Occurrence, Interconversion, and Perception of Topolins in Poplar

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Contents

Abstract

The poplar species *Populus* × *canadensis* cv. Robusta was the first organism found to contain aromatic cytokinins. Screening of the content of aromatic cytokinins in leaves of 12 *Populus* species revealed that the capacity to produce aromatic cytokinins is widespread among *Populus* accessions. The major aromatic metabolites are *ortho*-topolin and *ortho*-topolin riboside. Their levels transiently increase after daybreak and are much higher in older plants. Poplar species contain five genes coding for functional CHASE-containing histidine kinases acting as cytokinin receptors. Poplar genome further contains nine isopentenyl transferase genes coding for enzymes responsible for the biosynthesis of isoprenoid cytokinins, two genes coding for adenosine kinase, two genes of nucleoside *N*-ribohydrolase, and one gene encoding purine nucleoside phosphorylase. These enzymes contribute to interconversion of cytokinin ribosides.

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Trans-Zeatin is the most abundant cytokinin in poplar and displays the highest variation in abundance. It shows the strongest affinity to all five cytokinin receptors and activates the cytokinin signaling via A-type response regulators. Among aromatic cytokinins, *meta*-topolin is efficiently bound to all receptors, while *ortho*-topolin binds only at micromolar concentrations. The origin of topolins in poplar remains unclear, and it is possible that they are not products of poplar metabolism but indeed endophyte-derived products.

Keywords

Activity · Aromatic cytokinin · *Ortho*-topolin · Poplar · Cytokinin receptor

Abbreviations

ADK	Adenosine kinase
BA	N^6-benzyladenine
*c*Z	*cis*-zeatin
HK	Histidine kinase
iP	N^6-isopentenyladenine
iPR	N^6-isopentenyladenosine
*m*T	*meta*-topolin
NRH	Nucleoside N-ribohydrolase
*o*T	*ortho*-topolin
PNP	Purine nucleoside phosphorylase
*t*Z	*trans*-zeatin
*t*ZR	Zeatin riboside

Plant signaling systems that integrate and control cellular responses are, at least partially, based on active chemical substances called phytohormones. Cytokinins represent a group of phytohormones defined as the compounds that in the presence of auxin (another phytohormone) induce cell division in a suitable assay material grown on a defined medium (Shaw 1994). Among other functions, they also regulate morphogenesis and cambial development; modulate the activity of root, shoot, and reproductive meristems; and inhibit leaf senescence. All native cytokinins are derivatives of adenine (free bases, sugar conjugates, or nucleotides) with at least one substituent at the exocyclic N^6 position. Depending on the side chain structure, two main subclasses of cytokinins are recognized – isoprenoid and aromatic. Isoprenoid cytokinins, comprising N^6-isopentenyladenine (iP), *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z) and dihydrozeatin, are ubiquitous in plants, whereas their aromatic counterparts, which consist of N^6-benzyladenine (BA) and *ortho*- and *meta*-topolin (*o*T and *m*T), are very rare. For this reason, much is known about the function, metabolism, and perception of isoprenoid cytokinins, while the origin and fate of aromatic cytokinins remain largely unexplained (Frébort et al. 2011).

Several aromatic cytokinins have been identified in crown gall tumors of tomato (Nandi et al. 1989a, b), calla (Chaves dasNeves and Pais 1980), palm oil (Jones et al. 1996), and red goosefoot (Doležal et al. 2002). However, poplar (*Populus* ×

canadensis cv. Robusta) appears to be the best model plant for studying aromatic cytokinins. Mainly hydroxylated derivatives of BAP, *o*T, and *m*T as well as their sugar conjugates have been identified in extracts of poplar leaves (Horgan et al. 1975; Strnad et al. 1992, 1994, 1997; Tarkowská et al. 2003). Recently, we performed screening for aromatic cytokinins in a collection of poplar genetic resources (Forestry and Game Management Research Institute, Kunovice, Czech Republic). UHPLC-MS/MS analyses of leaf extracts from 12 gene bank accessions revealed no BAP-, *methoxy-o*T-, *m*T-, or *para*-topolin-type cytokinins (Jaworek et al. 2019). On the other hand, *ortho*-topolin riboside (*o*TR) was present in all accessions and especially abundant in *P.* × *canadensis*, *P.* × *berolinensis*, *P. laurifolia*, *P. maximovici*, and *P.* × *oxford*. Surprisingly, apart from *P.* × *canadensis*, *o*T was present in low quantities or not at all in the accessions tested. A concentration of aromatic cytokinins is reported to vary according to the length of exposure to light and the quality of light (Hewett and Wareing 1973). Time-dependent accumulation of aromatic cytokinins was observed in *P.* × *canadensis*, with the highest levels of *o*T (253 pmol g^{-1} FW) and *o*TR (156 pmol g^{-1} FW) occurring 4 h after daybreak. Interestingly, only negligible fluctuations of *t*Z and its riboside (*t*ZR) were observed, with average concentrations of 2.2 pmol g^{-1} FW and 1.2 pmol g^{-1} FW, respectively (Jaworek et al. 2019). Annual and seasonal fluctuations were also observed in *P. tremula* (Edlund et al. 2017).

Three cytokinin moieties were identified in tRNA hydrolysates of *P.* × *canadensis* and *P.* × *deltoides*—N^6-isopentenyladenosine (iPR) (50 pmol g^{-1} FW), *cis*-zeatin riboside (*c*ZR) (19 pmol g^{-1} FW) and *o*TR (4 pmol g^{-1} FW) (Jaworek et al. 2019). While the dynamics of free *o*TR was observed in both *P.* × *canadensis* and *P.* × *deltoides*, there were no significant changes in levels of tRNA-bound *o*TR, either in a the short time (day) or throughout the season. In addition, tissues of mature *P.* × *canadensis* and *P.* × *deltoides* trees contained much higher free aromatic cytokinin levels than those detected in leaves from young suckers. It seems to be likely that hybrid *P.* × *canadensis* inherited the trait to synthesize aromatic cytokinins from *P.* × *deltoides* (Jaworek et al. 2019, 2020).

The presence of *o*TR and *o*T in various poplar cultivars raises the question on their mutual interconversion. Nucleosides can be synthesized or de novo or transported from the vacuole or apoplast into the cytosol after RNA degradation by members of the equilibrative nucleoside transporter (ENT) family exhibiting broad substrate specificity toward purine, pyrimidine, and cytokinin ribosides with affinities in the micromolar range (Wormit et al. 2004; Hirose et al. 2008; Girke et al. 2014). In the cytosol, purine ribosides can be hydrolyzed by calcium-dependent nucleoside N-ribohydrolases (NRHs, E.C. 3.2.2.-) to corresponding nitrogenous bases and ribose (Jung et al. 2009, 2011; Kopečná et al. 2013) or phosphorylated to monophosphates by adenosine kinases (ADK, E.C. 2.7.1.20, Moffatt et al. 2000; Schoor et al. 2011). Purine nucleoside phosphorylase in plants (PNP, E.C. 2.4.2.1, Chen and Petschow 1978; Bromley et al. 2014) preferentially catalyzes a ribosylation reaction of adenine/isoprenoid cytokinin with ribose-1-phosphate to release adenosine/cytokinin riboside and phosphate moiety. Although phosphorolytic reaction can also appear in the presence of phosphate, lower K_m

Table 4.1 A list of genes coding for a nucleosidase (NRH), adenosine kinase (ADK), and purine nucleoside phosphorylase (PNP) in *Populus trichocarpa*

Gene	Phytozome ID number	Citation
NRH		
PtNRH1	Potri.007G144600	–
PtNRH2	Potri.006G083400	–
AtNRH1	At2g36310	Jung et al. (2009)
AtNRH2	At1g05620	Jung et al. (2011)
ZmNRH1a	GRMZM2G029845	Kopečná et al. (2013)
ZmNRH1b	GRMZM2G134149	Kopečná et al. (2013)
ZmNRH2a	GRMZM2G085960	Kopečná et al. (2013)
ZmNRH2b	GRMZM2G015344	Kopečná et al. (2013)
ZmNRH3	GRMZM2G104999	Kopečná et al. (2013)
ADK		
PtADK1	Potri.010G224300	–
PtADK2	Potri.008G038100	–
AtADK1	At3G09820	Moffatt et al. (2000)
AtADK2	At5G03300	Moffatt et al. (2000)
PNP		
PtPNP	Potri.T096300	–
AtPNP	At4g28940	–
StPNP	Sotub08g016060	Bromley et al. (2014)

The table shows phytozome gene ID numbers and genes studied in detail are provided for comparison (*Pt* v *Populus trichocarpa*, *Zm Zea mays*, *At Arabidopsis thaliana*, *St Solanum tuberosum*)

values for bases favor the ribosylation reaction as shown for potato enzyme (Bromley et al. 2014). ADKs found in *Arabidopsis* and tobacco are known to catalyze phosphorylation of adenosine and isoprenoid cytokinins with K_m values in low micromolar range (Moffatt et al. 2000; Kwade et al. 2005). NRHs hydrolyze various ribosides including isoprenoid cytokinin ribosides. NRHs contain calcium ions in the active site which is coordinated by aspartate residues from the conserved N-terminal DXDXXXDD motif. Calcium ions are essential for the ribose moiety binding. Plant NRHs belong to nonspecific inosine-uridine containing NRHs as they are able to act on a wide range of ribosides. K_m values of *Arabidopsis* and maize NRHs for iPR and *t*ZR are in high micromolar range (Jung et al. 2009; Kopečná et al. 2013).

The genome of poplar (https://phytozome.jgi.doe.gov/pz/portal.html) contains at least two *NRH* genes, two *ADK* genes, and one *PNP* gene (Table 4.1). Two most active NRHs from maize, namely, ZmNRH2b and ZmNRH3, hydrolyze not only isoprenoid cytokinin ribosides (Kopečná et al. 2013) but also aromatic cytokinins including *o*TR to *o*T (Fig. 4.1a, b). Both isoforms display specific activities of 0.3 and 0.5 nkat mg^{-1} with iPR. Their activities with *o*TR are in similar range and attain values of 0.5 and 0.4 nkat mg^{-1}, respectively. For this reason, it is very likely that ADK and PNP reactions will also catalyze the conversion of *o*T and *o*TR in addition to isoprenoid cytokinins.

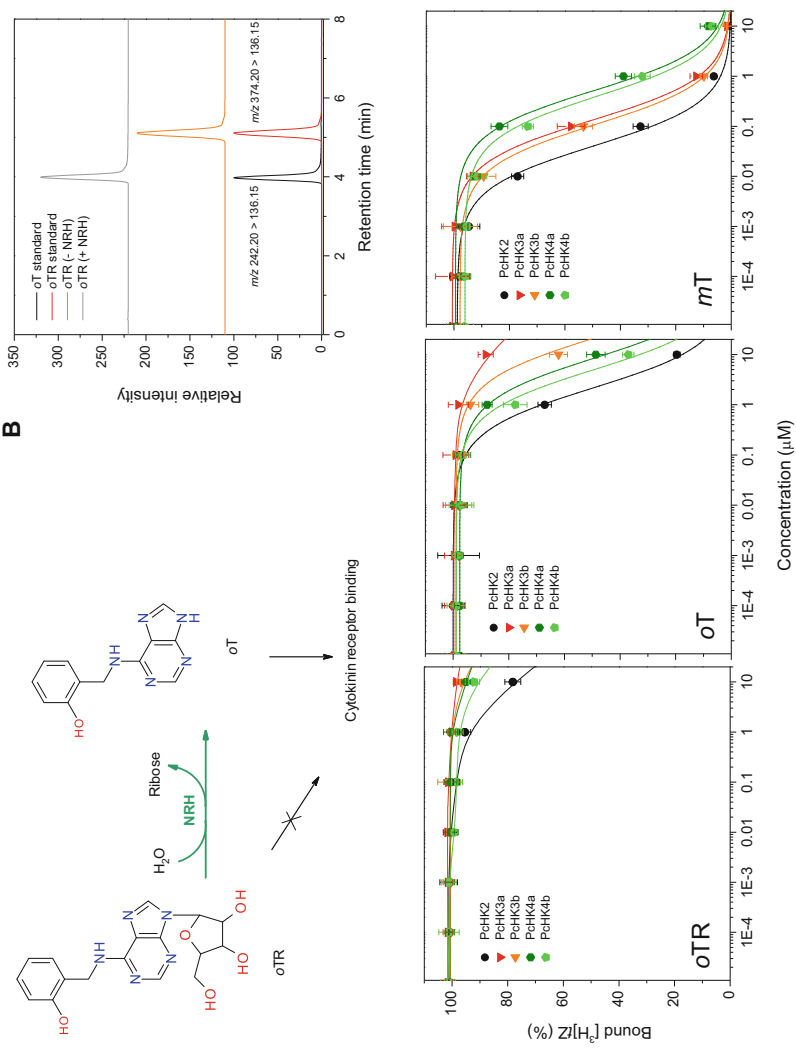

Fig. 4.1 (**a**) A scheme of the catalytic conversion of oTR to oT catalyzed by plant NRHs. (**b**) Confirmation of *o*TR conversion by ZmNRH2b using UHPLC-MS/MS with *m/z* transitions indicated for each peak. (**c**) Competitive binding curves for *o*TR, *o*T, and *m*T versus [^{3}H]-*t*Z using five poplar cytokinin receptors in live cell assay. Binding was studied at pH 7.0 (adapted from Jaworek et al. 2020)

The poplar genome contains five genes coding for CHASE-containing histidine kinases (HKs), which are known to function as cytokinin receptors. The abbreviation "CHASE" denotes a cyclase/histidine kinase-associated sensory extracellular domain, which comprises a region of ~270 amino acids and represents the cytokinin binding site located at the N-terminus of HK (Heyl et al. 2007). HKs from *Populus* × *canadensis* cv. Robusta, namely, PcHK2, PcHK3a, PcHK3b, PcHK4a and PcHK4b, display kinase activity and are able to bind both isoprenoid and aromatic cytokinins (Jaworek et al. 2020). All five PcHKs display strong *t*Z binding affinities ranging from 1.8 nM to 5.5 nM concentrations.

While both PcHK3a and PcHK3b display the strongest binding at pH 7.5, binding to both PcHK4 increases steadily toward pH 5.5 in line with their putative membrane localization in the endoplasmic reticulum and plasma membrane. Eventual intra- and intercellular transport of *o*T to the receptor can be mediated by several transporter families including purine permeases, ureide permeases, and nucleobase: cation symporter families 1 and 2 (reviewed in Girke et al. 2014). Of the tested aromatic cytokinins, *m*T binds more strongly than BAP. However, it is unlikely that these appear in vivo in poplar. K_i values for *m*T range between 23 nM and 300 nM (Jaworek et al. 2020). Conversely, *o*T, the metabolite found in poplar, is a much weaker ligand for PcHKs. The lowest K_i value of 1.1 μM and thus the highest sensitivity for *o*T are displayed by PcHK2 (Fig. 4.1c) (Jaworek et al. 2020). Its riboside is inactive and not bound below 10 μM concentrations. As it has been shown that leaves of mature *P.* × *canadensis* trees exhibit diurnally fluctuating levels of *o*T under physiological conditions and the concentration can peak at around 250 nM, it is possible that the local concentration may be even higher and trigger cytokinin signaling via PcHK2 (Jaworek et al. 2019).

A chlorophyll retention bioassay was used to assess the biological activity of *o*T and *m*T in poplar. The leaf discs floated on the micromolar cytokinin solution in the dark for 3 weeks at 25 °C. Data are presented in Fig. 4.2 as chlorophyll content relative to the initial value. Unlike the results outlined by Holub et al. (1998), the assay does not permit satisfactory determination of biological activity, mainly due to extremely high biological variability. While winter wheat plants were cultivated under controlled conditions in the original assay, poplar leaves were harvested from adult trees in an urban environment. The activity of *m*TR is higher and delays chlorophyll degradation. The effect of the *o*T is comparable with other three cytokinins *m*T, *t*Z, and BAP, and the values range between 60% and 80%. However, the differences are not significant enough from the control. Interestingly, chlorophyll degradation in poplar leaf discs is very slow compared with winter wheat. Bioassay with winter wheat requires 96 h, while bioassay with poplar needs 3 weeks or more.

To conclude, levels of aromatic cytokinins found in *P.* × *canadensis* and measured in leaves from the same tree are very variable between years and during the season (Jaworek et al. 2019, 2020). While *o*T derivatives are found, those of *m*T are not. Moreover, *m*T is more active in bioassays and activates cytokinin receptors at nanomolar concentrations. Currently, we cannot exclude the possibility that topolins are not products of poplar metabolism but indeed endophyte products

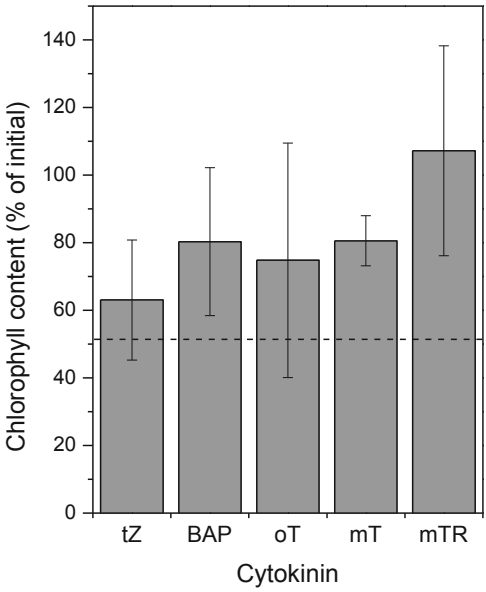

Fig. 4.2 A biological activity of topolins in poplar analyzed by a chlorophyll retention bioassay. The chlorophyll content was measured in leaf discs left in the dark for 3 weeks at 25 °C. It is shown relative to the initial value. The content in the absence of cytokinin is shown by a dashed line and corresponds to 51.5%

(Wang et al. 2019). Observed fluctuations could account for the changes in the endophyte growth. The origin of topolins in poplar remains to be identified.

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