Phytochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus

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ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 29 November 2015
Accepted 2 December 2015
Available online xxxx

Keywords:
Aromatic cytokinin
Ribosides
Synthesis
Cytokinin activity
Photosynthesis
Photosystem
Senescence delay
Gene expression

ABSTRACT

Cytokinins are plant hormones with biological functions ranging from coordination of plant growth and development to the regulation of senescence. A series of 2-chloro-N^6-(halogenobenzylamino)purine ribosides was prepared and tested for cytokinin activity in detached wheat leaf senescence, tobacco callus and *Amaranthus* bioassays. The synthetic compounds showed significant activity, especially in delaying senescence in detached wheat leaves. They were also tested in bacterial receptor bioassays using both monocot and dicot members of the cytokinin receptor family. Most of the derivatives did not trigger cytokinin signaling *via* the AHK3 and AHK4 receptors from *Arabidopsis thaliana* in the bacterial assay, but some of them specifically activated the ZmHK1 receptor from *Zea mays* and were also more active than the aromatic cytokinin BAP in an *ARR5::GUS* cytokinin bioassay using transgenic *Arabidopsis* plants. Whole transcript expression analysis was performed using an *Arabidopsis* model to gather information about the reprogramming of gene transcription when senescent leaves were treated with selected C2-substituted aromatic cytokinin ribosides. Genome-wide expression profiling revealed that the synthetic halogenated derivatives induced the expression of genes related to cytokinin signaling and metabolism. They also prompted both up- and down-regulation of a unique combination of genes coding for components of the photosystem II (PSII) reaction center, light-harvesting complex II (LHCII), and the oxygen-evolving complex, as well as several stress factors responsible for regulating photosynthesis and chlorophyll degradation. Chlorophyll content and fluorescence analyses demonstrated that treatment with the halogenated derivatives increased the efficiency of PSII photochemistry and the abundance of LHCII relative to DMSO- and BAP-treated controls. These findings demonstrate that it is possible to manipulate and fine-tune leaf longevity using synthetic aromatic cytokinin analogs.

1. Introduction

Cytokinins (CKs) are an important group of plant hormones that control many physiological, metabolic and developmental processes in plants (for a review, see e.g. Spichal, 2012). They stimulate cell division and differentiation in the presence of other phytohormones, such as auxins. In addition, they promote the formation and activity of shoot apical meristems, participate in *de novo* bud formation, control apical dominance, inhibit root growth and branching, delay leaf senescence, and stimulate seed germination (Mok et al., 2000; Riefler et al., 2006). It has also been shown that CKs can modulate plants' responses to various pathogenic infections (Bari and Jones, 2009). Naturally occurring CKs are

Abbreviations: EA, elemental analysis; ESI–MS, electrospray ionization–mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; BAP, 6-benzylaminopurine; MS medium, Murashige–Skoog medium; CKs, cytokinins; iP, N^6-isopentenyladenine; BAPR, 6-benzylaminopurine riboside; ArCKs, aromatic cytokinins; tZ, 6-(4-hydroxy-3-methylbut-2-enylamino)purine; *meta*-topolin, 6-(3-hydroxybenzylamino)purine; AHK, *Arabidopsis* histidine kinase; ARR, *Arabidopsis* response regulator; GUS, β-glucuronidase; RMA, robust multi-array average; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; CKX, cytokinin dehydrogenase; LHCII, light harvesting complex II; PSII, photosystem II; kinetin, 6-furfurylaminopurine; P_N, net photosynthetic rate; F_M, maximal fluorescence; F_V, variable fluorescence; ZmHK, *Zea mays* histidine kinases; qP, photochemical quenching.

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http://dx.doi.org/10.1016/j.phytochem.2015.12.001
0031-9422/

purine derivatives with an isoprenoid or aromatic side chain bound to the N6 atom of the adenine moiety. N^6-isopentenyladenine (iP) and *trans*-zeatin (tZ) are typical isoprenoid CKs; notable aromatic CKs include 6-benzylaminopurine (BAP), the topolins (hydroxylated BAP derivatives), and kinetin (6-furfurylaminopurine) (Mok and Mok, 2001). In plant tissues, CKs may be present in the form of free bases but they occur more frequently as nucleosides or nucleotides. In addition, they can be conjugated to glucose or xylose at various positions of the adenine ring (Auer, 1997; Frebort et al., 2011).

N^6-(2-hydroxybenzylamino)purine-β-D-riboside, the first known aromatic CK, was initially isolated from fully expanded poplar (*populus* × *robusta* Schneid) leaves (Horgan et al., 1975). For a long time, it was believed that aromatic CKs (ArCKs) were merely biologically inactive degradation products (Strnad et al., 1997). However, Strnad and co-workers isolated and identified various natural ArCKs from mature poplar leaves, including 6-(3-hydroxybenzylamino)purine and its 9-β-D-ribofuranosyl or 9-β-D-glucopyranosyl conjugates (Strnad et al., 1992; Strnad, 1997). Hydroxylated ArCK derivatives were subsequently prepared and their activity was tested in three classical cytokinin bioassays: the tobacco callus, *Amaranthus*, and wheat leaf senescence bioassays (Holub et al., 1998). Structure–activity relationship analyses revealed that the biological activity of these aromatic CKs was heavily dependent on the presence and position of the hydroxyl groups on the benzyl ring, and on their ribosylation or glucosylation at the N9 position of the purine moiety (Holub et al., 1998). A large family of BAP and BAP riboside (BAPR) analogs with various substituents on the benzyl ring was also prepared and screened in the standard cytokinin bioassays. Some of these compounds exhibited particularly high activities in the senescence bioassay, being up to twofold more efficient than BAP itself at delaying the onset of senescence (Dolezal et al., 2006, 2007).

Leaf senescence is one of the final stages of plant development. It is a highly regulated degradation process that involves changes in cell structure, metabolism, gene expression, and also environmental signals. In particular, it is associated with substantial changes in the expression of genes involved in degrading proteins into amino acids and membrane lipids into fatty acids and sugars (Sarwat et al., 2013). Plant senescence involves enhanced chlorophyll catabolism activity and the degradation of leaf proteins, membrane lipids and RNA; these processes are largely responsible for the color changes of autumn leaves. There is extensive crosstalk between plant hormones such as CKs, auxins, ethylene and abscisic acid, which establishes an endogenous regulatory pathway for age-controlled senescence (Lim et al., 2007). CKs play an essential role in this process by delaying the onset of senescence. In *Arabidopsis*, the AHK3 receptor seems to play a major role in regulating cytokinin-mediated leaf longevity because it promotes the phosphorylation of the cytokinin response regulator ARR2 (Kim et al., 2006). In this context it is interesting that bacterial receptor assays have shown that the AHK3 and AHK4 receptors have important differences in their ligand preferences (Lomin et al., 2015; Spichal et al., 2004). While iP and tZ are preferred ligands for both receptors, only AHK3 is activated by CK ribosides and ribotides. In addition, AHK3 also has a higher affinity for BAP and other ArCKs, which generally exhibit low activity in bacterial receptor tests despite their significant biological activity *in planta* (Spichal et al., 2004).

The anti-senescent activity of CKs has been recognized since 1957, when Richmond and Lang showed that treatment with exogenous kinetin delayed chlorophyll breakdown and extended the lifespans of detached cocklebur leaves and cut carnation flowers (Eisinger, 1977; Richmond and Lang, 1957). These effects were subsequently found to be heavily dependent on the light conditions: CKs delayed leaf senescence under dark conditions but may have accelerated chlorophyll degradation under strong illumination (Gan and Amasino, 1996; Vlckova et al., 2006). The onset of leaf senescence may be related to the quantity of reactive oxygen species in the plant cells, which increases as the cells age (BuchananWollaston, 1997; Prochazkova and Wilhelmova, 2009). It was reported that CKs may retard senescence by scavenging or interfering with free radicals (Synkova et al., 2006). In keeping with this hypothesis, treatment with exogenous BAP reduced superoxide radical production, improved the quenching of hydrogen peroxide, protected the photosynthetic system and supported carbohydrate production (Xiaotao et al., 2013). It was also shown that treatment with synthetic kinetin derivatives significantly protected lipid membranes against the negative influence of accumulated reactive oxygen species in the dark phase (Mik et al., 2011). Recently, Li et al. developed a database of genes potentially associated with leaf senescence (the Leaf Senescence Database, a gene network for identifying common regulators of leaf senescence in *Arabidopsis thaliana*). It is now widely accepted that phytohormones such as CKs play a critical role in regulating senescence (Li et al., 2012; Sarwat et al., 2013).

As mentioned above, CKs are also involved in the control of photosynthesis. Treatment with exogenous CKs usually delays senescence-induced changes such as the decline in chlorophyll content, and reduces the values of photosynthetic parameters such as the net photosynthetic rate (P_N) (Čatský et al., 1996; Gan and Amasino, 1995; Rulcová and Pospíšilová, 2001), the photochemical chlorophyll fluorescence quenching (qP), and the maximal photochemical efficiency of photosystem II (PSII) as measured by the variable-to-maximal fluorescence ratio (F_V/F_M) (for a review, see e.g. Synková et al., 1997). Moreover, a recent study showed that adding aromatic cytokinins (BAP, BAPR, or *meta*-topolin) to culture media induced changes in the chlorophyll a and b contents of apple leaves *in vitro* and also affected the capacity of the photosynthetic apparatus as determined by fluorescence measurements conducted after three weeks of cultivation (Dobránszki and Mendler-Drienyovszki, 2014). Importantly, Cortleven et al. showed that plants with reduced cytokinin levels are more susceptible to photodamage due to the malfunctioning of photosystem II and deregulation of the associated photoprotective mechanisms (Cortleven et al., 2014).

Here we report the synthesis of a library of 2-chloro-N^6-(halogenobenzylamino)purine riboside ArCK sugar conjugates, tests of their activity in standardized cytokinin bioassays, and analyses of their effects on the photosynthetic apparatus. To clarify the molecular mechanisms underpinning the observed physiological changes in senescent *Arabidopsis* leaves, we performed a gene expression study using the two most active of these compounds. The results revealed that treatment with active ArCKs alters the expression of several genes that are closely related to photosynthesis and which encode important anti-stress factors.

2. Results and discussion

2.1. Chemistry

A series of 2,6-disubstituted ArCK derivatives (see Table 1) was prepared by the reaction of 2,6-dichloropurine riboside or 2,6-dichloropurine with suitable benzylamines in the presence of triethylamine in *n*-propanol. The yields of these reactions along with the purities of the prepared substances and their ESI–MS and NMR data are given in the supplementary data section. All of the tested compounds were dissolved in DMSO and subsequently diluted in water or buffer for use in the biological experiments. Consequently, a 0.05% solution of DMSO in water was used as a negative control in all of the assays.

Please cite this article in press as: Vylíčilová, H., et al. C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus. Phytochemistry (2015), http://dx.doi.org/10.1016/j.phytochem.2015.12.001