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Design, synthesis and perception of fluorescently labeled isoprenoid cytokinins

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ABSTRACT

Isoprenoid cytokinins play a number of crucial roles in the regulation of plant growth and development. To study cytokinin receptor properties in plants, we designed and prepared fluorescent derivatives of 6-[(3-methylbut-2-en-1-yl)amino]purine (N^6-isopentenyladenine, iP) with several fluorescent labels attached to the C2 or N9 atom of the purine moiety *via* a 2- or 6-carbon linker. The fluorescent labels included dansyl (DS), fluorescein (FC), 7-nitrobenzofurazan (NBD), rhodamine B (RhoB), coumarin (Cou), 7-(diethylamino)coumarin (DEAC) and cyanine 5 dye (Cy5). All prepared compounds were screened for affinity for the *Arabidopsis thaliana* cytokinin receptor (CRE1/AHK4). Although the attachment of the fluorescent labels to iP *via* the linkers mostly disrupted binding to the receptor, several fluorescent derivatives interacted well. For this reason, three derivatives, two rhodamine B and one 4-chloro-7-nitrobenzofurazan labeled iP were tested for their interaction with CRE1/AHK4 and *Zea mays* cytokinin receptors in detail. We further showed that the three derivatives were able to activate transcription of cytokinin response regulator *ARR5* in Arabidopsis seedlings. The activity of fluorescently labeled cytokinins was compared with corresponding 6-dimethylaminopurine fluorescently labeled negative controls. Selected rhodamine B C2-labeled compounds **17**, **18** and 4-chloro-7-nitrobenzofurazan N9-labeled compound **28** and their respective negative controls (**19**, **20** and **29**, respectively) were used for *in planta* staining experiments in *Arabidopsis thaliana* cell suspension culture using live cell confocal microscopy.

Abbreviations: ABA, abscisic acid; 2-AmEtAm, 2-aminoethylamino-; 6-AmHexAm, 6-aminohexylamino-; AHK, histidine-kinase receptor from *A. thaliana*; AFCS, Alexa Fluor 647 labeled castasterone; ARR5:GUS, Arabidopsis response regulator 5: β-glucuronidase; ARCKs, aromatic cytokinins; BRI1, protein brassinosteroid insensitive 1; Cou, coumarin; Cou-OH, coumarin-3-carboxylic acid; CK(s), cytokinin(s); Cy5, cyanine 5 dye; Cy5-NHS, NHS ester; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; DMSO, dimethylsulfoxide; DEAC, 7-(diethylamino)coumarin; DEAC-OH, 7-(diethylamino)coumarin-3-carboxylic acid; DAP, 6-dimethylaminopurine; DS, dansyl; DS-Cl, dansyl chloride; NBD, 7-nitrobenzofurazan; NBD-Cl, 7-nitrobenzofurazan chloride; ESI^+-MS, electrospray ionization mass spectrometry (positive mode); EtOAc, ethyl acetate; FC, fluorescein; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; ISCK, isoprenoid cytokinins; iP, 6-[(3-methylbut-2-en-1-yl)amino]purine, N^6-isopentenyladenine; MeOH, methanol; NAA, naphthalene acetic acid; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; PrOH, *n*-propanol; RhoB, rhodamine B; RhoB-NHS, rhodamine B NHS ester; RT, room temperature; TFA, trifluoroacetic acid; TLC, thin layer chromatography; THF, tetrahydrofuran; *tZ*, *trans*-zeatin.

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1. Introduction

Naturally occurring isoprenoid cytokinins (ISCK), such as 6-[(3-methylbut-2-en-1-yl)amino]purine (iP), *trans*-zeatin (*tZ*) and *cis*-zeatin, are plant signaling molecules. For this reason, they have attracted the attention of biologists owing to their importance in numerous aspects of plant growth and development, cell division, seed germination, the formation and activity of shoot and root meristems, apical dominance, auxiliary bud release, nutrition mobilization, leaf senescence and responses to pathogens (Davies, 2007). Fluorescently labeled ISCK may be a useful alternative tool for research into cytokinin perception and signaling in plants. Although several cytokinin receptors have been already described, e.g., in species such as Arabidopsis (Inoue et al., 2001; Suzuki et al., 2001), maize (Yonekura-Sakakibara et al., 2004), legumes *Medicago truncatula* (Gonzalez-Rizzo et al., 2006), *Lotus japonicus* (Murray et al., 2007; Tirichine et al., 2007) and rice (Du et al., 2007), there remains the need for mapping the receptor domain in order to understand the relation between the chemical structure and activity of cytokinin derivatives. This approach is indispensable developing new strategies in plant biotechnology, such as plant tissue culture, modern agriculture and plant protection against stress (Plíhalová et al., 2016). Fluorescent labeling is an important tool in cell biology research, e.g., staining and immunostaining techniques (Doskočilová et al., 2013; Mason, 1999; Ovečka et al., 2014; Šamajová et al., 2014), and for visualizing of small bioactive molecules. It offers several advantages over traditional radio-ligand binding techniques, i.e., fluorescence labels are relatively safe and inexpensive compared to tritiated or iodinated compounds and a wide range of fluorophores are available to suit different experimental setups (McGrath et al., 1996; Daly and McGrath, 2003). Fluorescent ligands are continually being developed to meet the demands of the pharmacological community and are being used to study pharmacological receptor systems (Daly and McGrath, 2003). Hiratsuka and Kato used a fluorescent analogue of colcemid with 7-nitrobenzo-furazan (NBD, NBD-colcemid) to visualize tubulin (Hiratsuka and Kato, 1987). Fluorescent labeling of small active molecules has been shown to be effective for visualizing plant hormones, such as auxins, abscisic acid, jasmonates, gibberellins, brassinosteroids and even strigolactones. In one such study, abscisic acid (ABA) was coupled with fluorescein isothiocyanate (FITC) and used to study direct interaction of ABA with the plasma membrane as although ABA receptors were unknown at the time, they were predicted to lie in the membrane (Asami et al., 1997). Fluorescent brassinosteroid was prepared by labeling castasterone with Alexa Fluor 647 (AFCS) and the endocytosis of BRI1-AFCS complexes in living cells was visualized (Irani et al., 2012). Fluorescent labeling at the cellular level has also been done using gibberellins labeled with FITC (Pulici et al., 1996). 1,4-Dithiobutylene and 1,3-dithiopropylene spacers were employed between the fluorescent label and gibberellin, particularly for the compound 17-mercaptobutylthio-3α,10-dihydroxy-20-norgibberella-7,19-dioic acid-19,10-lactone. It was shown that derivatives with longer spacers between gibberellin and FITC were more active in the ability to induce α-amylase activity in the embryoless half grain, a process known to be specifically induced by active Gas synthesized by the embryo. However, an approximately 10-fold higher concentration of the fluorescent probe than GA_3 was needed to obtain a comparable biological effect (Pulici et al., 1996; Lace and Prandi, 2016). Synthesis of fluorescently labeled strigolactone analogs (DS, FC, BODIPY) has been used to search for possible strigolactone receptors *in vivo* (Prandi et al., 2013). Rhodamine and fluorescein auxin derivatives have been synthesized by direct conjugation of FITC and rhodamine B to the NH group of IAA (Sokolowska et al., 2014). Both fluoroprobes were shown to retain auxin activity in three different bioassays (Sokolowska et al., 2014). Tsuda and Hayashi introduced an NBD label into 5-hydroxy-IAA and 7-hydroxy-NAA but the prepared auxin analogs were found to be inactive toward auxin receptors (Tsuda et al., 2011; Hayashi et al., 2014; Lace and Prandi, 2016). Fluorescently labeled jasmonate has been synthesized by bonding jasmonoyl-L-isoleucine to coumarin 343 *via* the carboxyl group of isoleucine (Liu et al., 2012). The fluorescent probe was examined in cabbage using a root growth inhibition bioassay and the effect of fluorescently labeled probe on the root growth of cabbage seedlings was similar to that of the methyl jasmonate, the standard bioactive jasmonate. Like approaches to other plant growth regulators, in preparing a fluorescent probe for visualizing a cytokinin receptor, the compound has to possess cytokinin activity and high affinity for the receptor while nonspecific binding to other cellular structures needs to be minimized. When the first attempts to prepare a cytokinin fluorescent probe failed in the 1970s, a different strategy based on the construction of mimetic adenine-like molecules was developed (Skoog et al., 1975; Specker et al., 1976). Modifications of cytokinins, particularly in the purine moiety, has led to the preparation of fluorescent imidazo[4,5-*g*]- and imidazo[4,5-*f*]-quinazolines, 4-substituted 2-methylthiopyrido[2,3-*d*]pyrimidines and 7-phenylethynylimidazo[4,5-*b*]pyridines and their ribosides, which were shown to have only weak or negligible cytokinin activity in a tobacco callus bioassay (Specker et al., 1976; Hamaguchi et al., 1985; Nishikawa et al., 2000). Zawadski's group prepared synthetic cytokinin *N*-phenyl-*N*′-(4-pyridyl) urea labeled with 4-chloro-7-nitrobenzofurazan and rhodamine B fluorescent labels and detected binding of the cytokinin-specific protein VrCSBP by fluorescence correlation spectroscopy (Zawadski et al., 2010). It has been suggested that the loss of biological activity could be prevented by separation of the pharmacophore from the fluorescent moiety through the introduction of a spacer or linker (Leopoldo et al., 2009). However, so far, only a few studies have systematically evaluated spacer length for fluorescent probes and none have directly evaluated purine based cytokinins. Spacer length and position of the spacer (label) in the purine moiety can both have a large impact on the biological activity of such cytokinin derivatives. Appropriate positional attachment of fluorophores to small molecule ligands is critical for retaining both receptor binding affinity and efficacy (Leopoldo et al., 2009). In addition to standard fluorophores such as fluorescein and rhodamine, we have also endeavoured to find new efficient fluorolabels with fewer limitations for use in biological systems and during confocal microscopy imaging. For example, fluorescein is known to self-quench after bioconjugation (Lace and Prandi, 2016; Sjöback et al., 1995) as the emission properties of fluorescein greatly depend on environmental pH (Lavis et al., 2007) and it often exists as an equilibrium between lactone and quinoid forms (Lace and Prandi, 2016). Although rhodamine dyes are less sensitive to pH than fluorescein, they are poorly soluble in water (Lace and Prandi, 2016). Despite these limitations, both are widely used for labeling bioactive molecules. We have also used the small heterocyclic molecule 4-chloro-7-nitrobenzofurazan (NBD-Cl), which is a benzoxadiazole compound with low molecular weight. Derivatives of NBD-Cl have been used for the preparation of novel kinase substrates, lipid probes and fluorescent analogs of native lipids and the study of a variety of processes (Chattopadhyay, 1990; Lavis and Raines, 2008; Lace and Prandi, 2016).

In this work, we prepared fluorescently labeled iP derivatives because iP is known to bind to *Arabidopsis thaliana* CRE1/AHK4, *Zea mays* ZmHK1 and other cytokinin receptors. 6-Dimethylaminopurine (DAP) analogs with no cytokinin activity were also prepared to obtain fluorescent negative controls for receptor bioassays. The prepared compounds contained a 2- or 6-