FEBS 25874 FEBS Letters 515 (2002) 39–43

A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signalling

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Received 28 September 2001; revised 24 December 2001; accepted 31 January 2002

First published online 25 February 2002

Edited by Marc Van Montagu

Abstract **Seedlings of *Arabidopsis thaliana* harboring a fusion of the cytokinin-responsive *ARR5* gene promoter and the *GUS* reporter gene were used for a pharmacological approach to study cytokinin signal transduction. The assay was shown to be rapid, sensitive, dose-dependent and highly specific for cytokinins, both adenine and phenylurea derivatives. Numerous inhibitors of known signalling pathways were tested and some were shown to suppress reporter gene induction. Particularly, primary alcohols that specifically inhibit phospholipase D (PLD) partially prevented cytokinin-induced GUS activity and reduced the accumulation of *ARR5* gene transcripts. This indicates a role for PLD early during cytokinin signalling.**

Key words: Cytokinin; Phospholipase D; Signalling; *Arabidopsis*

1. Introduction

Cytokinins play central roles in plant cell division and in different plant developmental processes, such as chloroplast maturation, regulation of root growth and branching and plant senescence [1]. Recently, some elements of the cytokinin signalling chain have been discovered. The cytokinin receptors CRE1/AHK4/WOL, AHK2 and AHK3 are sensor histidine kinases [2–5]. The only known class of rapid cytokinin response genes of *Arabidopsis* encode response regulator (ARR) proteins [6–8] and there is increasing evidence that cytokinin signalling proceeds through components of the two-component system [9,10]. Recently it has been demonstrated that *Arabidopsis* histidine phosphotransmitters (AHPs) and response regulators have distinct roles during cytokinin signalling: the former shuttle between the cytoplasm and the nucleus in a cytokinin-dependent manner, while the latter (type B response regulators ARR1, ARR2 and ARR10) act as transcriptional activator of cytokinin target genes [11,12]. Earlier investigations have shown that classical components of signal transduction chains, e.g. Ca^{2+} and protein phosphorylation, participate also in cytokinin signalling and it could well be that more than one cytokinin signalling system exists [13–16]. However, these studies did not use primary response genes but relied on relatively slow responses to cytokinins as output reaction. Therefore, it is unclear whether these components act early during signalling or at a later stage. We used one particular member of the primary response gene family of *Arabidopsis*, *ARR5*, as a tool for a pharmacological approach to study a rapid cytokinin signalling process. The *ARR5* mRNA is almost undetectable in the absence of cytokinin and accumulates in less than 10 min following cytokinin application [6,17]. It has also been demonstrated that the reaction to cytokinin of the promoter–reporter gene fusion used in this study is similar to the resident gene. This implies that the regulation of *ARR5* is mediated at the transcriptional level [17]. We show that inhibitors of phospholipase D (PLD) block partially the accumulation of *ARR5* gene transcript and *ARR5* promoter-dependent *GUS* reporter gene activity suggesting that PLD has a role early in cytokinin signalling.

2. Materials and methods

2.1. Plant material

Transgenic *Arabidopsis* plants (*Arabidopsis thaliana* (L.) Heynh. accession Col-0) harboring the *GUS* reporter gene fused to 1.6 kb of the *ARR5* (P_{ARR5}-*GUS*) gene promoter were described [17]. Seeds of homozygous plants were surface-sterilized and soaked for 2–3 days at 4°C in sterilized distilled water. Then cold water was replaced by sterile water at room temperature and seeds were placed in a growth chamber at 24°C, 16 h light/8 h dark cycles. Seedlings grown for 3–4 days were used for the experiments.

2.2. Chemicals

Phytohormones and inhibitor substances were from Sigma (Deisenhofen, Germany), Calbiochem (Schwalbach, Germany) or Duchefa (Haarlem, The Netherlands). Roscovitin was a kind gift of Dr. M. Strnad and ethrel was a gift of Dr. Y.V. Rakitin. Stock solutions were prepared in H_2O, Me_2SO or MeOH and stored as recommended by the manufacturer.

2.3. Assay procedure

Three to four-day-old seedlings were placed into wells of a sterile cell culture cluster (Costar, New York, USA) containing 0.2 ml of sterilized distilled water. For each data point 10 plants of similar size were selected and treatments were carried out at least in duplicate. Compounds to be tested were added as microaliquots or predissolved in H_2O to the desired final concentration. Final concentrations of Me_2SO or methanol never exceeded 1%. The final assay volume was adjusted with water to 0.4 ml. The seedlings were immediately subjected to a mild vacuum infiltration for 30–40 s and then incubated for 4–7.5 h at 24°C in the dark. At the end of the incubation period solutions were removed from the wells and seedlings were washed

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0014-5793/02/$22.00
PII: S0014-5793(02)02415-8

once with 0.6 ml H_2O. Thereafter seedlings were placed into microtubes with 0.1 ml GUS extraction buffer and kept at −70°C until fluorometric assay.

2.4. GUS activity measurement

Quantitative GUS activity determinations were performed according to common protocols with only minor modifications using 4-methylumbelliferyl glucuronide as a substrate [18,19]. Fluorescence was measured with a F2000 spectrofluorometer (Hitachi, Japan) at excitation and emission wavelengths of 365 and 450 nm, respectively. Protein quantification was performed according to [20] using Roti-Q staining solution (Roth, Germany). GUS specific activity was expressed as nmol 4-methylumbelliferone (MU)/mg protein/h. Mean values, standard deviations and significance of experimental differences were calculated using a *t*-test statistical program. Generally, standard deviations did not exceed 3–4% of the mean value.

2.5. RNA blot analysis

Total RNA was extracted from plant tissues according to [21]. 25 μg RNA was separated in a denaturing 1.5% agarose–formaldehyde gel, transferred to nylon membranes (Amersham Hybond N) and hybridized with DNA probes labelled with ^{32}P using Random Primer Labelling kit (Stratagene, USA). The lowest stringency wash was performed in 0.2×SSC, 0.1% SDS at 65°C. The *ARR5* gene specific probe was a full length cDNA of *A. thaliana* accession Col-0. As a control for loading, the blot was rehybridized with an *actin 2* probe. The Northern blot was repeated once. Quantification of signals was done with the BIO-1D programme (Bio-Profil Image Analysis Software, Vilbert Lourmat, Marne-la-Vallée, France).

3. Results

3.1. Characteristics of the assay system

In order to make early events in cytokinin signalling processes amenable to pharmacological studies we aimed to establish an assay system that should be specific for cytokinins, fast, easy to use and highly reproducible with a low background. Testing of the reaction of different P_{ARR5}-*GUS* transgenic tissues at different developmental stages showed that seedlings that were germinated in distilled water responded uniformly to cytokinin application with a strong increase in GUS activity. Histochemical staining in untreated seedlings was mainly confined to the meristematic regions of the shoot and root [17]. About 2 h after gene induction an increase in staining was noted in the vasculature in all plant parts. After 6 h almost all cells of the seedling were stained. An exception was cells of the basal half of the hypocotyl and the cortex and epidermis of the primary root, which showed only a weak response or no response at all, even after 24 h treatment with 5 μM 6-benzyladenine (BA; data not shown). Fluorometric assays of the reaction kinetics in 3–4-day-old seedlings showed that the initial lag period lasted about 1 h followed by a quasi linear increase in activity for at least 24 h (Fig. 1A and data not shown). After only 2 h the GUS specific activity of BA-treated plants exceeded that of control plants by nearly two-fold, and after 7–8 h the increase was in the range of 5–6-fold. A quantitative dose–response curve (Fig. 1B) showed that with long incubation times (20 h) the induction of GUS activity was detected at BA concentrations as low as 10 nM. At higher concentrations the activation strongly increased and finally reached a plateau at about 5 μM BA. 5 μM BA was used in most subsequent experiments. The cytokinin induction of GUS activity proved to be stable between pH 5 and pH 8, with a slight decrease of induction at higher pH values (data not shown). Cytokinin induced GUS activity in the presence or absence of light (data not shown), therefore incubations were carried out in the dark to exclude light effects on inhibitor substances.

3.2. Specificity of the assay system

To prove the specificity of the assay system for cytokinins, different phytohormones and related compounds were tested in parallel with BA. Results showed that different types of cytokinins – BA, *trans*-zeatin, isopentenyladenine (2iP) and kinetin – were similarly active in the induction of GUS activity (Fig. 2). Cytokinin riboside as exemplified by 2iP-riboside was only slightly less active than the corresponding free base. Structural analogs of adenine-derived cytokinins, namely adenine and adenosine, were inactive. Not only iP- and Z-type cytokinins, but also the phenylurea derivative thidiazuron induced a strong activation of the *ARR5* promoter (Fig. 2).

None of several different other phytohormones including the auxins naphthalene-1-acetic acid and 2,4-dichlorophenoxyacetic acid, abscisic acid, gibberellic acid, the ethylene releasing compound ethrel (not shown), epibrassinolide, jasmonic acid and salicylic acid induced GUS activity in the assay system. Similarly, none of these compounds, except spermine, interfered significantly with the induction by BA (Fig. 2). Other known effector molecules such as sucrose and nitrate were also unable to induce P_{ARR5}-*GUS* gene expression. These results demonstrate the very high specificity of the *ARR5-GUS* assay for cytokinins.

3.3. Inhibitors of transcription and translation

Next we tested whether the assay system could be used for inhibitor studies. The system relies on two basic processes of gene expression: transcription and translation. Therefore known inhibitors of RNA and protein synthesis were tested for their ability to suppress the cytokinin-mediated induction of GUS activity. Indeed, cycloheximide (CHX) totally prevented the cytokinin induction of P_{ARR5}-*GUS* at the low concentration of 2–4 μg/ml (Fig. 3). Actinomycin D (ActD) inhibited the transgene expression, especially when added some time before BA (Fig. 3). These data show that *Arabidopsis* seedlings are permeable to inhibitors that act, presumably, in the cytoplasm (CHX) or cell nucleus (ActD) under the assay conditions.

3.4. Inhibitor screening identifies a role for PLD in cytokinin signalling

We used the reporter gene to screen a series of known specific pharmacological inhibitors of various signal transduction processes for their influence on the cytokinin-dependent induction of GUS activity. Most inhibitors were applied in a wide concentration range according to published data. With one exception (see below), no single compound influenced GUS expression on its own. These experiments yielded a first indication about antagonists and agonists of the reaction which would be tested further for their influence on RNA accumulation.

The results of these investigations are summarized in Table 1. The primary alcohols butan-1-ol and propan-1-ol strongly inhibited the gene induction whereas the corresponding secondary alcohols butan-2-ol and propan-2-ol did not. Primary, but not secondary alcohols are known to be specific inhibitors of PLD-catalyzed formation of phosphatidic acid [22]. Mastoparan, a known activator of heterotrimeric G-proteins,

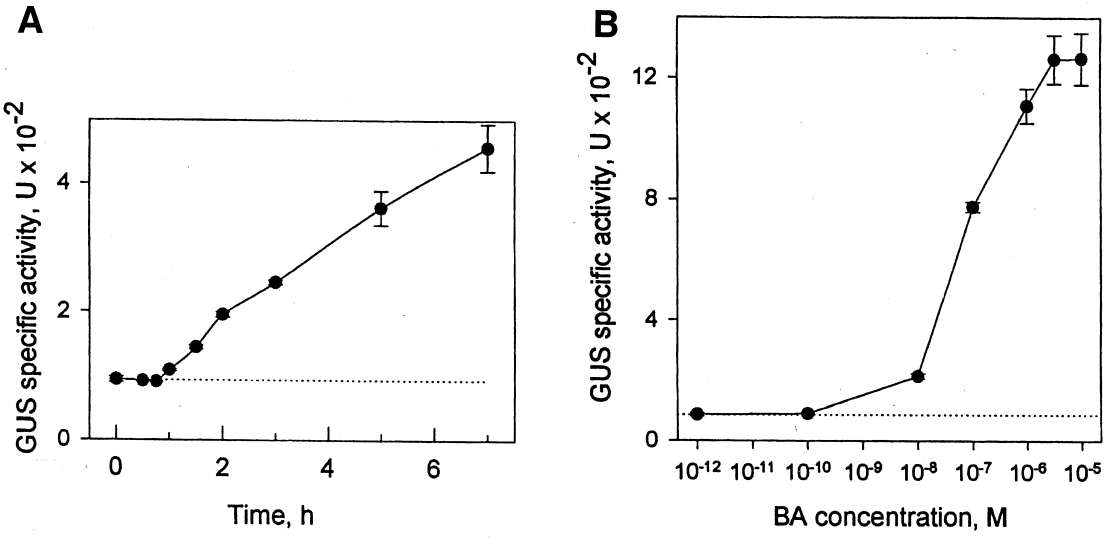

Fig. 1. Induction of the P_{ARR5}-*GUS* gene by cytokinin. A: Kinetics of induction of GUS activity. P_{ARR5}-*GUS* transgenic *Arabidopsis* seedlings were incubated with 5 μM BA for the indicated time. The GUS activity in non-induced seedlings (H_2O control) is indicated by the dotted line. B: Dose dependence of cytokinin-induced GUS activity. P_{ARR5}-*GUS* transgenic *Arabidopsis* seedlings were incubated for 20 h in different BA concentrations. The GUS activity in non-induced seedlings (H_2O control) is indicated by the dotted line.

which often act in animals upstream of phospholipases [23,24], did not activate GUS expression (data not shown).

The Ca^{2+} chelator BAPTA and the calmodulin antagonist W7 completely inhibited GUS activation by cytokinin, indicating that Ca^{2+}/calmodulin are important for this cytokinin response. Among different inhibitors of protein phosphatases [25], calyculin A, a very potent inhibitor of protein phosphatases 1 and 2A, was a strong inhibitor, while okadaic acid and tautomycin were less effective or not effective at all (Table 1). K252a, a broad spectrum inhibitor of protein kinases [25], partially inhibited the reaction. Genistein, an inhibitor of tyrosine kinases [26], and roscovitin, an inhibitor of cyclin-dependent kinases [27], lacked any inhibitory effect. Roscovitin was the only compound tested that induced by itself the accumulation of GUS activity, probably due to its chemical resemblance to adenine-derived cytokinins.

3.5. RNA blot analysis indicates an early role for PLD

Next we tested inhibitors of PLD and Ca^{2+} signalling for their ability to prevent transcript accumulation of the *ARR5* gene. Suppression of accumulation of *ARR5* mRNA would indicate an involvement in an early step of cytokinin signalling. Fig. 4 shows that in the presence of butan-1-ol only 42% of the *ARR5* transcript accumulated compared to cytokinin treatment alone. In contrast, treatment with BAPTA did not inhibit *ARR5* transcript accumulation, indicating that this compound does not act prior to transcription of the target gene.

4. Discussion

The most important result of our study is that specific inhibitors of PLD reduced the accumulation of GUS activity and *ARR5* mRNA by 60–70%, indicating that PLD has a role in cytokinin signalling prior to transcription. Similarly, a kinetic study performed in *Amaranthus*, where PLD antagonists inhibit the cytokinin-induced accumulation of amaranthin, has indicated that PLD antagonists act prior to transcription

Table 1
Influence of compounds on the cytokinin-induced GUS activity in P_{ARR5}-*GUS* transgenic *Arabidopsis*

Compound[a]	Concentration	Induction of GUS activity (%)[b]	Target(s)
Butan-1-ol	1.0%	22 ± 2	PLD
Propan-1-ol	1.0%	29 ± 3	PLD
Butan-2-ol	1.0%	100	Analog of butan-1-ol
Propan-2-ol	1.0%	100	Analog of propan-1-ol
EDTA	10 mM	0	Divalent cations
BAPTA	10 mM	2 ± 2	Calcium
W7	500 μM	0	Calmodulin
Calyculin A	2 μM	9 ± 1	Protein phosphatases (1 = 2A)
Okadaic acid	2 μM	67 ± 6	Protein phosphatases (2A > 1)
Tautomycin	0.5–2 μM	100	Protein phosphatases (1 > 2A)
K252a	4 μM	40 ± 19	Protein kinases
Genistein	50–500 μM	100	Tyrosine kinases
Roscovitin[c]	10–100 μM	100	Cyclin-dependent kinases

[a]Seedlings were treated with nothing or drugs at indicated concentrations for 15–20 min and subsequently with BA (5 μM) as described in Section 2.
[b]Data show GUS activity above water control (0). Activity with BA (5 μM) and without drug was set to 100.
[c]100 μM roscovitin alone induced 39% of the GUS activity induced by 5 μM BA.

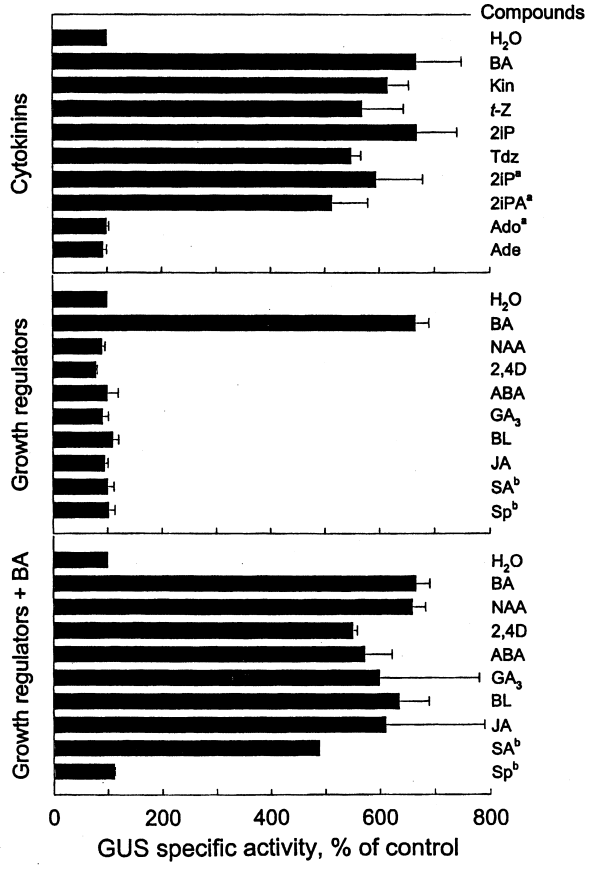

Fig. 2. Influence of different cytokinins and other growth regulators alone or in combination with cytokinin (5 μM BA) on the GUS activity in P_{ARR5}-*GUS* transgenic seedlings. Seedlings were incubated for 7.5 h in the presence of 5 μM, 1 μM[(a)] or 50 μM[(b)] of the indicated compound. Water control was treated as 100% (86 ± 5.4 nmol MU/mg protein/h). Abbreviations: BA, 6-benzyladenine; Kin, kinetin; *t*-Z, *trans*-zeatin; 2iP, isopentenyladenine; Tdz, thidiazuron; 2iPA, isopentenyladenosine; Ado, adenosine; Ade, adenine; NAA, naphthalene-1-acetic acid; 2,4D, 2,4-dichlorophenoxyacetic acid; ABA, abscisic acid; GA_3, gibberellic acid; BL, epibrassinolide; JA, jasmonic acid; SA, salicylic acid; Sp, spermine.

[16]. At least seven putative PLD genes belonging to four different clusters have been identified in *Arabidopsis* [22,28]. PLD releases phosphatidic acids and hydrophilic head groups as second messenger molecules and is, in animal systems, often activated via different G-proteins. PLD has been shown to be involved in different signalling processes in plants [23,24], including abscisic acid signalling and during the wound reaction. The inability to mimic the activity of cytokinins with mastoparan indicates that the PLD involved is not activated through a seven-transmembrane receptor–heterotrimeric G-protein–PLD chain but that the PLD(s) involved in cytokinin signalling might be coupled to another system. However, it cannot be excluded that a mastoparan insensitive G-protein is involved in cytokinin signalling nor that an additional G-protein-independent pathway needs to be activated in parallel to achieve *ARR5* gene activation.

The inhibitory activity of the PLD antagonists was incomplete (Table 1). This could on the one hand be due to the competitive nature of the inhibition reaction, which leads to only partial inhibition of the enzyme [22]. Another reason might be that different independent cytokinin signalling pathways to the *ARR5* promoter exist. One pathway is likely to

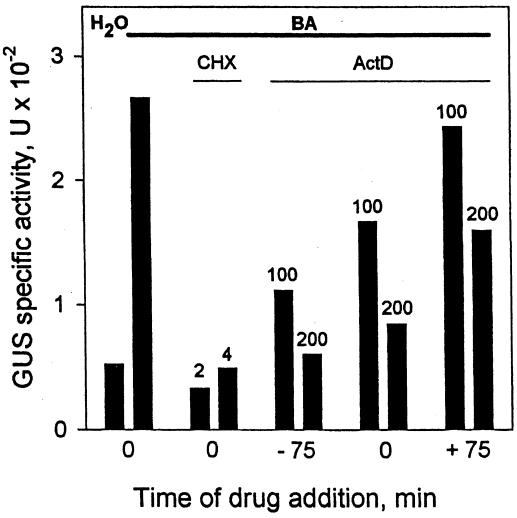

Fig. 3. Effect of inhibitors of RNA and protein synthesis on cytokinin-induced GUS activity. P_{ARR5}-*GUS* transgenic *Arabidopsis* seedlings were incubated for 7.5 h in the presence or absence of 5 μM BA. CHX or ActD were added to the samples at different indicated times relative to the addition of BA. Numbers above bars indicate drug concentration (μg/ml).

operate through the known cytokinin receptors [2–5] and the two-component signalling system of *Arabidopsis* [9,10,29]. All elements that characterize eukaryotic two-component systems and could act downstream of His kinase cytokinin receptors are present in *Arabidopsis* [9,10,29]. Parallel action of a two-component signalling path and a PLD-dependent path that converge on the *ARR5* promoter may explain the results.

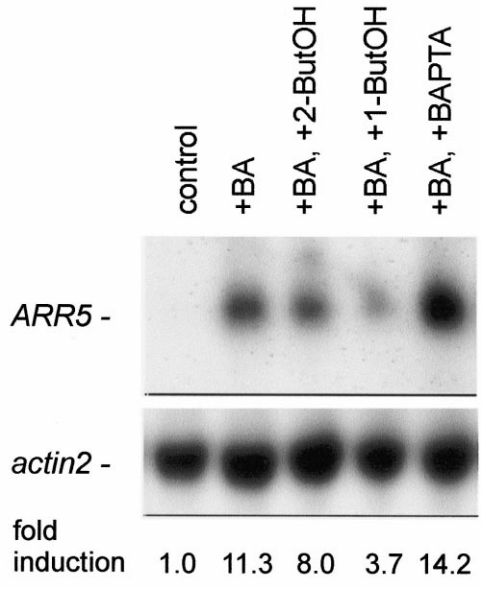

Fig. 4. RNA blot analysis of *ARR5* gene induction by cytokinin in the absence or presence of different inhibitors. Ca. 2000 seedlings were incubated in 5 μM BA. Inhibitors were added 15 min prior to BA. Seedlings were harvested 35 min after addition of BA. 25 μg total RNA was used for each lane. Northern blots were probed with a *ARR5* specific probe and an *actin 2* probe to standardize signal strength. Relative signal strength obtained without cytokinin was set as 1. Lane 1, no treatment; lane 2, 5 μM BA; lane 3, 5 μM BA plus 1% butan-2-ol; lane 4, 5 μM BA plus 1% butan-1-ol; lane 5, 5 μM BA plus 10 mM BAPTA.

Parallel pathways could act independently or might be required simultaneously to activate the promoter. The observation that overexpression of AHPs has no marked effect by themselves on cytokinin-induced gene expression [11] is consistent with this hypothesis. Alternatively, full activity of the two-component signalling system might depend on PLD or a signalling branch downstream of the receptor may require PLD activity.

This study suggests a possible link between cytokinin and polyamine signalling. The antagonistic action of cytokinins and the polyamines spermine and spermidine was previously shown using an *Amaranthus* bioassay [30]. These previous results and the data presented here suggest that some polyamines can exert their biological effects in plant cells at least in part by modulating cytokinin signalling.

An inhibitor of serine/threonine kinases and inhibitors of class 1 and class 2A phosphatases partially suppressed the cytokinin-dependent accumulation of reporter gene product (Table 1). This is in agreement with previous studies implicating Ser/Thr protein kinases and phosphatases in cytokinin signalling [14–16]. Neither the enzymes involved nor their targets with regard to cytokinin signalling are presently known.

The result that Ca^{2+}/calmodulin inhibitors suppressed cytokinin-dependent GUS accumulation but not accumulation of *ARR5* mRNA is not trivial as these components often act downstream of PLD and prior to target gene activation. The result is supported by recent kinetic studies in *Amaranthus* where a Ca^{2+} chelator was shown to inhibit cytokinin-dependent amaranthin accumulation at a later time than the onset of transcript induction [16]. This also demonstrates that reporter gene assays are not sufficient to interpret early signalling events if they are not combined with kinetic studies or studies on mRNA accumulation. Nevertheless, the reporter gene-based assay described here is indeed a valuable tool to screen a wide range of compounds for inhibitors of early cytokinin action. Furthermore, this test has potentially numerous applications in the study of cytokinin signalling, for example for the definition of the target sequences of the PLD-dependent pathway through promoter deletion constructs.

Acknowledgements: This work was supported by a Grant of the Deutsche Forschungsgemeinschaft (Schm 814/13-1), the Russian Foundation of Basic Research (NN 99-04-04015/01-04-04002) and NATO Grant LST-EV977817. We thank Dr. Ulrike Zentgraf for densitometric measurements and Carola Scholz for skillful technical assistance.

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