Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots

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The plant hormones cytokinins are a convenient target of genetic manipulations that bring benefits in biotechnological applications. The present work demonstrates the importance of the subcellular compartmentalization of cytokinins on the model dicot plant *Arabidopsis thaliana* and monocot crop *Hordeum vulgare*. The method of protoplast and vacuole isolation combined with precise cytokinin analysis and recovery assay of a vacuolar marker protein were used to quantify the contents of individual cytokinin forms in the leaf extracellular space, cell interior and vacuole. The data obtained for wild type plants and in each case a specific mutant line allow comparing the effect of genetic manipulations on the hormone distribution and homeostatic balance of cytokinins in the modified plants.

Introduction

Cytokinins, constituting a specific group of plant hormones, are involved in the regulation of plant morphogenesis, such as shoot and root formation, stem branching or apical dominance. In coordinated action with other hormones, especially auxins, they promote cell division; stimulate seed germination and delay senescence (reviewed in [1,2]). In biotechnological applications, cytokinins are valued for their ability to mediate defense responses towards environmental stresses such as salinity or drought [3]. Chemically, cytokinins are adenine derivatives substituted at the *N*6 position by an isoprenoid or aromatic side chain. The most abundant naturally occurring plant cytokinins are isopentenyl adenine (iP) and its hydroxylated derivatives *trans*-zeatin (tZ) and *cis*-zeatin (cZ). However the aromatic cytokinins such as benzyladenine derivatives and kinetin are used in plant propagation and various biotechnological applications due to their higher stability against conversion or degradation by plant endogenous enzymes.

Natural cytokinins [4] occur in four principal forms: (1) the nucleotides, which are produced during the *de novo* biosynthesis and then converted to other derivatives; (2) the free bases, which are considered to have the highest activity [5]; (3) the ribosides, which are preferred transport form [6] and (4) the glucosides that are storage/inactivated forms [7]. Structural modifications of cytokinins directly influence their biological activities and binding ability to receptors; for example, glycosylation at the zeatin hydroxyl group or at *N9* position leads to clear reduction of activity [8].

The direct involvement of diverse forms of cytokinins in important physiological processes such as seed ripening and stress responses and a good knowledge of their metabolism make them suitable target for artificial manipulations with the plant phenotype. The overexpression of the gene coding for the cytokinin dehydrogenase (CKX, EC 1.5.99.12) in tobacco, *Arabidopsis thaliana* and barley causes an enlargement of the root system, but unfortunately also a significant reduction of aerial parts of plants

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and decrease or loss of fertility [9–12]. Even though an extended root system gives plants advantages in water and nutrients uptake, well grown upper parts are often important from the agronomical point of view, especially in crop plants. Modifying cytokinin degradation by expression of *CKX* gene in a tissue specific manner increases the root biomass without affecting the development and function of the aerial parts [13,14]. An alternative approach to modify the active cytokinin levels in plants is based on gene silencing or knock-out. RNAi silencing of *CKX* genes resulted in higher grain number and improved yield in rice [15] and barley [16].

The biosynthesis of cytokinins involves a transfer of dimethylallyl chain from dimethylallyl pyrophosphate (DMAPP) or (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) to *N6*-amino group of free or tRNA-bound adenosine phosphates catalyzed by adenylate isopentenyl transferase (EC 2.5.1.27) and tRNA isopentenyl transferase (EC 2.5.1.75), respectively. The major share of cytokinins in plants is synthesized by adenylate isopentenyl transferases, but cZ type of cytokinins, which may constitute a considerable portion of the cytokinin pool, appears to be exclusively generated from the tRNA mediated pathway [17].

The biosynthetic enzymes adenylate isopentenyl transferases (EC 2.5.1.27) are localized in chloroplasts, mitochondria or cytosol (for review see [18]). Remarkably, the activating phosphoribohydrolase LOG [19] and cytokinin *O*- and *N*-glycosyltransferases are cytosolic [20,21]. Thorough analysis of cytokinin content in tobacco and wheat leaves shows a whole spectrum of cytokinins present in chloroplast with moderately elevated contents of free bases, ribosides and nucleotides and significantly increased zeatin *N9*-glucosides compared to the whole leaf [22]. Cytokinins are transported between the cytosol and apoplast by purine permeases [23], equilibrated nucleoside transporters [6] and/or tZ-type specific ABC transporter [24,25]. However the way of subcellular transport and organelle localization of cytokinins is still largely unknown. Although substantial evidence was given that zeatin-*O*-glucoside is stored in vacuoles in tobacco by retargeting specific β-glucosidase [26], there is only single experimental study directly quantitating vacuolar contents of cytokinins. After exogenous application of radioactively labeled dihydrozeatin to photoautotrophic suspension cultures of *Chenopodium rubrum*, *O*-glucosides were mainly found in vacuoles, whereas free bases and ribosides, which comprised only a minor portion of total labeled cytokinins, were found outside the vacuole [27]. The hypothesis that some plant hormones are stored in vacuoles is further supported by more recent findings, the vacuolar targeting of some Arabidopsis cytokinin degrading enzymes CKX [9,28] and the identification of auxin vacuolar transporter WAT1 [29]. Arabidopsis *wat1-1* mutant plants show a retarded growth typical for auxin/cytokinin hormonal imbalance. WAT1 is a tonoplast-localized transporter that exports auxin from the vacuole to the cytoplasm, most likely operating as a proton symporter [29]. Cytokinins and auxins have long time been known to interact antagonistically and therefore changes in cytokinin profile incurred by blocked auxin export from vacuoles are of interest.

In this work, we prepared the protoplast and vacuolar fractions from the leaves of model dicot plant *A. thaliana* and monocot crop plant barley (*Hordeum vulgare*), extracted, purified and quantified contents of whole range of cytokinin metabolites, thus determining total, intracellular and vacuolar distribution of the hormone.

Materials and methods

Plant preparation

A. thaliana plants *wat1-1* mutant [30,31] of the WAT1 gene (GenBank accession no. AT1G75500) that harbors a T-DNA insertion situated 55 bp upstream from the ATG translation start codon and wild type (ecotype Col-0) were used as model of dicots. The WAT1 gene (Walls Are Thin1) is required for the secondary cell wall deposition and codes for the vacuolar auxin transport facilitator needed for auxin homeostasis [29,31].

The wild type barley (*H. vulgare*, cv. Golden Promise) and a transgenic line overexpressing cytokinin dehydrogenase gene from *A. thaliana* under a root specific β-glucosidase promoter [32] with vacuolar targeting of the protein (bGLU::vAtCKX1 [14]), referred herein as AtCKX1 line, were selected as model of monocots.

Barley seeds were sterilized in 70% ethanol and germinated for 5 days in the dark, at 15°C. Then, the plantlets were moved to soil containers and placed in a growth chamber under 8 hours light/16 hours dark cycle at 21/19°C and 55% of humidity. *Arabidopsis* seeds were sown directly to the soil in the growth chamber and the plants were grown under the same conditions as barley. Harvested plants of *Arabidopsis* (45 days-old) and barley (30 days-old) were used for further experiments.

Isolation of protoplasts and vacuoles

Arabidopsis protoplasts and vacuoles were isolated using slightly modified published protocol [33]. Shortly, approx. 2 g of rosette leaves were cut to small strips and incubated in an enzyme solution 1 (1% macerozyme, 1% cellulase, 0.4 M mannitol, and 0.8 mM $CaCl_2$ in 10 mM MES, pH 5.6) in the dark for 4 hours. Obtained protoplasts were gently spun down and collected. Vacuoles were then released from protoplasts by the osmotic and temperature shock and fractionated on the Ficoll density gradient (0–10%) by ultracentrifugation at 71,000 × *g* for 1 hour at 10°C. The pink-colored layer of vacuoles was collected by a pipette on the interface between 0 and 4% Ficoll.

Preparation of barley protoplasts and vacuoles was newly developed based on an old protocol originally used for tobacco samples [34]. Protoplasts were obtained from approx. 2 g of cut leaves by incubating samples in an enzyme solution 2 (1% macerozyme, 2% cellulase, 0.6 M mannitol, and 2.5 mM $CaCl_2$ in 10 mM MES) for 4 hours. Vacuoles were released from the protoplasts by a lysis solution containing 0.35 M mannitol in 5 mM Tris/HCl, pH 7.5, and loaded onto a 1.5–12.5% Ficoll gradient in 0.6 M mannitol and 5 mM Tris/HCl, pH 7.5, by ultracentrifugation at 90,000 × *g* for 2 hours at 4 °C. Vacuoles were collected from the interface between 1.5 and 7% Ficoll. All collected samples were stored at −80°C.

Quality control of isolated protoplasts and vacuoles by western blot with vacuolar V-ATPase

The protein extracts were prepared from leaves and protoplasts by adding an extraction buffer (1 mM EDTA, 1 mM ascorbic acid, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton-X, in 50 mM phosphate buffer, pH 7.6). The extracts were concentrated by columns (Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane, cut-off 10 kDa) to 1/8 and 1/20 of original volumes for Arabidopsis and

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