella* wild type and *ove*ST25 is summarised in Fig. 17. In both pools, cZ-type cytokinins were found to be major cytokinin constituents (in wild type: 99% in whole culture-pool and ca. 92% in the tRNA- pool; in *ove*ST25: 97% and 81%, respectively). tZ-type cytoknins were only found in traces in both pools. If free tZ-cytokinins are not formed by the activity of adenylate-IPTs in *Physcomitrella* (see Miyawaki et al. 2006), the traces of the *trans*-isomer could be also the result of a *cis-trans* isomerisation (Bassil et al. 1993).

With respect to the comparison presented in Fig. 17, it should be taken into account, that the cytokinin distribution in the whole culture pool is influenced by various enzymatic activities and metabolic changes multiplying the number of cytokinin forms and modulating their concentrations.

Despite this limitation in the comparative approach, the resemblance in the distribution profiles of tRNA- cytokinins and whole culture- cytokinins from *Physcomitrella* are in favour of the hypothesis that cytokinins might originate from the tRNA in *Physcomitrella*.

Homologous changes in cytokinin distributions of oveST25 tRNA as well as whole culture relatively to wild type

In *ove*ST25 both cytokinin pools, the one from the whole culture as well as the one from the tRNA, contain more iP-cytokinins and concomitantly lower cZ-cytokinins compared to wild type (s. Fig. 17).

When comparing the relative amount of cytokinins in tRNA of *ove*ST25 and wild type differences were obvious: iPRMP represented 18.8% of total cytokinin content in tRNA of *ove*ST25 compared to 8% of wild type; the relative amount of cZRMP was 81% and 91.6 %, respectively.

In the whole culture- pool the relative proportion of iP-cytokinins was found to be 2.3% in *ove*ST25 compared to 0.8% in wild type, while concentrations of cZ-type compounds were 97.2% and 99%, respectively.

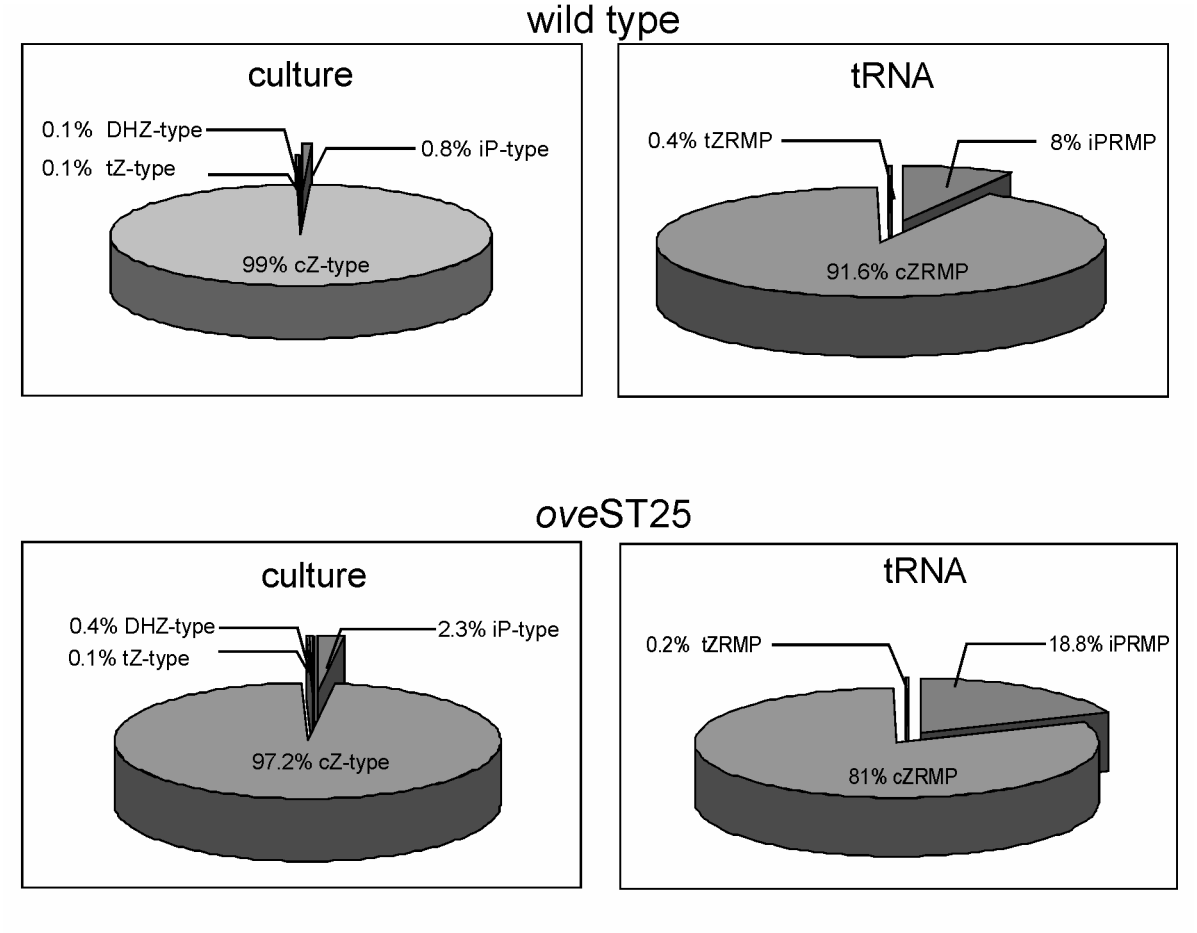

Fig. 17 Cytokinin distribution in whole culture (sum of intracellular and extracellular cytokinins) and tRNA of wild type and *ove*ST25. Both strains were cultured at 25°C. Data are summarised from Tab. 2, Tab. 3 (whole culture) and Tab. 4 (tRNA).

When expressing the HPLC-MS results as amounts of cytokinins produced by a whole culture of *Physcomitrella* the wild type revealed the following relative order of cytokinin groups: cZ (99%) > iP (0.8%) > DHZ (0.1%) = tZ (0.1%) (s. Fig. 17). A similar order can be established for the cytokinin constituents of tRNA: cZRMP (91.6%) > iPRMP (8%) > tZRMP (0.4%). DHZ was not detected in the tRNA-pool.

In summary, not only the similarities in cytokinin distribution of tRNA and whole culture cytokinins point towards a tRNA involvement in cytokinin biosynthesis. Under thermoinduction the cytokinin distributions in *ove*ST25 tRNA as well as in whole culture show similar changes relatively to wild type. These homologous changes, which were measured in both cytokinin pools for the two genotypes, add evidence for the tRNA- involvement in cytokinin biosynthesis in *Physcomitrella*.

4.2.3 Proposed possibilities of cZR synthesis in tRNA

cZ-type cytokinins were found to be dominant in tRNA of both *Physcomitrella* genotypes (91% of total cytokinin amount in tRNA of wild type and 81% in tRNA of *ove*ST25, s. Fig. 17). This finding raises the question how this cytokinin type is formed in the tRNA molecule.

In case of the indirect cZR^{37} biosynthesis employing DMAPP as a side chain donor for isopentenylation of the adenine in position 37 (A^{37}), iPA^{37} is the first biosynthetic product (Fig. 18). The formation of iPA^{37} is catalysed by *MiaA* in *E.coli* and *A. tumefaciens* (Rosenbaum and Gefter 1972, Gray et al.1992) and by *ScMOD5* in *S. cereviciae* (Golovko et a. 2002). Also for the *AtIPT9* gene product in *Arabidopsis* the catalysis of DMAPP-modification of tRNA was hypothesised on basis of studies of *AtIPT9* knockout mutants (Miyawaki et al. 2006).

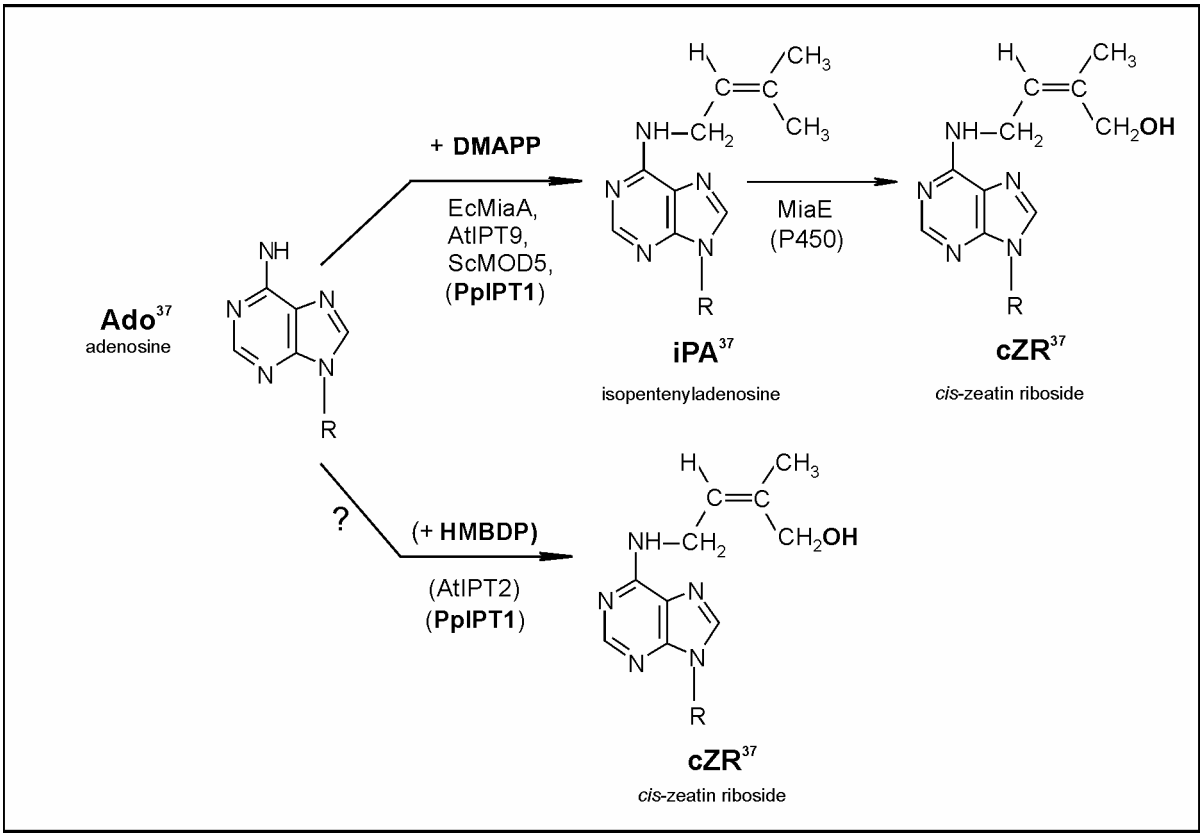

Fig. 18 Proposed possibilities of the synthesis of tRNA- bound cZR. Above: indirect pathway with a hypothesised hydroxylation of iPA^{37}. Below: direct formation of cZR^{37} using an hydroxylated side chain donor in isopentenylation step (see text from 4.2.3).

In order to generate cZR^{37} a subsequent hydroxylation on the level of tRNA has to be postulated. For free Z-type cytokinins Takei et al. (2002) showed that cytochrome P450 monooxygenases catalyse the hydroxylation of iPA-nucleotides in *Arabidopsis.* So far, there is no information published on the hydroxylation of iPA bound to tRNA in plants. In the

bacterium *Salmonella typhimurium* the hydroxylase MiaE was demonstrated to be involved in synthesis of 2-methylthio-*cis*-zeatin in tRNA (Persson et al. 1993).

tRNA- bound cZ-type cytokinins (cZR^{37}) might also be directly synthesised when employing already a *cis*- hydroxylated side chain donor (HMBDP) for isopentenylation of tRNA (s. Fig. 18); this was hypothesised for the *AtIPT2* gene product of *Arabidopsis* (Miyawaki et al. 2006) .

The question, whether tRNA- bound cZ is directly or indirectly synthesised in *Physcomitrella*, is subject of further investigations and can be addressed by enzymological studies of substrate specificity of PpIPT1 (and possibly PpIPT2) with respect to DMAPP or HMBDP.

4.3 Conclusion

From this work there are several arguments, which all suggest, that in the moss *Physcomitrella,* as an ancient land plant, the cytokinins are of tRNA origin:

(1) tRNA-*IPT*s were found to be the only *IPT*s in the *Physcomitrella* genome, and *PpIPT1* was shown to functionally complement the devective tRNA-IPT gene *ScMOD5* of *S. cerevisiae*. No adenylate- *IPT*s were found in the *Physcomitrella* genome;

(2) Transcription of *Physcomitrella* tRNA-*IPT PpIPT1* is strongly increased in temperature-sensitive cytokinin overproducing mutant *ove*ST25;

(3) cZ- type cytokinins, as reaction products of tRNA-IPT, dominate in *Physcomitrella* and accumulate in the mutant *ove*ST25;

(4) tZ- type cytokinins, as adenylate-IPT reaction products in seed plants, occur in *Physcomitrella* only as traces*;*

(5) The profile of cytokinins found in *Physcomitrella* tRNA resembles to the cytokinin profile established for whole cultures of *Physcomitrella*;

(6) In *ove*ST25 cytokinin distribution in tRNA as well as in whole culture shows similar changes relatively to wild type.

The occurrence isopentenyl (iPA^{37}) derivatives in tRNA of evolutionary early organisms, such as bacteria and yeast, suggests that the first cytokinins which appeared in living cells were those in tRNA.

Cytokinins were reported to influence the growth of some bacteria and algae (see Skoog and Armstrong 1970) but their action as hormones only became biologically significant with the evolution of multicellular plant organisms. It can be assumed that the first adenylate- IPTs emerged during the evolution of the seed plant line (Sakakibara et al. 2006). A control of hormone concentration on the level of biosynthesis became an essential condition for the regulation of growth and development especially in higher plants.

Possibly, the replacement of iPR in tRNA molecules with cZR and adaptation of site(s) of hormone action in such a way, which preferentially accept the tZ-isomer as product of adenylate- IPTs reaction, removed several limitations and extended the possibilities of utilization of cytokinins as plant hormones (Kaminek 1974).

During the evolution of higher plants the substrate range of cytokinin biosynthetic enzymes was extended including not only tRNA but also free adenylic nucleotides (Kakimoto 2001, 2003; Takei et al. 2001). In seed plants the existence of both adenylate- and the tRNA-IPTs as well as the production of different cytokinin species (tZ, iP and cZ) by these two different routes allows more flexibility. Generally, it can be assumed that the uncoupling

of cytokinin biosynthesis from the tRNA route in seed plants allowed new regulatory possibilities and made control of endogenous hormone level more precise.

The partial elucidation of cytokinin biosynthesis in *Physcomitrella* sheds light on an intermediate step in the evolution of cytokinin physiology, where the role of these substances was extended from a factor enabling precise translation to a role as a phytohormone already governing developmental processes such as budding.

4.4 Perspectives

To clarify the physiological importance of tRNA-dependent cytokinin biosynthesis in *Physcomitrella* the generation of knockout-mutants of the tRNA-IPT gene *PpIPT1* is of first priority. This plant provides major advantages for such experiments due to the occurrence of highly efficient homological recombination.

Also establishment of the full length sequence of the second identified tRNA-*IPT*-candidate, *PpIPT2,* and its knockout-mutation in *Physcomitrella ove* mutants (where its expression was shown) would provide more information about the regulation of endogenous cytokinins in *Physcomitrella*.

Future work is also aimed at determining the mechanisms of hydroxylation occuring in tRNA to clarify the biosynthesis of cZ- (and tZ-type) cytokinins in tRNA-dependent pathway. Whether DMAPP or HMBDP is (are) used as a side chain can be identified via demonstration of substrate specificity for PpIPT1 (as well as for PpIPT2).

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List of Publications

during the Ph.D.- study (2003-2007)

Articles:

1. Natalya A. Yevdakova, Klaus von Schwartzenberg (2007) **CHARACTERIISATION OF A PROKARYOTE-TYPE tRNA-ISOPENTENYLTRANSFERASE GENE FROM THE MOSS *PYSCOMITRELLA PATENS***. Planta 226: 683-695.

2. Natalya A. Yevdakova , Václav Motyka, Jiri Malbek, Ondrej Novák, Miroslav Strnad, Klaus von Schwartzenberg (2007) **EVIDENCES FOR IMPORTANCE OF tRNA-DEPENDENT CYTOKININ BIOSYNTHETIC PATHWAY IN MOSS *PYSCOMITRELLA PATENS***. (manuscript submitted)

Published abstracts for posters on conferences:

1. Fernandez M., N. Yevdakova, P.Schulz, V. Motyka, , K. v. Schwartzenberg (2003) Poster: **LOW CYTOKININ TRANSFORMANTS OF *PHYSCOMITRELLA* VIA CYTOKININ OXIDASE OVEREXPRESSION**. Moss 2003. The Annual International Conference for Moss Experimental research. Sept. 7th – 10th. St. Louis, USA. P. 9.

2. Yevdakova N., K. v. Schwartzenberg. (2004) Poster: **IDENTIFICATION OF PUTATIVE CYTOKININ BIOSYNTHETIC GENES FROM THE MOSS *PHYSCOMITRELLA.*** Botanikertagung Sept. 5th.-10th. Braunschweig, Germany. P.25.

3. Yevdakova N., K. v. Schwartzenberg (2004) Poster: **STUDIES OF CYTOKININ BIOSYNTHETIC GENES FROM *PHYSCOMITRELLA***. Moss 2004. The Annual International Conference for Moss Experimental research. Sept. 12th – 15th. Freiburg, Germany. P. 38.

4. Schwartzenberg K. v., M. Fernandez, H. Blaschke, N. Yevdakova, V. Motyka (2005) Poster: **EFFECTS OF ALTERED CYTOKININ BIOSYNTHESIS AND METABOLISM IN *PHYSCOMITRELLA***. Moss 2005. The Annual International Conference for Moss Experimental research. July 23rd – 26th. Brno, Czech Republic. P. 47.

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