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Nebularine Affects Plant Growth and Development but does not Interfere with Cytokinin Signaling

Hana Pospíšilová · Jaroslav Nisler · Lukáš Spíchal · Ivo Frébort

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Abstract Nebularine is known for its high cytotoxicity in animals, whereas in plants it was originally believed to be an anticytokinin. In this study we show that in classical cytokinin bioassays, nebularine antagonized cytokinin function in senescence and callus biotests but not in the *Amaranthus* bioassay. Nebularine reversed the inhibitory effect of cytokinin on lateral root formation in *Arabidopsis* seedlings, and when applied alone caused increased lateral root formation and shortening of the main root. Systematic spraying of *Arabidopsis* plants with nebularine led to yellowing and formation of purple pigments, local drying, and withering, although younger plants showed a greater resilience. Comparison of nebularine cytotoxicity in plant and animal cells showed that the growth of tobacco BY-2 cells was inhibited with only about tenfold lower efficacy than mammalian cell lines. Most importantly, direct binding assay with *Arabidopsis* cytokinin receptors AHK3 and CRE1/AHK4 showed that nebularine did not compete for binding with the natural cytokinin *trans*-zeatin. Although nebularine reduced cytokinin-induced expression of the cytokinin reporter *ARR5:GUS in planta*, the same effect was observed for *DR5:GUS*, an auxin reporter gene. Taken together, the results indicate that the mode of action of nebularine does not involve cytokinin signaling and that the anticytokinin-like effect is rather a consequence of the inhibition of various processes as described for animal systems.

Keywords Anticytokinin · *Arabidopsis thaliana* · Cytokinin receptor · Nebularine · Purine riboside · Cytokinin bioassay

H. Pospíšilová (✉) · I. Frébort
Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 78371 Olomouc, Czech Republic
e-mail: h.pospisilova@upol.cz

J. Nisler · L. Spíchal
Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany AS CR, Šlechtitelů 11, 78371 Olomouc, Czech Republic

Introduction

Nebularine is a purine riboside (9-β-D-ribofuranosylpurine, Fig. 1) first isolated from the fungus *Lepista nebularis* (Löfgren and others 1954), with antibiotic (Ehrenberg and others 1946), antiamebal (Das and Baer 1991), antiviral (Tamm and others 1956), and antiparasitic (el Kouni and Cha 1987) effects toward certain targets.

The cytotoxic action of nebularine on animal cells is fairly well described and includes mitotic aberrations in embryonic fibroblasts and epithelial cells of embryonic skin (Biesele and others 1955), neoplastic cell lines (Biesele and others 1955; Lynch and others 1981), and rat thymocytes (Kozlowska and others 1999), where it leads to the depletion of ATP and formation of purine riboside phosphates by adenosine kinase. Nebularine inhibits the synthesis of nucleic acids in different cancer ascites cells (Paterson and others 1979), which is not caused by nebularine incorporation into the nucleic acid as proved for *E. coli* mRNA (Bohr 1978). Studies on mismatch repair (Hoffman and others 2005; Wood and others 1986) and other effects of nebularine incorporated into DNA revealed that nebularine does not block DNA replication but shows apparent template characteristics resembling adenine (Rahman and Humayun 1997).

Nebularine has been found to be an inhibitor of adenosine deaminase (Lupidi and others 1982),

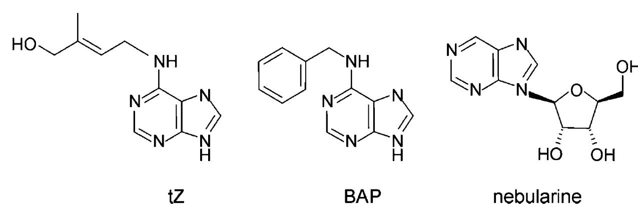

Fig. 1 Structure of the active cytokinins and nebularine used in this study. *tZ*, *trans*-zeatin; BAP, 6-benzylaminopurine; nebularine, 9-*β*-D-ribofuranosylpurine

S-adenosylhomocysteine hydrolase (Guranowski and others 1981), herpes simplex DNA polymerase (Frank and Cheng 1986), xanthine oxidase (Brown and Konuk 1994), NAD^+-dependent glucose dehydrogenase (Brink 1953), adenine-hypoxanthine and guanine-hypoxanthine phosphoribosyltransferase, 5-phosphoribose 1-pyrophosphate amidotransferase, ribose phosphate pyrophosphokinase (Henderson 1968; Smith and others 1974), and adenylyl cyclase (Backer and Khan 2004). Inosine nucleosidase from *Lupinus luteus* cleaves off the ribosyl moiety of nebularine (Guranowski 1982). In animal cell tissue cultures, nebularine cytoxicity is blocked by adenosine, AMP, and ATP applied at 100-fold excess (Biesele and others 1955).

Nebularine inhibits the growth of plant seedlings and at higher concentrations causes mitotic aberrations in root tips (Löfgren and others 1954). However, relatively little is known about the mechanism of nebularine action in plants and there is no evidence of a direct effect of nebularine on nucleic acid function. Brown and Konuk (1994) reported that nebularine, despite its structural similarity to plant hormones cytokinins, does not exhibit any cytokinin-like activity; however, at low concentrations it blocks the action of exogenously applied cytokinin in the *Amaranthus* and leaf senescence bioassays. In addition, 1-week-old wheat seedlings treated with 0.1–2 mM nebularine showed significant changes in the axis, root, and shoot length, with marcescence in a few days. In carrot, inhibition of direct somatic embryogenesis from the hypocotyl by 10 μM nebularine was observed, whereas application of zeatin riboside reversed the effect (Tokuji and Kuriyama 2002). Finally, application of nebularine to attached leaves of *Bryophyllum marnierianum* eliminated the inhibition of plantlet development caused by cytokinins. Nebularine also completely inhibited the growth of axillary buds (Kulka 2006).

A number of compounds structurally similar to cytokinins have been previously classified as anticytokinins, but recently established assays based on a direct binding to cytokinin receptors have revealed that some of them do not interact with cytokinin signaling but influence other mechanisms such as cell cycle machinery (Spíchal and others 2007). The first known molecule that antagonizes the activity of the plant hormone cytokinin at the receptor level has been identified recently (Spíchal and others 2009). In this study, the effect of nebularine on the development and growth of *Arabidopsis* plants was studied and the capability of nebularine to act as a cytokinin antagonist was tested in direct cytokinin-binding assays as well as in classical cytokinin bioassays.

Materials and Methods

Biological Material

Wild-type *Arabidopsis thaliana* (L.) Heynh ecotype Col-0 was grown in an environmental test chamber (Sanyo, Tokyo, Japan) or in a greenhouse. *Escherichia coli* strain KMI001 harboring the plasmids pIN-III-CRE1/AHK4 and pSTV28-AHK3, which expressed the *A. thaliana* histidine kinases CRE1/AHK4 and AHK3 (Suzuki and others 2001; Yamada and others 2001), respectively, was used in cytokinin receptor studies. Transgenic Col-0 *Arabidopsis* plants harboring the *ARR5:GUS* (D'Agostino and others 2000) or *DR5:GUS* (Ulmasov and others 1997) reporter gene construct were used. Tobacco BY-2 cells (*Nicotiana tabacum* L., cv. Bright Yellow 2) (obtained from Prof. Z. Opatrný, Charles University, Prague) and human cell lines K562, CEM, and MCF-7 purchased from American Type Culture Collection (Manassas, VA, USA) were used for cell viability assays. For cytokinin bioassays, *Nicotiana tabacum* L. cv. Wisconsin 38, seeds of *Amaranthus* (*Amaranthus caudatus* var. Atropurpurea), and excised wheat leaves of *Triticum aestivum* cv. Hereward were used. Human CDK2-cyclin E and mouse ABL kinases were produced in the insect cell line Sf9 and used for determination of nebularine inhibitory effect on these kinases.

Chemicals

Extra-pure agar for plant growth and casamino acids were obtained from Merck (Darmstadt, Germany), sucrose was from Penta (Chrudim, Czech Republic), minimal M9 medium was prepared according to a published protocol (Sambrook and Russell 2001), and Murashige & Skoog medium, including vitamins (MS), 2-(*N*-morpholino)ethanesulfonic acid (MES), and DMSO, were obtained from Duchefa Biochemie (Haarlem, The Netherlands). Nebularine was synthesized according to a published protocol (Nair and Chamberlain 1984) and its identity confirmed by NMR. [2-^{3}H]*tZ* of high specific activity and radiochemical purity greater than 99% was obtained from Dr. Jan Hanuš (Isotope Laboratory, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Calcein AM solution was obtained from

Invitrogen (Carlsbad, CA, USA). Olomoucine II was obtained from OlChemIm (Olomouc, Czech Republic). Silwet L-77 was purchased from Agro Bio (Opava, Czech Republic). 6-Benzylaminopurine (BAP), ampicillin, chloramphenicol, 4-methylumbelliferone, 2,4-dichlorophenoxyacetic acid, fluorescein diacetate, propidium iodide, and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

Cell Viability Assays

Human cell lines K562, CEM, and MCF-7 were used to determine the cytotoxicity of nebularine by means of a Calcein AM assay, as previously described (Doležal and others 2006). The suspension cultures of tobacco BY-2 cells and cell viability assays (performed using double staining with fluorescein diacetate and propidium iodide) were according to Mlejnek and Procházka (2002).

CDK2 and ABL Kinases Assays

Human CDK2-cyclin E and mouse ABL kinases were produced in Sf9 insect cells infected with appropriate baculoviral constructs and the kinase assays were done as previously described (Havlíček and others 2005).

Determination of Nebularine Effect on Adenine/Adenosine Deaminases

Nebularine was tested as an inhibitor of recombinant adenine deaminases (EC 3.5.4.2) AAH1 (from *Saccharomyces cerevisiae*) and SPBC1198.02 (from *Schizosaccharomyces pombe*), putative *A. thaliana* adenosine deaminase (EC 3.5.4.4) At4g04880 overexpressed in modified *E. coli* (Pospíšilová and others 2008), and adenosine deaminase ADA1 from human erythrocytes (certified reference material BCR-647, Sigma-Aldrich). The enzymatic activity was measured as an increase in hypoxanthine/inosine concentration with 0.067 mM adenine/adenosine as substrate in 0.2 M potassium phosphate buffer as described earlier (Pospíšilová and others 2008), with the addition of 0.067 mM nebularine. DMSO, used for dissolving nebularine, was added to all reaction mixtures in a final concentration of 0.7%.

Spraying of *A. thaliana* Plants with Nebularine

A. thaliana plants, grown in a greenhouse for 25 and 38 days, were sprayed with various concetrations of nebularine. Older plants, bearing mainly fully developed leaves, were divided into two groups that were sprayed either on about one half of their area or over their entire area. Young plants were divided into groups of five and treated with 0.01–1 mM nebularine solution containing 0.01% Silwet L-77; the control group was sprayed with only the surfactant water solution.

Evaluation of Nebularine Effect on the Roots of *A. thaliana*

A. thaliana seeds were sterilized with 70% ethanol and 0.1% Triton X-100 and then washed with 70% ethanol containing 0.01% Triton X-100. Then the seeds were transferred to vertical square petri dishes (14 seeds per dish) on a solid medium consisting of MS (2.15 g/l), MES (0.5 g/l), sucrose (1 g/l), agar (11 g/l), and various concentrations of nebularine, BAP, or both, all in triplicate. The compounds were dissolved in DMSO, thus 0.05% DMSO was used as a control solution. After incubation for 3 days in darkness at 4°C, the *A. thaliana* seeds were germinated and grown in the environmental test chamber (brightness 15,000 1×, humidity 55%, 16 h day/8 h night, 22°C). After 3 days, the length of the primary root tips was marked and the germinated seeds were grown for an additional 7 days. The plants were then photographed and the images were evaluated using Scion Image software (Scion Corporation, Frederick, MD, USA). The number of lateral roots was scored under a stereomicroscope.

Cytokinin Bioassays

Classical cytokinin bioassays (tobbaco callus, *Amaranthus* assay, and senescence assay with excised wheat leaves) were performed as described in Holub and others (1998).

Bacterial Cytokinin Assays

In receptor bioassays, transgenic bacterial strains expressing cytokinin receptor CRE1/AHK4 or AHK3 were grown overnight (17 h) in M9 medium (Sambrook and Russell 2001) with 1–50 μM nebularine and *trans*-zeatin (*t*Z), respectively. Activation of the cytokinin signaling pathway was detected as a fluorescence of the product 4-methylumbelliferone (MU), which is produced by degradation of 4-methylumbelliferyl galactoside (MUG) by bacterial β-galactosidase. The activity was expressed as pmol MU/h per OD_{600} as described previously (Spíchal and others 2004).

Live Cell Cytokinin-Binding Assay

The same bacterial strains and growing conditions were used as described above. The live cell cytokinin-binding assay was performed essentially as described in Romanov and others (2005), albeit with slight modification. Aliquots of 1 ml of homogeneous bacterial suspension, with OD_{600}

around 1.0, were dialyzed against the same volume of M9 medium containing 2 nM [2-^{3}H]*tZ* in the presence or absence of 10 μM nebularine, 10 μM *tZ* (positive control), and 10 μM adenine (negative control) in a Dianorm equilibrium dialyzer (Dianorm–Geräte, Munich, Germany) for 2 h at 4°C. Radioactivity of the dialysates was measured by scintillation counting using a Beckman LS 6500 scintillation counter (Beckman, Ramsey, MN, USA).

Arabidopsis ARR5:GUS and *DR5:GUS* Reporter Gene Assays

Arabidopsis ARR5:GUS or *DR5:GUS* transgenic seeds were surface-sterilized and sown on half-strength MS medium supplemented with 0.1% (w/v) sucrose and 0.05% (w/v) MES-KOH (pH 5.7) in 6-well plates. After pretreatment at 4°C for 3 days in darkness, the seedlings were grown under long-day conditions (16 h light/8 h dark) at 22°C in a growth chamber. To the wells containing 3-day-old seedlings, BAP, 2,4-dichlorophenoxyacetic acid (2,4-D), nebularine, or DMSO (solvent control, final concentration 0.1%) was added and the seedlings were grown for an additional 16 h. Quantitative determination of GUS activity was performed according to a published protocol (Romanov and others 2002) by measuring fluorescence on a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 365 and 450 nm, respectively. GUS specific activity was expressed as nmol 4-methylumbelliferone/h/mg protein. Determination of protein content was done by bicinchonin acid reagent (Smith and others 1985).

Results

Cytotoxic Effect of Nebularine on Human and Plant Cells

First, we compared the cytotoxic effect of nebularine on animal and plant cells. Selected human cell lines were treated with increasing concentrations of nebularine and the number of viable cells was determined by the Calcein AM assay (Weston and Parish 1990) to estimate concentrations lethal to 50% of the cells (IC_{50} values). As shown in Table 1 for all tested human cell lines, nebularine showed a 3-4-fold higher toxicity than olomoucine II, a typical cell cycle inhibitor (Kryštof and others 2002). To study its toxicity to plant cells, a tobacco BY-2 cell suspension was treated with nebularine. Figure 2 shows the dose- and time-dependent increase in the proportion of dead cells in the BY-2 tobacco suspension grown in the presence of various concentrations of nebularine. In as little as 24 h the number of dead cells increased to more than 60% when 100 μM nebularine was present in the growth medium, and after an additional 24 h almost all the cells were dead. The IC_{50} value of 20 μM estimated after 48 h of incubation indicated that the inhibitory effect of nebularine on the growth of the tobacco BY-2 cells was only about ten times less than that on mammalian cell lines (Table 1).

In further tests, inhibition of human CDK2-cyclin E and mouse ABL kinases by nebularine was studied in vitro to investigate whether the inhibition of protein kinases utilizing ATP as a substrate can be the origin of the toxic effect. In both assays, significant inhibition of kinase activity was not observed, even at 100 μM concentration (results not shown).

Table 1 Toxicity of nebularine for human cell lines K562, CEM, and MCF-7 and tobacco BY-2 cells

Cell line	IC_{50} (μM)	
	Nebularine	Olomoucine II
K562	2.2	9.1
CEM	1.1	4.9
MCF-7	3.6	12
BY-2	20	–

The effect of nebularine was evaluated after 72 and 48 h for mammalian cell lines and BY-2 cells, respectively

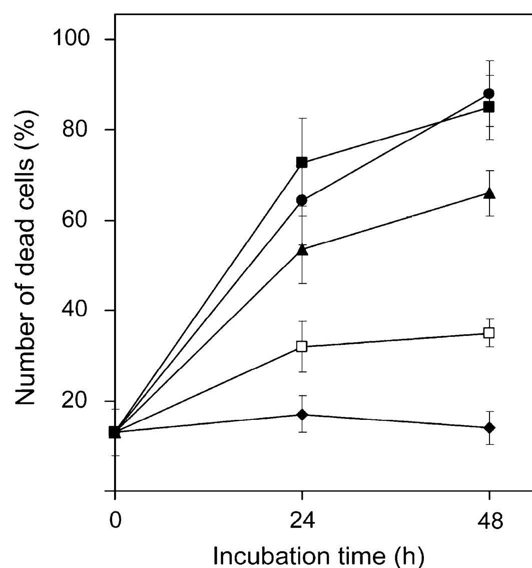

Fig. 2 Cytotoxic effect of nebularine on BY-2 cell suspensions. Cells were treated with the indicated concentrations of nebularine, 10 μM (-□-), 50 μM (-▲-), 100 μM (-●-) for 24 and 48 h. 50 μM BAPR (6-benzylaminopurine-9-riboside) was used as positive control (-■-); cells cultured with 0.1% DMSO were used as negative solvent control (-◆-). The number of dead cells was examined microscopically in at least 400 cells. Values represent mean from two replicate experiments. Error bars represent SD ($n = 2$)

Inhibitory Effect of Nebularine on Adenine/Adenosine Deaminases

Human adenosine deaminase is known to be competitively inhibited by nebularine (Osborne and Spencer 1973). For this reason the nebularine effect on the activity of putative *A. thaliana* adenosine deaminase and yeast adenine deaminases AAH1 and SPBC1198.02 was tested in this study. As a positive control, human adenosine deaminase was used. Indeed, addition of a nebularine concentration equimolar to the substrate (0.067 mM) decreased inosine production to 60%. Despite the structural similarity of eukaryotic adenine deaminases with adenosine deaminases, the activities of yeast deaminases were not inhibited (Pospíšilová and others 2008). Inhibition of putative *A. thaliana* adenosine deaminase by nebularine was not observed as well.

Spraying of *A. thaliana* Plants with Nebularine

Next, we studied the impact of nebularine application on the development of plants in vivo. *A. thaliana* plants (38 days old) with fully developed leaves (Fig. 3a1) were sprayed with 1 mM nebularine. When sprayed on only half of the leaf area, yellowing and formation of purple pigments were observed on the treated leaves. From the day 8 onward, local drying was detected (Fig. 3a2), which progressed into wilting after 2 weeks (Fig. 3a3). In 3 weeks, the sprayed leaves were withered, but the rest of the plant remained intact (Fig. 3a4) and was able to resume development. On the other hand, fully sprayed *A. thaliana* plants of the same age were completely withered within this period (Fig. 3b4) and dead in the next few days. Younger leaves were more resistant to the nebularine effect (Fig. 3b3).

For further experiments, 25-day-old *A. thaliana* plants were selected (Fig. 3d1). Plants sprayed with 1 mM nebularine showed damaged leaves from day 6 (Fig. 3d2). The plants were almost completely withered on day 12 (Fig. 3d3) and dead on day 17 (Fig. 3d4). Nebularine in 0.1 mM concentration caused bending of the leaves on day 17 (Fig. 3c2) in comparison with the control plants (Fig. 3c1). In the following days, older leaves started accumulating purple pigments (Fig. 3c3) and wilting (Fig. 3c4), but the youngest leaves retained their green color until 47 days after nebularine application (Fig. 3c5). Plants treated with 0.1 mM nebularine were still alive 2 months after spraying. After the next 20 days of

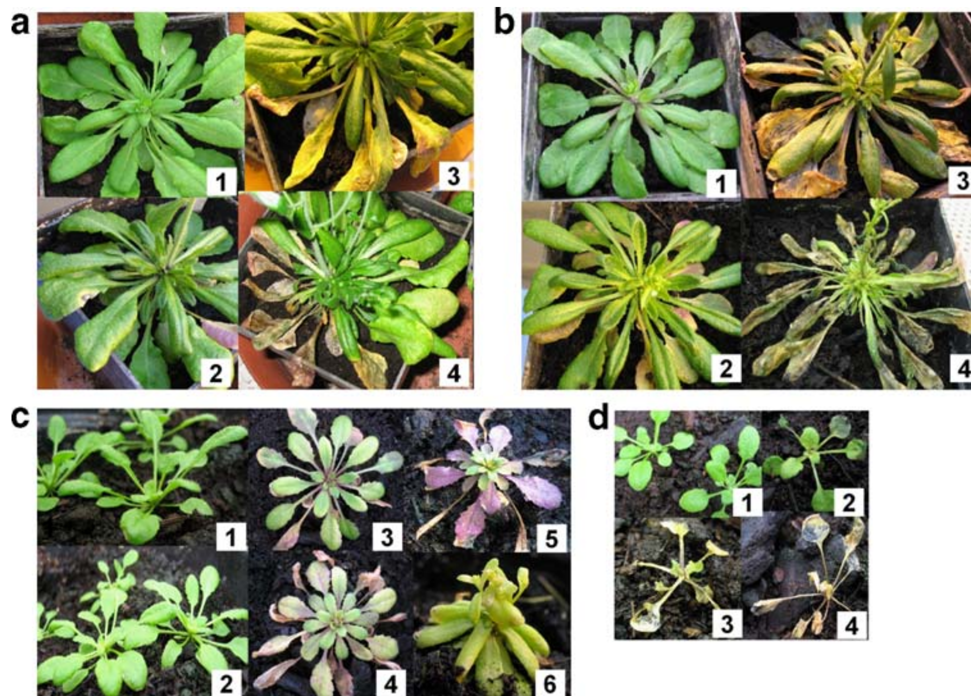

Fig. 3 Effect of nebularine application on *A. thaliana* plants. **a** Partially (only half of the leaf area) sprayed, 38-day-old *A. thaliana* plants (1 mM nebularine, 0.01% Silwet): (1) day 1, (2) day 8, (3) day 14, (4) day 21. **b** Fully sprayed, 38-day-old *A. thaliana* plants (1 mM nebularine, 0.01% Silwet): (1) day 1, (2) day 8, (3) day 14, (4) day 21. **c** Fully sprayed, 25-day-old *A. thaliana* plants (0.1 mM nebularine, 0.01% Silwet): (1) control plant sprayed only with 0.01% Silwet solution, day 17; nebularine sprayed plants: (2) day 17, (3) day 29, (4) day 39, (5) day 47, (6) plants after 61 days of nebularine application followed by 20 days of regeneration without nebularine. **d** Fully sprayed, 25-day-old *A. thaliana* plants (1 mM nebularine, 0.01% Silwet): (1) control plant (0.01% Silwet), nebularine-sprayed plants: (2) day 6, (3) day 12, (4) day 17

regeneration without nebularine, these dwarfed plants started to flower (Fig. 3c6). *A. thaliana* plants sprayed with 0.01 mM nebularine were not affected at all (not shown).

Nebularine Effects on Root Length and Lateral Root Formation

The effect of nebularine on *A. thaliana* roots was tested with approximately 100 seedlings per treatment. Seedlings grown in the presence of BAP exhibited a shorter primary root and inhibiton of lateral root formation. In agreement with a previous report (Laplaze and others 2007), the phenotype became more severe with increased concentration of BAP (Fig. 4a, b). Lateral root formation was completely blocked in plants grown on a medium containing 5 µM BAP (Fig. 4d). In the case of nebularine-treated plants, root length shortening was very similar to that caused by BAP, but not so profound (Fig. 4a). On the other hand, increasing nebularine concentration led to increased lateral root formation (Fig. 4a, b). When the plants were treated with both compounds, nebularine reversed the effect of BAP on lateral root formation (Fig. 4a).

Cytokinin Bioassays

The cytokinin bioassays evaluate various biological effects of exogenously applied cytokinins and can be used for the determination of cytokinin agonistic or antagonistic activities of tested compounds. Standard bioassays based on the stimulation of cytokinin-dependent tobacco callus growth, senescence assay measured as the retention of chlorophyll in excised wheat leaves (tip section of the first leaf of 7-day-old *Triticum aestivum* cv.), and dark induction of betacyanin synthesis in *Amaranthus* cotyledons were used to confirm the antagonistic effect of nebularine described previously by Brown and Konuk (1994).

In our experiments, nebularine antagonized the effect of BAP in senescence and tobacco callus assays but not in the *Amaranthus* bioassay (Fig. 5). In the senescence assay, nebularine at a concentration equimolar to BAP reduced the cytokinin effect to 55%, but the effect was not more pronounced with increasing concentration (Fig. 5a). In contrast, we could not repeat the results published by Brown and Konuk (1994) for the competition of nebularine with kinetin in the *Amaranthus* assay, where we did not observe any antagonistic activity, even when nebularine was applied at a 100-fold higher concentration than BAP (Fig. 5b). In the callus assay, where the effect of the tested compound on proliferation of cytokinin-dependent tobaco callus is scored, we observed a concentration-dependent decrease in callus growth that might point to an antagonistic activity (Fig. 5c). However, keeping in mind the IC_{50} of nebularine (20 µM) in the BY-2 tobacco cell suspension cultures, which is equivalent to a concentration causing 50% reduction of callus growth stimulated by 1 µM BAP, this effect cannot be attributed to cytokinin antagonism but rather to the cytotoxic effect of nebularine on plant cells in general.

Interaction of Nebularine with Cytokinin Receptors

To investigate whether nebularine can be classified as a cytokinin antagonist, we studied its interactions with the CRE1/AHK4 and AHK3 cytokinin receptors of *Arabidopsis* using *E. coli* strains expressing these receptors and the

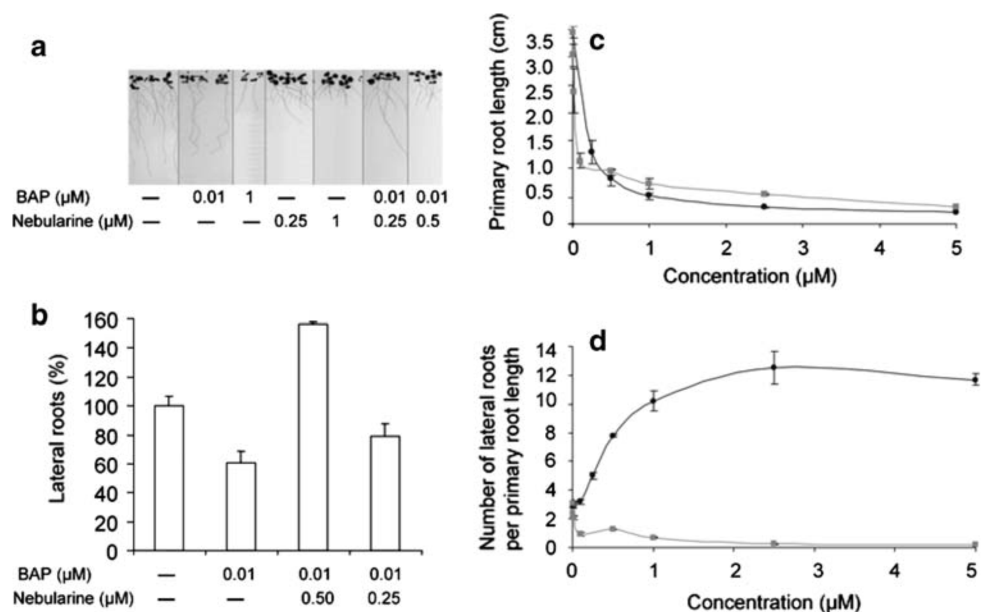

Fig. 4 Effect of nebularine and BAP on root formation in *A. thaliana*. Sterilized seeds were grown on MS medium for 7 days with various concentrations of the compounds to be tested. **a** Phenotype of the seedling. **b** Number of lateral roots related to the primary root length. **c**, **d** Effect of nebularine (-●-) on the primary root length and lateral root formation in comparison with BAP (-■-). Error bars show SD ($n = 100$)

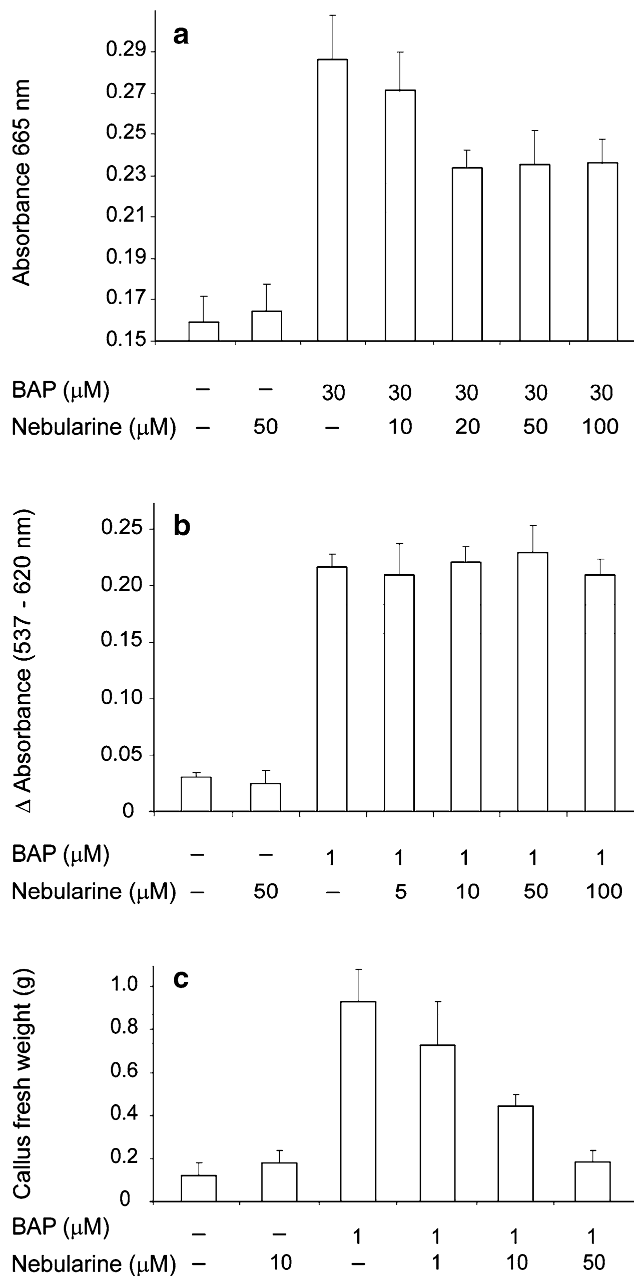

Fig. 5 Cytokinin bioassays. **a** Senescence assay. Retention of chlorophyll in detached leaves was measured spectrophotometrically after 6 days of cultivation with BAP and different concentrations of nebularine. For the control (measured immediately after detaching the leaves), the mean value of A_{665} was 0.867 ± 0.04. **b** *Amaranthus* assay. Monitoring of pigment production in *Amaranthus caudatus* treated with 1 μM BAP and increasing concentrations of nebularine. **c** Callus assay. Tobacco callus weight accumulation at different concentrations of nebularine and/or BAP. Error bars show SD ($n = 5$)

cytokinin-activated reporter *cps::lacZ* (Spíchal and others 2004; Yamada and others 2001). As shown in Fig. 6a, nebularine did not activate the receptors AHK3 and CRE1/AHK4, even at a concentration 500-fold higher than that required for receptor activation by *tZ*. Furthermore, the direct binding assay based on the competition of nebularine with radiolabeled natural cytokinin [2-^{3}H]-zeatin was carried out using unlabeled *tZ* and adenine as positive and negative controls, respectively. Data presented in Fig. 6b clearly show that nebularine was not able to block the binding of natural ligand, even at 5000-fold excess.

Arabidopsis ARR5:GUS and *DR5:GUS* Reporter Gene Assay

To find out whether the proposed antagonistic effect of nebularine takes place in the signaling pathway downstream of the cytokinin receptor, the effect of nebularine on cytokinin-induced expression of the response regulator gene *ARR5* (D'Agostino and others 2000) was investigated *in planta*. For this experiment, transgenic *Arabidopsis* plants harboring the *ARR5:GUS* reporter were used and the results are shown in Fig. 7a. Interestingly, nebularine in concentrations above 10 μM decreased the expression of the *ARR5:GUS* gene induced by 1 μM BAP to about 70%. To confirm that this effect can be attributed to a specific inhibition of cytokinin signal transduction, we performed the same assay with transgenic *Arabidopsis* seedlings harboring the auxin-responsive gene *DR5:GUS* (Ulmasov and others 1997) under the same conditions. Surprisingly, nebularine also decreased the expression of *DR5:GUS* induced by 1 μM 2,4-D to a level comparable to that in the *ARR5:GUS* assay (Fig. 7b). This indicates that nebularine does not act as an anticytokinin, at least in terms of inhibition of cytokinin signaling.

Discussion

As described before, nebularine inhibits several enzymes in a variety of tissues. Whereas adenosine deaminase from many sources is well known to be competitively inhibited by nebularine (Osborne and Spencer 1973), adenine deaminases from lower eukaryotes, frequently also marked as adenosine deaminases due to their amino acid sequence similarity (Pospíšilová and Frébort 2007), were not inhibited by nebularine at all. Inhibition by nebularine was also not detected for putative adenosine deaminase from *A. thaliana* prepared recently (Pospíšilová and others 2008).

It has been reported that some pyrrolo[2,3-*d*]pyrimidines and pyrazolo[4,3-*d*]pirimidines, previously classified as potent anticytokinins, do not interact with cytokinin receptors but inhibit cyclin-dependent kinases (Spíchal and others 2007). However, inhibitory effects of nebularine on mammalian CDK2 and ABL kinases were not observed in the present work.

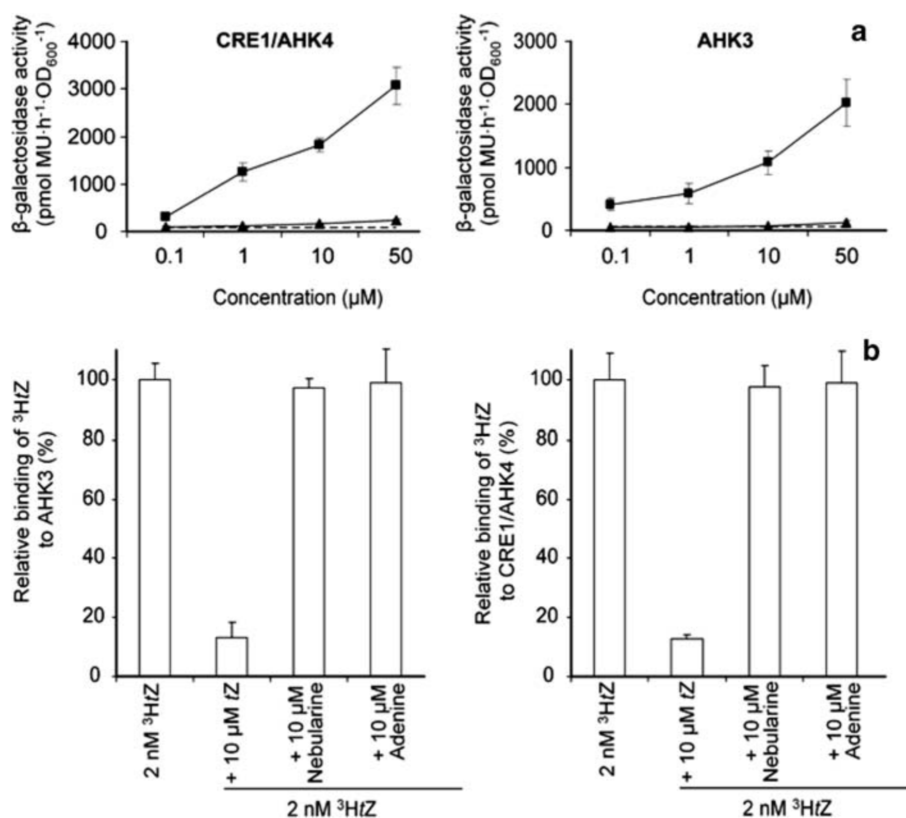

Fig. 6 Interaction of nebularine with cytokinin receptors. **a** Activation of cytokinin receptors CRE1/AHK4 and AHK3 by nebularine (-▲-) compared to cytokinin *tZ* (-■-). **b** Competition of [2-^{3}H]*tZ* with nebularine for the binding to cytokinin receptors. Unlabeled *tZ* and adenine were used as positive and negative controls, respectively. Error bars show SD ($n = 3$)

The effect of 1 mM nebularine on plants resembles that described in animals (Biesele and others 1955; Kozlowska and others 1999; Price and Murray 1969). Interestingly, younger leaves remained green and healthy, which may be a reflection of the presence of higher concentrations of cytokinins in developing tissues (Schmülling 2002), which agrees with previous studies (Biesele and others 1955; Tokuji and Kuriyama 2002). Application of 0.1 mM nebularine led to dwarfing and withering of the older leaves, but regenerated plants were still capable of flowering.

Nebularine, like BAP, showed concentration-dependent shortening of the primary roots. BAP also inhibited lateral root formation, whereas nebularine complemented this inhibition and increased the number of lateral roots per centimeter of primary root length in a hyperbolic manner, reaching a limit at 2.5 μM, probably because of nebularine triphosphate accumulation and inhibition of some enzymes of purine metabolism (Fig. 4d). The nebularine effect did not occur below the threshold of 0.25 μM that may result from the level of adenine derivatives in plants, as described above. In this experiment, nebularine behaves as an anticytokinin in accordance with an earlier report (Brown and Konuk 1994). In the senescence assay, we observed a significant antagonistic effect of nebularine in even equimolar concentrations toward BAP. However, in contrast to the results of Brown and Konuk (1994), we did not observe any antagonistic effect of nebularine towards BAP in the *Amaranthus* bioassay. Nebularine inhibited cytokinin-dependent tobacco callus growth induced by 1 μM BAP in a dose-dependent manner, but this effect is likely to be due to nebularine toxicity that was found with tobacco BY-2 cells at the same concentrations.

Tests with *Arabidopsis* cytokinin receptors AHK3 and CRE1/AHK4 indicated that nebularine does not interact with the receptors and is not able to compete with natural cytokinins at the receptor level. Interestingly, nebularine blocked the cytokinin primary response gene *ARR5*, but also an auxin reporter *DR5*.

The results described in this work clearly show that nebularine does not act as a cytokinin antagonist specifically interacting with a cytokinin signaling pathway. The anticytokinin-like effect of nebularine is thus probably a consequence of the inhibiton of the same enzymes as those it inhibits in mammals (Guranowski and others 1981; Lupidi and others 1982).

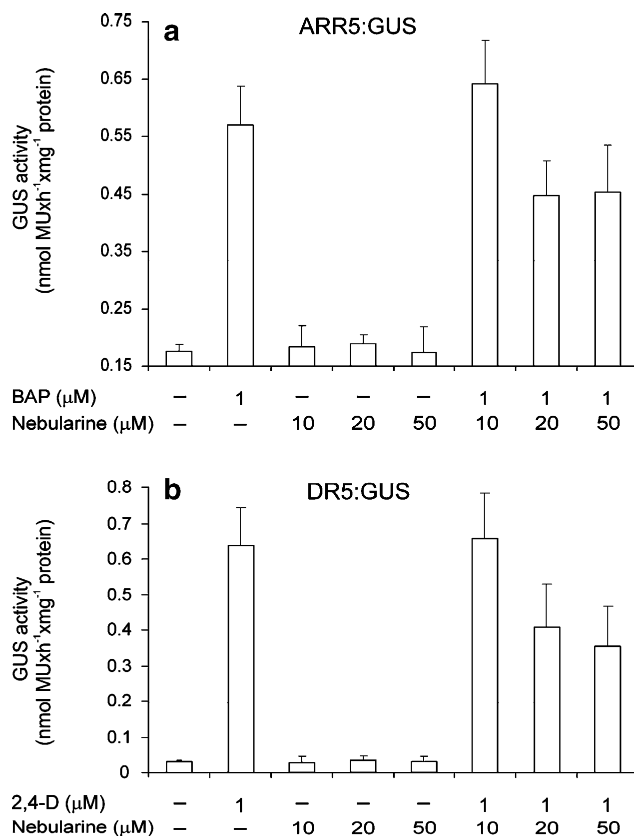

Fig. 7 Effect of nebularine on the expression of cytokinin and auxin-inducible reporters *in planta*. *A. thaliana ARR5:GUS* (**a**) and *DR5:GUS* (**b**) seedlings were grown for 16 h in the presence of nebularine (10-50 μM) and 1 μM BAP or 2,4-D and the activity of produced β-glucuronidase was measured. Error bars show SD ($n = 2$)

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References

Backer WS, Khan JA (2004) Effects of pharmacological agents on the activity of rat kidney adenylyl cyclase. Pak J Med Sci 20:41–45

Biesele JJ, Slautterback MS, Margolis M (1955) Unsubstituted purine and its riboside as toxic antimetabolites in mouse tissue cultures. Cancer 8:87–96

Bohr V (1978) Effects of purine riboside on nucleic acid synthesis in ascites cells. Biochim Biophys Acta 519:125–137

Brink NG (1953) Beef liver glucose dehydrogenase. 1. Purification and properties. Acta Chem Scand 7:1081–1089

Brown EG, Konuk M (1994) Plant cytotoxicity of nebularine (purine riboside). Phytochemistry 37:1589–1592

D'Agostino IB, Deruère J, Kieber JJ (2000) Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. Plant Physiol 124:1706–1717

Das SR, Baer HP (1991) Inhibition of axenically grown *Entamoeba histolytica* by purine nucleoside analogs and actions of natural nucleosides. Trop Med Parasitol 42:161–163

Doležal K, Popa I, Kryštof V, Spíchal L, Fojtíková M, Holub J, Lenobel R, Schmülling T, Strnad M (2006) Preparation and biological activity of 6-benzylaminopurine derivatives in plants and human cancer cells. Bioorg Med Chem 14:875–884

Ehrenberg L, Hedström H, Löfgren N, Takman B (1946) Antibiotic effect of agarics on tubercle bacilli. Svensk Kem Tidskr 58:267

el Kouni MH, Cha S (1987) Metabolism of adenosine analogues by *Schistosoma mansoni* and the effect of nucleoside transport inhibitors. Biochem Pharmacol 36:1099–1106

Frank KB, Cheng YC (1986) Inhibition of herpes simplex virus DNA polymerase by purine ribonucleoside monophosphates. J Biol Chem 261:1510–1513

Guranowski A (1982) Purine catabolism in plants: Purification and some properties of inosine nucleosidase from yellow lupin (*Lupinus luteus* L.) seeds. Plant Physiol 70:344–349

Guranowski A, Montgomery JA, Cantoni GL, Chiang PK (1981) Adenosine analogues as substrates and inhibitors of *S*-adenosylohomocysteine hydrolase. Biochemistry 20:110–115

Havlíček L, Fuksová K, Kryštof V, Orság M, Vojtěšek B, Strnad M (2005) 8-Azapurines as new inhibitors of cyclin-dependent kinases. Bioorg Med Chem 13:5399–5407

Henderson JF (1968) Purine nucleoside inhibitors of purine biosynthesis *de novo*. Cancer Chemo Rep 2:375–382

Hoffman PD, Wang H, Lawrence CW, Iwai S, Hanaoka F, Hays JB (2005) Binding of MutS mismatch repair protein to DNA containing UV photoproducts, "mismatched" opposite Watson-Crick and novel nucleotides, in different DNA sequence contexts. DNA Repair 4:983–993

Holub J, Hanuš J, Hanke DE, Strnad M (1998) Biological activity of cytokinins derived from *ortho*- and *meta*-hydroxybenzyladenine. Plant Growth Regul 26:109–115

Kozlowska M, Smolenski RT, Makarewicz W, Hoffmann C, Jastorff B, Swierczynski J (1999) ATP depletion, purine riboside triphosphate accumulation and rat thymocyte death induced by purine riboside. Toxicol Lett 104:171–181

Kryštof V, Lenobel R, Havlíček L, Kuzma M, Strnad M (2002) Synthesis and biological activity of olomoucine II. Bioorg Med Chem Lett 12:3283–3286

Kulka RG (2006) Cytokinins inhibit epiphyllous plantlet development on leaves of *Bryophyllum* (Kalanchoë) *marnierianum*. J Exp Bot 57:4089–4098

Laplaze L, Benková E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, Offringa R, Graham N, Doumas P, Friml J, Bogusz D, Beeckman T, Bennett M (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 19:3889–3900

Löfgren N, Lüning B, Hedström H (1954) The isolation of nebularine and the determination of its structure. Acta Chem Scand 8:670–680

Lupidi G, Riva F, Cristalli G, Grifantini M (1982) Inhibition of adenosine deaminase by deaza derivatives of adenosine and purine riboside. Ital J Biochem 31:396–403

Lynch TP, Jakobs ES, Paran JH, Paterson AR (1981) Treatment of mouse neoplasm with high doses of tubercidin. Cancer Res 41:3200–3204

Mlejnek P, Procházka S (2002) Caspase activation and isopentenyladenosine-induced apoptosis in tobacco BY-2 cells. Planta 215:158–166

Nair V, Chamberlain SD (1984) Reductive deamination of aminopurine nucleosides. Synthesis 1984:401–403

Osborne WR, Spencer N (1973) Partial purification and properties of the common inherited forms of adenosine deaminase from human erythrocytes. Biochem J 133:117–123

Paterson AR, Paran JH, Yang S, Lynch TP (1979) Protection of mice against lethal dosages of nebularine by nitrobenzylthioinosine, an inhibitor of nucleoside transport. Cancer Res 39:3607–3611

Pospíšilová H, Frébort I (2007) Aminohydrolases acting on adenine, adenosine and their derivatives. Biomed Papers 151:3–10

Pospíšilová H, Šebela M, Novák O, Frébort I (2008) Hydrolytic cleavage of N^6-substituted adenine derivatives by eukaryotic adenine and adenosine deaminases. Biosci Rep 28:335–347

Price CE, Murray AW (1969) Purine metabolism in germinating wheat embryos. Biochem J 115:129–133

Rahman MS, Humayun MZ (1997) Nebularine (9–2′-deoxy-β-D-ribofuranosylpurine) has the template characteristics of adenine in vivo and in vitro. Mutat Res 377:263–268

Romanov GA, Kieber JJ, Schmülling T (2002) A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signalling. FEBS Lett 515:39–43

Romanov GA, Spíchal L, Lomin SN, Strnad M, Schmülling T (2005) A live cell hormone-binding assay on transgenic bacteria expressing a eukaryotic receptor protein. Anal Biochem 347: 129–134

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, vol 3, 3rd edn. Cold Spring Harbor Press, Cold Spring Harbor, p A2.2

Schmülling T (2002) New insights into the functions of cytokinins in plant development. J Plant Growth Regul 21:40–49

Smith CM, Snyder FF, Fontenelle LJ, Henderson JF (1974) Improved methods for the study of drug effects on purine metabolism and their application to nebularine and 7-deazanebularine. Biochem Pharmacol 23:2023–2035

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Garmer FH, Provenzano MD, Fujimoto FK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchonic acid. Anal Biochem 150:76–85

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol 45:1299–1305

Spíchal L, Kryštof V, Paprskářová M, Lenobel R, Stýskala J, Binarová P, Cenklová V, De Veylder L, Inzé D, Kontopidis G, Fischer PM, Schmülling T, Strnad M (2007) Classical anticytokinins do not interact with cytokinin receptors but inhibit cyclin-dependent kinases. J Biol Chem 282:14356–14363

Spíchal L, Werner T, Popa I, Riefler M, Schmülling T, Strnad M (2009) The purine derivative PI-55 blocks cytokinin action via receptor inhibition. FEBS J 276:244–253

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001) The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol 42:107–113

Tamm I, Folkers K, Ch Shunk (1956) Certain benzimidazoles, benzenes, and ribofuranosylpurines as inhibitors of influenza B virus multiplication. J Bacteriol 72:59–64

Tokuji Y, Kuriyama K (2003) Involvement of gibberellin and cytokinin in the formation of embryogenic cell clumps in carrot (*Daucus carota*). J Plant Physiol 160:133–141

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9:1963–1971

Weston SA, Parish CR (1990) New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Methods 133:87–97

Wood SG, Ubasawa A, Martin D, Jiřičný J (1986) Guanine and adenine analogues as tools in the investigation of the mechanisms of mismatch repair in *E. coli*. Nucleic Acids Res 14:6591–6602

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42:1017–1023