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Sensitive quantification of isoprenoid cytokinins in plants by selective immunoaffinity purification and high performance liquid chromatography–quadrupole-time of flight mass spectrometry

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ABSTRACT

In this work, we developed a novel class-specific immunoaffinity column for the natural isoprenoid cytokinins (CTKs) by using *trans*-zeatin riboside as the hapten to generate a complete antigen. By combination with a mixed-mode solid phase extraction step for pre-cleanup and a high performance liquid chromatography–quadrupole-time of flight mass spectrometry for the quantification, an efficient analytical protocol was established which allowed simultaneous quantification of eight endogenous isoprenoid CTKs in *Arabidopsis thaliana* leaves with a wide linear range from 25 to 500 pg/g fresh weight and a detection limit of 12.5 pg/g fresh weight. The method will be very useful for comprehensive research on the networks of signaling interactions of the active phytohormones and their regulation of the plant functions.

1. Introduction

Cytokinins (CTKs) are a group of phytohormones that exhibit cell division activity [1]. They play important roles in many biological processes such as chloroplast formation, seed germination and pathogeny resistance [2–4]. Naturally occurring CTKs are derivatives of adenine [5] and usually classified as isoprenoid, isoprenoid-derived and aromatic CTKs depending on the N^6 substituent. Previous studies demonstrated that phosphorylated isopentenyladenine riboside (iPR) is the first CTK in biosynthesis [5–8]. Subsequently, phosphate *trans*-zeatin riboside (tZR) and *cis*-zeatin riboside (cZR) were formed by side-chain hydroxylation. The chemical structures and possible biosynthetic ways of different isoprenoid cytokinins are shown in Fig. 1. To further clarify these isoformation reactions and elucidate the network of signaling interactions of these active phytohormones, there is an increasing interest in the development of specific, sensitive and reproducible analytical methods for these compounds.

CTKs are often present in plant tissues at ultra-trace levels (pmol/g of fresh weight) [9]. Due to the extremely complex matrices of plant tissues, identification and quantitative analysis of endogenous CTKs in plant tissue samples have been a great challenge. Traditional methods for CTKs detection such as bioassay [10] and immunoassay [11,12] were sensitive and economic, but they could not provide the individual concentration data of multiple analytes in single analysis. Gas chromatography combined with mass spectrometry (GC–MS) shows the merits of high resolution and high sensitivity. However, due to the low volatility of CTKs, chemical derivatization is required before the GC–MS analysis [13]. As a powerful alternative, liquid chromatography combined with mass spectrometry (LC–MS) is nowadays widely used for CTKs detection because of to its high separation ability, high sensitivity and the capability in providing useful molecular structural information [5,14,15], which is especially crucial for identification of novel cytokinins [16]. Nevertheless, before the LC–MS detection, an efficient extraction and cleanup procedure is of vital importance to isolate the CTKs from most of the endogenous interferences in the plant tissue samples which are detrimental to both the instrumental performance and the analytical results.

Many efforts have been made to remove the complex interferences in plant tissue samples. Ge et al. [17] developed an LC–MS/MS method for analysis of kinetin and kinetin riboside in coconut water sample purified by C18 and mixed-mode solid phase extraction (SPE). In their study, however, it was also observed by HPLC-ultraviolet detector (UVD) that remarkable endogenous interferences were still present in the purified sample, indicating an insufficient cleanup capability of reversed phase SPE or ion-exchange extraction. In contrast, immunoaffinity purification methods are much more selective than conventional SPE and proved to be indispensable in the purification protocol for CTKs in plants [5].

The purpose of this study is to establish an efficient protocol for purification and quantitative detection of isoprenoid CTKs (as

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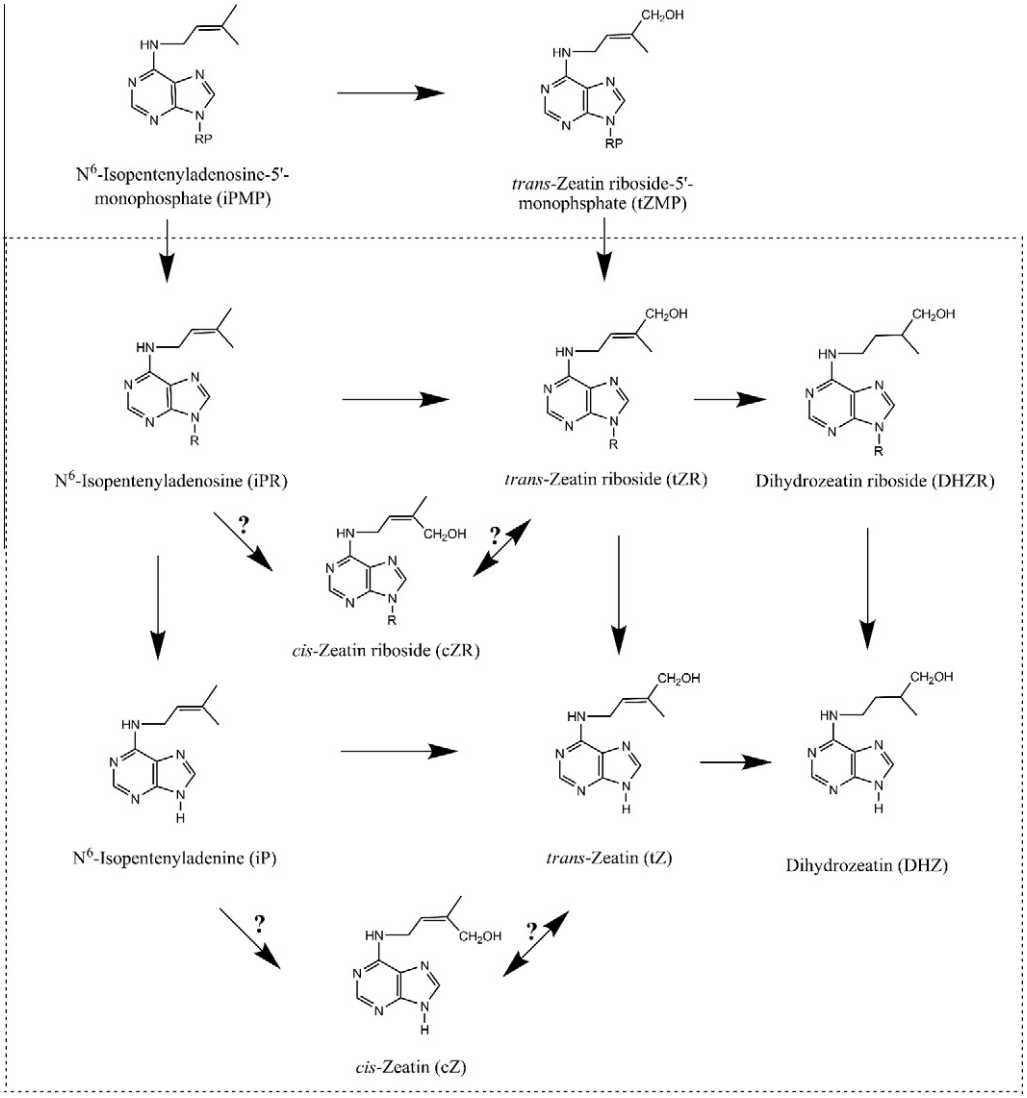

Fig. 1. Chemical structures and possible biosynthetic pathways of isoprenoid cytokinins in plants. *cis*-Hydroxylation and *cis–trans* isomerization were marked with '?' since the processes have not been confirmed experimentally or no related enzymes have been isolated. RP = β-d-ribofuranosyl-5′-monophosphate; R = β-d-ribofuranosyl.

shown in Fig. 1) in plant tissues. Though several groups have reported on immunoaffinity extraction methods for various CTKs [5,18], the antibodies were all prepared against aromatic CTKs such as ortho-Topolin riboside [5] or a synthetic benzylaminopurine (6-(2-hydroxy-3-methoxybenzylamino)purine riboside [18]. The monoclonal antibodies against 6-(2-hydroxy-3-methoxybenzylamino)purine riboside showed very weak cross-reactivity (<0.1%) with all natural isoprenoid CTKs. In this work, to obtain an antibody with specificity to the isoprenoid CTKs of interest, we employed *trans*-zeatin riboside (tZR) as the hapten and synthesized a complete antigen that well preserved the characteristics structures of the target analytes. With the antibody, we prepared a class-specific immunoaffinity column (IAC) for the natural isoprenoid CTKs. Since the complex matrix of the plant tissue extract may contaminate the IAC and influence the binding activity of the immobilized antibody, SPE was used for preliminary cleanup of the plant tissue extract before immunoextraction. We compared the efficacy of different alternatives such as reversed phase C18 SPE, strong cation exchange (SCX) and mix mode of them (Cleanet-PCX). The purified samples were finally analyzed by high performance liquid chromatography–quadrupole-time of flight mass spectrometry (HPLC–QTOF MS). The whole method achieved a detection limit of 12.5 pg/g CTKs and allowed simultaneous quantification of eight isoprenoid CTKs in *Arabidopsis thaliana* leaves.

2. Materials and methods

2.1. Chemicals and reagents

trans-Zeatin (tZ), *cis*-zeatin (cZ), *trans*-zeatin riboside (tZR), isopentenyladenine (iP), isopentenyladenine riboside (iPR), bovine serum albumin (BSA), ovalbumin (OVA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, tetramethylbenzidine (TMB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). *cis*-Zeatin riboside (cZR), dihydrozeatin (DHZ) and dihydrozeatin riboside (DHZR) were purchased from OlChemim (Olomouc, Czech Republic). CNBr activated Sepharose 4B from Pharmacia (Piscataway, USA) was used to prepare the immunoaffinity gel. All other reagents used in this study are of analytic grade or better.

Bieleski's solvent consists of methanol/chloroform/water/formic acid at volume ratios of 12/5/2/1 [19]. Cleanert PCX-SPE