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Evidence for Importance of tRNA-Dependent Cytokinin Biosynthetic Pathway in the Moss *Physcomitrella patens*

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Abstract To study cytokinin biosynthesis, we characterized a temperature-sensitive cytokinin-overproducing mutant, *ove*ST25, of the moss *Physcomitrella patens* with respect to changes in cytokinin content during thermal induction in comparison to wild type. Our findings, based on combined liquid chromatography-mass spectrometry (LC-MS) analyses, show that thermoinduction caused a strong increase of extracellular N^6-(Δ^2-isopentenyl)adenine (iP), N^6-(Δ^2-isopentenyl)adenosine (iPR), *cis*-zeatin (cZ), *cis*-zeatin riboside (cZR) and its O-glucoside cZROG in *ove*ST25. In contrast, no significant changes were measured in the wild type. To investigate the relevance of tRNA for cytokinin production in *Physcomitrella*, we determined cytokinins in tissue and culture medium as well as in tRNA hydrolysates. The analysis of cytokinins from whole-culture extracts of wild type revealed 56% of iP-type, 32% of cZ-type, and 11% of *trans*-zeatin (tZ)-type forms. In tRNA, 90% of cytokinins were represented by cZ-type and 8% by iP-type forms; tZ-type cytokinins were found only in trace amounts. The finding that the major free cytokinins are, albeit with altered proportions, also major forms in tRNA is compatible with the hypothesis of a strong tRNA-mediated biogenesis of cytokinins in this plant. Our RT-PCR-based studies on the expression of the tRNA-IPT gene, *PpIPT1*, revealed enhanced transcription levels in the cytokinin-overproducing *ove*ST25 mutant at the inducing temperature of 25°C, but not at noninducing conditions (15°C). A wild-type transgenic line with cytokinin deficiency due to heterologous cytokinin oxidase/dehydrogenase overexpression (*AtCKX2*) also exhibited enhanced *PpIPT1* expression levels, indicating that cytokinin deficiency might upregulate tRNA-mediated cytokinin biosynthesis. The evidence that the tRNA-mediated pathway might be mainly responsible for biosynthesis of isoprenoid cytokinins in *Physcomitrella* is strongly supported by the recent release of the *Physcomitrella* genomic sequence where only tRNA-IPTs but no adenylate-IPTs are present.

Keywords *Physcomitrella* · *ove* mutant · Cytokinin biosynthesis · tRNA-isopentenyl transferase · tRNA-bound cytokinins

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Introduction

The moss *Physcomitrella patens* (Funariales, Bryophyta) represents a plant lineage that is separated from seed plants by more than 400 million years. Mosses are especially useful in studies on hormonal action and homeostasis because of their simple developmental differentiation and sensitivity to growth substances (for reviews see Cove 2005; Decker and others 2006; von Schwartzenberg 2006).

Cytokinins affect the development of mosses at a single-cell level as protonema cells can be induced to switch from filamentous growth to three-dimensional growth resulting in the formation of buds and gametophores (Brandes and Kende 1968).

Most naturally occurring 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, including plants, whereby plants also possess significant amounts of free cytokinins. The most abundant class of cytokinins is that of the isoprenoid type: for example, N^6-(Δ^2-isopentenyl)adenine (iP) carries an unmodified dimethylallyl side chain, whereas *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ) carry terminally hydroxylated side chains. Many plant species also contain adenine derivatives carrying an aromatic substitution. 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, whereas the cZ isomer possesses a significantly lower activity (for review see Haberer and Kieber 2002).

Cytokinins exist in three interconvertible forms: free bases, ribosides (in which a ribose is attached to the N^9 of the purine ring), and nucleotides (containing one to three phosphate groups at the 5′ position of the ribose). Free cytokinin bases are readily converted to their respective riboside and nucleotide forms, both of which have less biological activity. Enzymes responsible for cytokinin interconversion are also involved in purine metabolism (Mok and Mok 2001), and cytokinin-specific phosphoribohydrolases that convert nucleotides directly to bases have recently been described (Kurakawa and others 2007).

Via glucosylation, cytokinins can be converted into inactive storage forms, *N*-glucosides and *O*-glucosides (the latter only for Z- and DHZ-type cytokinins). In general, a plant contains numerous species of cytokinin molecules modified in different ways, and the distribution of the various cytokinins may differ significantly between plant species. Recently, for *Physcomitrella* a total of 20 different isoprenoid and aromatic cytokinins have been described, and by creation of cytokinin-deficient transgenics it was revealed that extracellular iP and N^6-(Δ^2-isopentenyl)adenosine (iPR) are responsible for the morphogenetic process of budding (von Schwartzenberg and others 2007).

Physcomitrella provides unique *ove* mutants, which were shown to have drastically increased cytokinin amounts exceeding that of the wild-type plants up to 100-fold (Wang and others 1981; von Schwartzenberg 2006). In seed plants, mutants with such extreme cytokinin overproduction are unknown. For example, the cytokinin level in the *Arabidopsis* mutant *amp1* is only about fivefold higher than that of the wild type (Chaudhury and others 1993). Cytokinin-overproducing mutants of *Physcomitrella* were first selected by Ashton and others (1979) on the basis of bud-overproducing phenotypes.

Some cytokinin-overproducing mutants of *Physcomitrella* show a phenotypically visible temperature sensitivity: at 15°C these mutants produce wild-type-like gametophores, whereas abnormal bud formation occurs at 25°C, resulting in callus-like structures. A temperature-sensitive bud-overproducing phenotype was first described by Futers and others (1986) for the mutant *ove*409. Most *ove* mutations, including *ove*409, were shown to be recessive and are divided into three complementation groups (Featherstone and others 1990). Futers and others (1986) characterized *ove*409 in comparison with a mutant *ove*A78 (both belong to the same complementation group) and wild type. The authors summarized that the *ove* allele need not necessarily code for a temperature-sensitive gene product. Already at 15°C both *ove* mutants produced more cytokinin than the wild type. Due to a general effect of temperature on cytokinin production, all three strains showed higher cytokinin levels at 25°C than at 15°C. Cytokinin levels in wild type were low so that normal development continued at both temperatures, whereas those in *ove*A78 were high enough at both temperatures to cause increased production and abnormal development of the plant. In the case of *ove*409, the cytokinin production was high enough only at 25°C to cause abnormal bud formation. These studies showed that changes in the mutants' phenotype correlate with the higher production of iP. Schulz and others (2001) studied another temperature-sensitive mutant, *ove*ST25, and also demonstrated a strong increase of iP and iPR concentrations in the culture medium. These characteristics make temperature-sensitive *ove* mutants particularly useful for studies of cytokinin biosynthesis and homeostasis.

Schulz and others (2001) addressed the question of the metabolic mechanisms underlying the *ove* phenotype and carried out in vivo labeling with tritiated (^{3}H)-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, indicating that cytokinin overproduction is not caused by a deficiency in cytokinin breakdown. Thus, as *ove* mutants have been described as loss-of-function mutants (Featherstone and others 1990), it is assumed that the *ove* mutation concerns negative regulators of early cytokinin biosynthetic steps.

For cytokinin biosynthesis two different pathways have been described: (1) the direct *de novo* biosynthesis of free cytokinins, where adenylic nucleotides are prenylated to cytokinin nucleotides by adenylate isopentenyl transferases (IPTs) (EC 2.5.1.27); and (2) the liberation of cytokinins from tRNA, which contains cytokinins as hypermodified nucleotides. In tRNAs recognizing UNN codons, cytokinin

nucleotides are localized 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. For example, in *E. coli*, modification of tRNA-nucleotide A_{37} was shown to affect the codon-anticodon affinity (Konevega and others 2006). The posttranscriptional prenylation of the N^6 at A_{37} in the UNN recognizing tRNAs is catalyzed by tRNA-IPTs (EC 2.5.1.8). The N^6-dimethylallyl side chain is known to be derived from the mevalonate pathway (MVA) or methylerythritol phosphate pathway (MEP). Depending on whether DMADP (dimethylallyl diphosphate) or HMBDP (4-hydroxy-3-methylbut-2-enyl diphosphate) is used as a side chain donor, either iP or Z hypermodification of tRNA occurs (Kasahara and others 2004).

In seed plants both adenylate- and tRNA-IPT-mediated pathways contribute to cytokinin biosynthesis (Maaß and Klämbt 1981; Kakimoto 2001, 2003). The discovery of nine biosynthetic genes in *Arabidopsis thaliana* (Kakimoto 2001, 2003; Takei and others 2001) and the analyses of the corresponding mutants (Miyawaki and others 2004, 2006) clarified the roles of the different groups of biosynthetic genes for the generation of specific cytokinin forms, demonstrating that for *Arabidopsis* seven adenylate-IPT genes are responsible for biosynthesis of the bulk of iP- and tZ-type cytokinins, whereas two tRNA-IPT genes are required for cZ-type cytokinins. The question of substrate specificity of adenylate-IPTs and tRNA-IPTs was addressed by Kakimoto (2001), who showed that bacterial and plant tRNA-IPTs do not catalyze the transfer of radiolabeled DMADP to ATP, ADP, AMP, or adenosine. Because adenylate-IPTs do not catalyze the transfer of DMADP to tRNA (Yevdakova and von Schwartzenberg 2007), there seems to be a distinct and narrow substrate specificity of IPTs.

In microorganisms, tRNA is a common source of free cytokinins (Gray and others 1996; Koenig and others 2002). Mutations in tRNA-IPT have a crucial influence on the translation precision and lead to pleiotropic phenotypes in yeasts and bacteria (for review see Taller 1994). Microbial tRNA-IPT encoding genes have been identified in *E. coli* (Rosenbaum and Gefter 1972) and *A. tumefaciens* (Gray and others 1992) as *MiaA*, and *Saccharomyces cerevisiae* as *MOD5* (Martin and Hopper 1982; Dihanich and others 1987).

Recently, we identified and characterized a tRNA-IPT gene from *Physcomitrella*, *PpIPT1* (Yevdakova and von Schwartzenberg 2007). This gene functionally complements a *mod5-1* mutation in the *Saccharomyces cerevisiae* strain MT-8, and its gene product catalyzes the prenylation of tRNA-containing biologically active cytokinin (iPR).

Important conclusions on cytokinin biosynthesis in moss can be drawn from the *Physcomitrella* genomic resources, which have been expanded considerably by the recent release of the genome assembly (Rensing and others 2008). Screening of the *Physcomitrella* genome and transcriptome databases revealed no homologs of adenylate-IPTs (Yevdakova and von Schwartzenberg 2007). Based on functional analysis and on the searches in the *Physcomitrella* sequences databases, we therefore hypothesize that cytokinin biosynthesis in moss differs from seed plants.

To address this hypothesis, the present work assesses the cytokinin content in *ove*ST25 and wild-type cultures and that of corresponding tRNA fractions. For this purpose, we also monitored the kinetics of cytokinin production in transition from noninducing to inducing conditions during thermal induction. We present our findings of *PpIPT1* expression with respect to tRNA-mediated cytokinin biosynthesis in the cytokinin-overproducing *ove*ST25 mutant and in the transgenic line tCKX7, which exhibits enhanced cytokinin degradation (von Schwartzenberg and others 2007). Results from these approaches as well as from measurements of cytokinins in tRNA demonstrated, along with the absence of adenylate-*IPT* genes in the genomic sequence, the importance of the tRNA-dependent pathway for cytokinin biosynthesis in *Physcomitrella*.

Materials and Methods

Plant Material and Culturing Conditions

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

The cytokinin-overproducing mutant *ove*ST25 was obtained by UV mutagenesis of the thiamine auxotrophic wild-type *thiA1*d strain and provided by E. Russo and A. Hofmann (MPI für Molekulare Genetik, Berlin, Germany). At 15°C of cultivation *ove*ST25 produces normal leafy shoots analogous to 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 and others 2001; von Schwartzenberg 2006).

The transgenic *Physcomitrella* strain tCKX7, which exhibits enhanced cytokinin degradation, was derived 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 (von Schwartzenberg and others 2007).

All strains were grown in liquid culture as described by Schulz and others (2001) at 15°C or 25°C as stated in the Results section.

Preparation of Tissue and Culture Medium Samples for Cytokinin Measurements

Samples of tissue and culture medium from *Physcomitrella* cultures were separated by nylon mesh filtration. Tissue samples were dried by vacuum filtration, weighed, and frozen in liquid nitrogen. Samples of culture medium were prepurified by solid phase extraction (Sep-Pak®, C18 cartridge, Waters, Milford, MA, USA). After loading, the C18 cartridges were rinsed with water and eluted with 3 ml of 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.

Cytokinin Preparation from Tissue and Culture Medium Samples

Cytokinins from tissue and culture medium samples were extracted and purified according to Dobrev and Kaminek (2002). Deeply frozen plant material (equiv. 0.4–1.2 g FW and 50–200 ml for tissue and media, respectively) was homogenized 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 the endogenous iP-type cytokinins, 50 pmol of each [$^2H_6$]iP and [$^2H_6$]iPR (products of Apex Organics, Honigton, UK) were added.

The extracts were purified using Sep-Pak Plus tC18 cartridges (Waters) and Oasis® MCX 6-cc mixed-mode (cation exchange and reverse phase) columns (150 mg, Waters). After washing with 5 ml of 1 M formic acid and 5 ml of methanol, cytokinin nucleotides were eluted with 5 ml of 0.35 M NH_4OH and cytokinin bases, ribosides, and glucosides were eluted with 5 ml of 0.35 M NH_4OH in 60% (v/v) methanol. The separated cytokinin nucleotides were dephosphorylated by incubation with calf-intestine alkaline phosphatase (CIAP, Sigma, St. Louis, MO, USA) and determined as corresponding nucleosides.

LC-MS Determination of Cytokinins from Tissue and Culture Medium

LC-MS analysis was performed as described by Lexa and others (2003) using a Rheos 2000 HPLC quaternary gradient pump (Flux Instruments, Switzerland) and HTS PAL autosampler (CTC Analytics, Switzerland) coupled to a Finnigan LCQ™ ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray interface. 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 that were used as internal standards. The detection limit was calculated for each compound as 3.3 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve. Experiments were done in triplicates and each sample was injected at least twice.

Extraction and Purification of tRNA

First, tRNA was selectively extracted from 10 g of homogenized protonema tissue using a method based on phenol/*m*-cresol treatment (Maaß and Klämbt 1981). Then tRNA was purified by chromatography on DEAE cellulose columns according to Buck and others (1983). Purified tRNA samples were adjusted to an optical density (OD) of 5.0 at 260 nm and analyzed by agarose gel electrophoresis (TAE) for purity, that is, absence of high-molecular-weight RNA contamination. Samples were stored at −80°C before further treatments.

Cytokinin Preparation from tRNA

For alkaline hydrolysis, aliquots of purified tRNA (5 units OD_{260}) were incubated in 0.5 M KOH (37°C) for 14 h followed by neutralization with $HClO_4$ (Maaß and Klämbt 1981, modified). The hydrolysates were separated by centrifugation from the $KClO_4^-$ pellet and treated with 15 units CIAP, (Fermentas, Glen Burnie, MD, USA) for 14 h at 37°C. After precipitation of CIAP protein in 80% ethanol (final concentration) and centrifugation (15,000*g*, 10 min), the riboside-containing supernatant was dried by rotary film evaporation. The residue was dissolved in 10% methanol.

UPLC-MS/MS Determination of tRNA-Derived Cytokinins

Samples of dephosphorylated tRNA hydrolysates were prepared as described above. The procedure used for cytokinin purification was a modification of the method described by Faiss and others (1997). In brief, deuterium-labeled internal standards for cytokinins (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, [$^{13}C_5$]cZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_5$]tZOG, [$^2H_5$]tZROG, [$^2H_5$]tZRMP, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_7$]DHZOG, [$^2H_7$]DHZROG, [$^2H_3$]DHZRMP, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_6$]iPRMP, [$^2H_7$]BA, [$^2H_7$]BAR, [$^2H_7$]BA9G, [$^2H_7$]BARMP, [$^{15}N_4$]mT, and [$^{15}N_4$]oT. Samples were purified using immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against cytokinins (Faiss and others 1997). The metabolic eluates from the IAC columns were dried and dissolved in 20 µl of the mobile phase used for quantitative analysis.

Because of the lower sensitivity of ion-trap LC-MS, the cytokinin tRNA fractions were analyzed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC™; Waters, Milford, MA, USA) linked to a Quattro micro™ API (Waters) triple quadrupole mass spectrometer equipped with an electrospray interface. Purified samples were dissolved in 20 µl of MeOH/H_2O (30/70), and 10 µl of each sample was injected onto a C18 reversed-phase column (Acquity UPLC™ is based on a combination of high-pressure and small-bridged ethylsiloxane/silica hybrid particles; BEH Shield RP18; 1.7 µm; 2.1 × 150 mm; Waters). The column was eluted with a linear gradient of 15 mM 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 (MRM) 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 the collision cell corresponding to the 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 was calculated to obtain 16 scan points per peak during which time the interchannel delay was 0.1 s. In MRM mode, the limit of detection (LOD) for most cytokinins was below 5.0 fmol and the linear range was at least five orders of magnitude.

Analysis of *PpIPT1* Expression

Moss protonema from 10-day-old cultures of wild type, tCKX7, and *ove*ST25 was grown at 25°C. In addition, *ove*ST25 was cultured at 15°C.

Total RNA extracts were prepared using the plant RNA Kit (INNUSCREEN, AnalytikJena Group, Berlin, Germany). From the resulting RNA for each genotype, 4-µg aliquots were treated with RNase-free DNase (Fermentas, St. Leon-Rot, Germany).

Synthesis of first-strand cDNA was preformed in 20-µl reaction volume according to the Fermentas protocol using RevertAid™ M-MuLV reverse transcriptase by random nonamer priming.

The reverse transcription products (5 µl cDNA from each sample) were amplified in real-time PCR with SYBR® Green using JumpStart™ Taq ReadyMix™ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). ReadyMix (12.5 µl) was added to specific primers, cDNA templates, and water for a final reaction volume of 25 µl. For RT-PCR analysis of *PpIPT1* (accession No. EF512463), the following primers were used at an annealing temperature of 55°C for 30 cycles: pr-99 5′-AAG TGGTGAGCGATGTCCTG-3′; pr-98 5′-AGCAACGTTA CATAGAGGTC-3′. The constitutive control primers (Nakamura and others 2005) corresponding to the actin encoding gene *PpACT3* (AY382283) were used at an annealing temperature of 57°C: pr-214 5′-CGGAGAGGA AGTACAGTGTGTGGA-3′; pr-215 5′-ACCAGCCGTTA GAATTGAGCCCAG-3′.

For real-time monitoring of PCR, an iCycler iQ (Bio-Rad Laboratories GmbH, München, Germany) was used. Data were processed using iCycler iQ Optical System Software (version 3.0a).

Results

Temperature-Induced Bud Formation in *ove*ST25

To study morphologic changes in relation to cytokinin overproduction, the temperature-sensitive *ove*ST25 mutant and wild type were first grown at the noninducing temperature of 15°C and then transferred to the inducing temperature of 25°C. The appearance of buds in *ove*ST25 was observed on the fourth day after its transfer to 25°C. Three days later, the formed buds showed malformed callus-like structures, typical for the *ove* phenotype (Figure 1). The wild-type control did not phenotypically respond to the temperature increase.

Cytokinin Content and Kinetics of Temperature-Induced Cytokinin Overproduction

To assess differences and similarities in production of cytokinins by *ove*ST25 and wild type, we studied the changes in cytokinin content of both genotypes during the time course of thermoinduction by means of LC-MS focusing on the prevailing cytokinins. Previously, a broad spectrum of various isoprenoid cytokinins, including iP, cZ, tZ, and DHZ types, was detected in *Physcomitrella* cultures, and extracellular iP and iPR were found to be mainly responsible for bud induction (von Schwartzenberg and others 2007). The absolute contents of iP-type cytokinins in tissue and in culture medium are presented in Tables 1 and 2.

As expected, the temperature increase caused a rise of concentrations for iP-type cytokinins. In the tissue of both wild type and *ove*ST25, the thermoinduction led to mostly moderate increases of iP and iPR contents ranging between

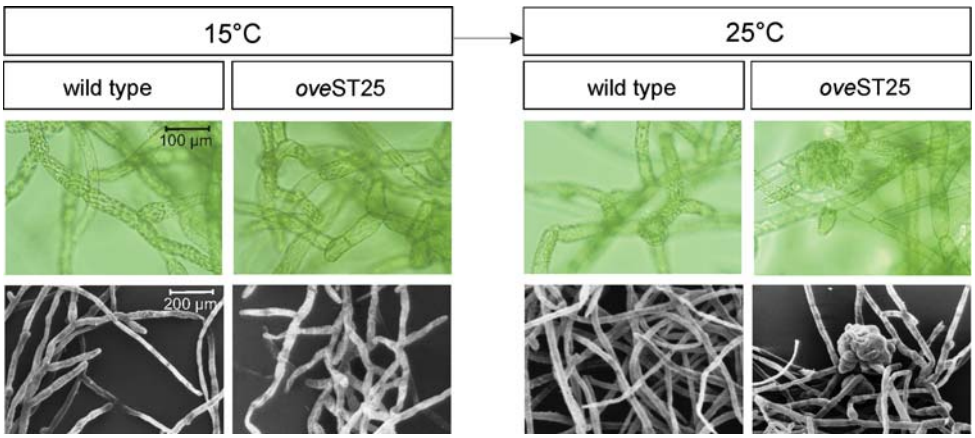

Fig. 1 Light microscopy (*top*) and scanning electron micrographs (*bottom*) demonstrating temperature-induced bud formation causing the *ove* phenotype of *ove*ST25 in comparison with wild type. Strains were cultivated in liquid culture for 4 days at 15°C and then transferred to 25°C for another 6 days

Table 1 iP-type cytokinins in Tissue (pmol/g FW) of Wild-type *Physcomitrella* and *ove*ST25 before and after Thermoinduction

Genotype	Temperature (°C)	iP		IPR		iPRMP	
		C (pmol/g)	$C_{25°C}/C_{15°C}$	C (pmol/g)	$C_{25°C}/C_{15°C}$	C (pmol/g)	$C_{25°C}/C_{15°C}$
Wild type	15	0.45 ± 0.06	3	0.65 ± 0.08	1.1	5.0 ± 0.33	0.7
	25	1.35 ± 0.08		0.75 ± 0.06		3.7 ± 0.64	
*ove*ST25	15	2.60 ± 0.31	1.4	0.13 ± 0.01	1.2	8.8 ± 0.81	1
	25	3.65 ± 0.47		0.16 ± 0.01		9.2 ± 1.27	

C = concentration

Mean values ± standard deviation from four cultures per genotype are presented

Liquid cultures were suspended in fresh medium at day 0 and cultured at 15°C for 4 days; then the cultivation temperature was raised to 25°C. The given data correspond to day 4 (15°C) and day 8 (25°C). Tissue from 50-ml culture samples was analyzed

Table 2 iP-type Cytokinins in Extracellular Fraction (pmol/ml culture medium) of Wild-type *Physcomitrella* and *ove*ST25 before and after Thermoinduction

Genotype	Temperature (°C)	iP		iPR		IPRMP	
		C (pmol/ml)	$C_{25°C}/C_{15°C}$	C (pmol/ml)	$C_{25°C}/C_{15°C}$	C (pmol/ml)	$C_{25°C}/C_{15°C}$
Wild type	15	0.010 ± 0.0025	1.5	0.007 ± 0.0001	0.9	nd	–
	25	0.015 ± 0.0010		0.006 ± 0.0001		nd	
*ove*ST25	15	0.012 ± 0.0013	10.8	0.009 ± 0.0001	1.5	nd	–
	25	0.130 ± 0.0270		0.014 ± 0.0013		nd	

For further information see footnotes to Table 1

nd = not determined

1.1- and 1.4-fold, as indicated by the ratio of concentrations measured at 25°C and at 15°C ($C_{25°C}/C_{15°C}$; Table 1). For iP in the wild type, a threefold increase was measured, although this rise did not initiate bud induction (see Figure 1). The intracellular level of iPRMP, however, remained either unchanged (*ove*ST25) or was even slightly decreased (wild type) during thermoinduction (Table 1). In contrast to the tissue, we observed dramatic changes of the iP concentration in the culture medium, where, during thermoinduction, a 10.8-fold increase was found for *ove*ST25 (Table 2). This rise can be correlated with the phenotypic changes of the mutant, which, in contrast to the wild type, responded with budding at 25°C (Figure 1), thus confirming the important role of extracellular iP in *Physcomitrella*. In the wild-type medium only a 1.5-fold increase of iP and even a slight decrease ($C_{25°C}/C_{15°C} = 0.9$) of iPR were observed.

The time course of extracellular cytokinin concentrations during thermoinduction is depicted in Figure 2. For this analysis no appropriate internal standards were available for the quantification of cZ, cZR, and cZROG. Concentrations for these cytokinin species were determined based on retention times and MS spectra of corresponding *trans*-isomers, and the kinetic data are

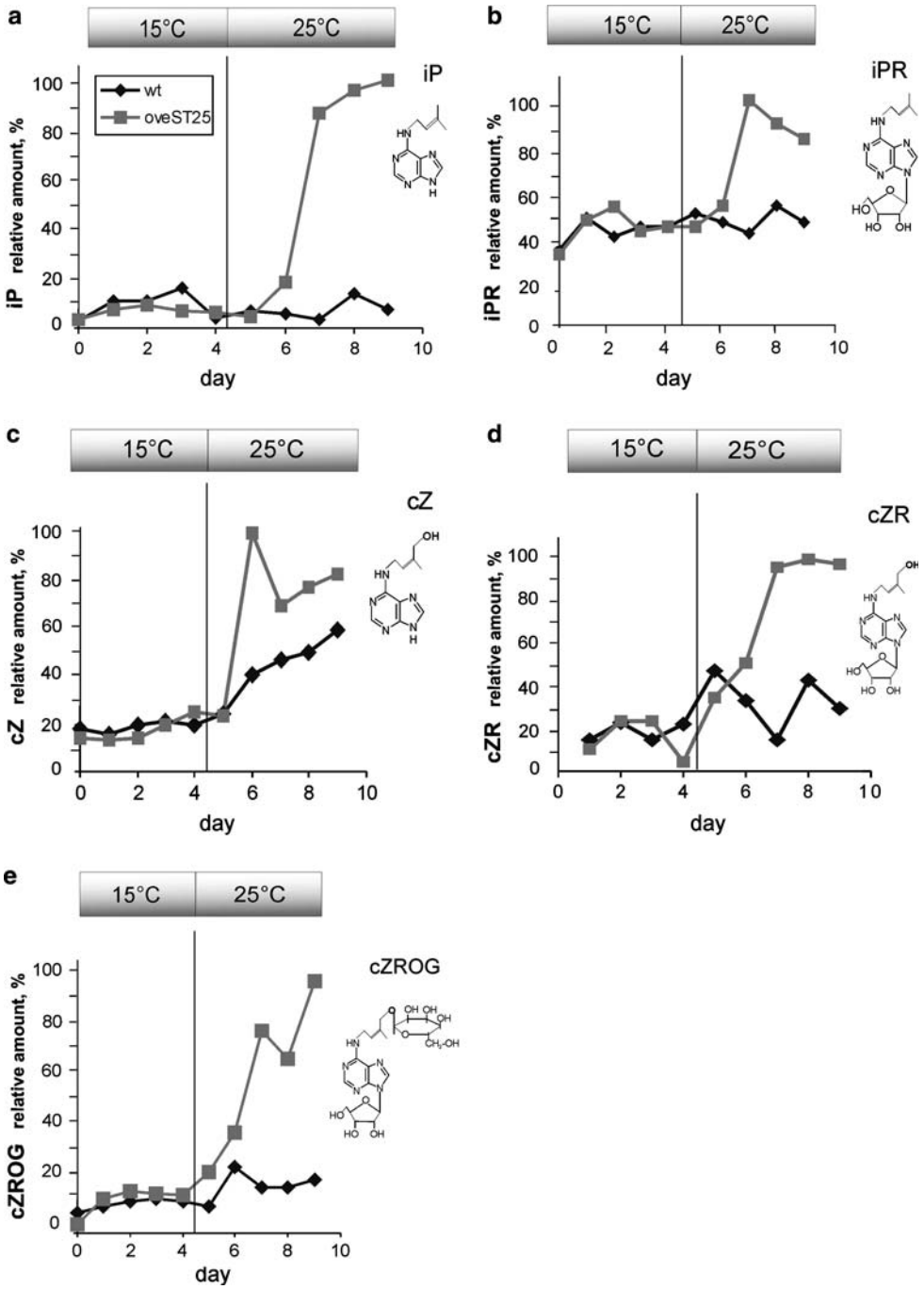

Fig. 2 Thermal dependence of extracellular cytokinin concentrations in the temperature-sensitive mutant *ove*ST25 and in the wild type during thermoinduction (liquid culture). On the fourth day of cultivation the temperature was raised from 15°C to 25°C. The LC-MS analysis revealed that extracellular cytokinins iP and iPR are rapidly affected already 36 h after temperature increase and accumulate in *ove* mutant

therefore presented as relative values (Figure 2). In the *ove*ST25 mutant, five cytokinin species were found to accumulate extracellularly as early as within 36 h after the beginning of the temperature increase. In the wild type, thermoinduction had no significant effect on iP and iPR concentrations (Figure 2a, b) although it caused slight accumulations for cZ forms (Figure 2c–e).

The strongest temperature-induced increase of cytokinin was measured for extracellular iP in *ove*ST25 (Table 2, Figure 2a).

Profiles of Free and tRNA-Bound Cytokinins

Based on the hypothesis that tRNA might be an important source for free cytokinins in *Physcomitrella* (Yevdakova and von Schwartzenberg 2007), we established a profile for free cytokinins as determined in whole-culture extracts and a profile of tRNA-bound cytokinins from *Physcomitrella* wild type. For that purpose, the ribosides obtained from dephosphorylated tRNA hydrolysates (3 A_{260} units ml^{-1}) and cytokinins from tissue and culture media were

Table 3 Cytokinin Content in Whole Culture (tissue plus medium) and in tRNA Hydrolysates

	iP-type	tZ-type	cZ-type	DHZ-type	Aromatic cytokinins
Whole culture	63.70 ± 2.1	12.61 ± 1.03	36.76 ± 2.8	0.24 ± 0.02	0.4 ± 0.01
tRNA	0.75 ± 0.03	0.03 ± 0.002	8.20 ± 0.16	0.13 ± 0	udl

udl = under detection limit

Cytokinin distribution is given in pmol produced per 200 ml of liquid culture. Data from repetitive cultures are presented as mean value ± standard deviation

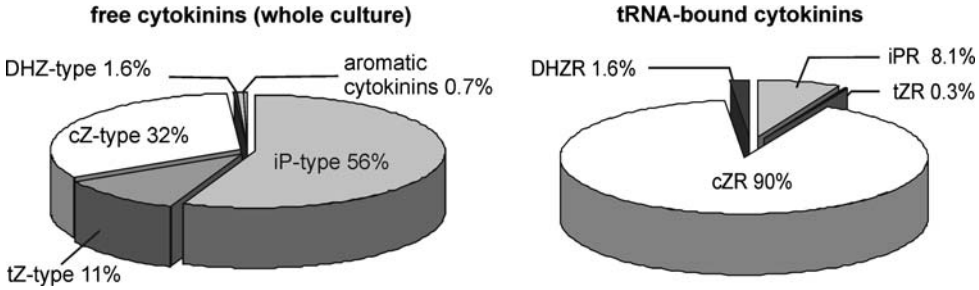

Figure 3 Cytokinin distribution in whole culture (sum of intracellular and extracellular cytokinins) and tRNA of *Physcomitrella* (wild type) based on data from Table 3

analyzed by reverse-phase UPLC-MS/MS. From a total of 40 measurable cytokinins, four main riboside forms, namely, cZR, tZR, DHZR, and iPR, were detected. UPLC-MS/MS quantification revealed that the dominant cytokinins occurring in wild-type tRNA were those of cZ type and iP type (Table 3). Comparison of total tRNA cytokinins to the sum of free cytokinins determined for *Physcomitrella* wild-type tissue and culture medium (data from von Schwartzenberg and others 2007; Table 3, Figure 3 herein) showed that cZ- and iP-type cytokinins represent major forms in both the fraction of free cytokinins (whole culture) and in the tRNA fraction. DHZ and tZ cytokinins were minor forms in both fractions (Figure 3).

Enhanced Transcription of *Physcomitrella* tRNA-IPT Gene in *ove*ST25

To further investigate whether the tRNA-mediated pathway contributes to cytokinin biosynthesis in *Physcomitrella*, an expression analysis of the tRNA-IPT gene *PpIPT1* (Yevdakova and von Schwartzenberg 2007) was carried out for wild type and *ove*ST25. In addition, a wild-type transgenic strain tCKX7 (25°C), which shows cytokinin deficiency due to overexpression of a heterologous cytokinin oxidase/dehydrogenase gene (*AtCKX2*) (von Schwartzenberg and others 2007), was included as a control for this experiment.

For *ove*ST25 cultured at the inducing temperature of 25°C, a low Ct value of 18, compared to 22 in the wild type, suggested a high expression level of *PpIPT1*. Under noninducing conditions the expression of *PpIPT1* in the *ove* mutant was low, as indicated by a high Ct value of 23 (Table 4). When the *PpIPT1* expression levels in all three analyzed genotypes were compared under the same conditions, the *ove*ST25 mutant and cytokinin oxidase/dehydrogenase-overexpressing strain tCKX7 displayed significantly higher expression levels of *PpIPT1* (Ct = 18) compared to the wild type (Ct = 22), indicating a correlation between cytokinin status and tRNA-IPT gene expression.

Table 4 RT-PCR Analysis of *PpIPT1* (EF512463) Expression in Temperature-sensitive *ove*ST25 at 15°C and 25°C Compared to Wild Type and tCKX7, Both Cultivated at 25°C

Temperature of cultivation	Genotype	Real-time RT-PCR Ct value	
		PpIPT1	PpACT3
15°C	*ove*ST25	23	17
25°C	*ove*ST25	18	17
25°C	tCKX7	18	17
25°C	Wild type	22	17

1 µg of RNA template was used in RT reactions. Real-time PCR was performed using pr-99/98, specific to *PpIPT1*, and pr-214/215, specific to the control gene *PpACT3* (AY382283). Four repetitive real-time RT-PCR reactions showed similar results. Threshold cycles (Ct values) of one representative experiment are given. Standard deviation of Ct values did not exceed 0.4

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

Discussion

In the following we discuss and evaluate our findings in detail, which all suggest that in the moss *Physcomitrella* tRNA-dependent cytokinin biosynthesis is of major

importance: (1) no adenylate-IPT-encoding genes are found in the *Physcomitrella* genome; (2) cZ- and iP- type cytokinins dominate in *Physcomitrella* whole culture and in tRNA extracts; (3) transcription of the tRNA-IPT gene *PpIPT1* is strongly increased in the cytokinin-overproducing mutant *ove*ST25.

No Adenylate-IPTs Are Found in the *Physcomitrella* Genome

The release of the draft assembly of the *Physcomitrella* genome has recently been published (Rensing and others 2008). The large coverage of the genomic sequence data comprising 480 Mb is supported by the fact that from a total of 251,086 expressed sequence tags (ESTs) more than 98% yielded hits to the assembly. When we performed extensive searches in the *Physcomitrella* genomic and EST databases (http://www.mossgenome.org/links.php), we found no adenylate-IPTs (Yevdakova and von Schwartzenberg 2007). However, the functionality of *PpIPT1* as tRNA-IPT was unequivocally proved in a yeast complementation assay. We demonstrated that tRNA was isopentenylated by the *PpIPT1* gene product and that tRNA of a complemented yeast strain contained iPR. The absence of adenylate-IPTs suggests that tRNA-IPTs might be essential for cytokinin biosynthesis in *Physcomitrella.* Furthermore, comparative genomic studies, including the genome of *Selaginella*, provide evidence that the tRNA-dependent pathway is the only cytokinin biosynthetic pathway in lower plants and that adenylate-IPTs have evolved later only in the seed plant line (Sakakibara and others 2006).

cZ- and iP-type Cytokinins Dominate in *Physcomitrella* Whole-culture and tRNA Extracts

The profiles we established for free and tRNA-bound cytokinins revealed iP and cZ forms to be major cytokinins in both fractions (summarized in Figure 3). This finding is consistent with our hypothesis that cytokinins in *Physcomitrella* might be of tRNA origin. cZ forms of cytokinins have been shown to be derived exclusively from the tRNA route in *Arabidopsis*, whereas tZ- and iP-type forms are generated by direct *de novo* synthesis using adenylate-IPTs (Kakimoto 2001; Miyawaki and others 2006). In evolutionary early plants, such as algae and the moss *Physcomitrella*, high or even prevailing amounts of cytokinins are of the cZ form (Stirk and others 1999, 2003; von Schwartzenberg and others 2007). For such plants cytokinin biosynthetic pathways differing from those of seed plants can be assumed.

The existing differences in the distribution of cytokinins that we found in the fractions of free and tRNA-bound cytokinins, for example, lower relative amount of free cZ-type forms than the tRNA-bound cZ-forms, are likely to be the result of the activities of cytokinin-metabolizing enzymes acting preferentially on free cytokinins. For example, our enzymatic studies on cytokinin oxidase/dehydrogenase activity from crude extracts of *Physcomitrella* wild-type tissue revealed that cZ is degraded at rates up to 7-fold and 4.5-fold higher than tZ and iP, respectively (von Schwartzenberg and others 2007), which could explain a shift in favor of the free iP (and tZ) forms (Figure 3).

Moreover, cZ and iP cytokinins were found to accumulate in the *ove*ST25 mutant when grown under inductive conditions (Tables 1 and 2; Figure 2). The importance of the extracellular space as a cytokinin-containing compartment has been reported previously for both bryophytes and seed plants (Reutter and others 1998; Schulz and others 2000, 2001; Petrášek and others 2002; Motyka and others 2003; von Schwartzenberg and others 2007). This was corroborated in this study, as the thermoinduction experiment showed that the extracellular iP concentration increased in *ove*ST25 10.8-fold in the medium, but only 1.4-fold in the tissue (Tables 1 and 2). As cytokinin accumulation in the culture medium is already detectable within 36 h after thermoinduction, we assume that iP and cZ derivatives are primary products from the cytokinin biosynthetic route. In *Physcomitrella*, the hydroxylation of iP- to Z-type cytokinins was not detected in in vivo labeling experiments (von Schwartzenberg and others 2003, 2007). As iP and cZ cytokinins accumulate, and both are major constituents of tRNA in *Physcomitrella* (Table 3), we hypothesize that all of these compounds originate from the tRNA biosynthetic route, which seems to be de-regulated (de-repressed) in *ove*ST25.

The dominant amounts of cZR and iPR that we determined in the tRNA of *Physcomitrella* agree well with comparable measurements in seed plants such as *Spinacea oleracea* and *Arabidopsis* (Kaminek 1974; Vreman and others 1978; Miyawaki and others 2006). In general, we found that the amount of cytokinins in the tRNA pool of *Physcomitrella* is low: approximately 4 pmol of cytokinins were detected per 400 pmol of tRNA, assuming that 1 µg of tRNA equals about 400 pmol. The low content of cytokinins is probably mainly because only certain tRNAs, those recognizing UNN codons, actually contain cytokinins (Edmonds and others 1991). Thus, it is concluded that the tRNA-mediated pathway in moss must possess a high capacity (turnover) for the production of free cytokinins. Unfortunately, to date only rough measurements of tRNA turnover in plants have been published (Maaß and Klämbt 1981; Perry and Cove 1987). Specific measurements of the turnover rates of individual UNN codon-recognizing tRNAs, as well as determinations of their degree of hypermodification by isopentenylation, are necessary to

describe the tRNA-mediated cytokinin biosynthesis in detail.

Transcription of tRNA-IPT *PpIPT1* Is Strongly Increased in Cytokinin-overproducing *ove*ST25 Mutant

A temperature increase from 15° to 25°C induces cytokinin overproduction in *ove*ST25 (Tables 1 and 2; Figure 2), and correlates with an increased expression of the gene *PpIPT1* (Table 4), which possesses tRNA-IPT activity (Yevdakova and von Schwartzenberg 2007). The difference of 5 Ct units between *ove*ST25 cultured at 15°C and at 25°C (Table 4) indicates an extremely high transcriptional level of *PpIPT1* under inducing conditions. Because *ove* strains have been shown to be loss-of-function mutants (Featherstone and others 1990), it is thought that increased cytokinin production is the result of a deregulated cytokinin biosynthesis (von Schwartzenberg 2006). Presumably, based on our findings here, we believe that a negative regulator of cytokinin biosynthetic gene(s), including *PpIPT1*, is affected, leading to derepression of gene activity.

Interestingly, in the wild-type-derived cytokinin oxidase/dehydrogenase transformant tCKX7, in which cytokinin degradation is increased due to *AtCKX2* being overexpressed, *PpIPT1* expression is at the same high level as that in *ove*ST25 cultured at 25°C. CKX-mediated cytokinin deficiency evidently leads to an upregulation of *PpIPT1*, involved in tRNA-mediated cytokinin biosynthesis. In this view, the upregulation of *PpIPT1* in tCKX7 is regarded as a counterreaction as 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* (Miyawaki and others 2006). In addition, in mutants with extremely low amounts of tZ-type cytokinins, the amount of cZ cytokinins was shown to increase significantly (Miyawaki and others 2006).

In summary, our *PpIPT1* expression data and the data obtained from genome analysis and cytokinin determinations suggest that the tRNA-dependent pathway is of central importance for biosynthesis of isoprenoid cytokinins in *Physcomitrella*. Further studies should reveal details of the regulation of tRNA-dependent cytokinin biosynthesis.

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