Plant Cell Rep (2016) 35:2181–2195
DOI 10.1007/s00299-016-2028-5

ORIGINAL ARTICLE

Nitric oxide–cytokinin interplay influences selenite sensitivity in *Arabidopsis*

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Received: 9 May 2016 / Accepted: 13 July 2016 / Published online: 23 July 2016

Abstract

Key message **Selenite oppositely modifies cytokinin and nitric oxide metabolism in *Arabidopsis* organs. A mutually negative interplay between the molecules exists in selenite-exposed roots; and their overproduction causes selenite insensitivity.**

Abstract Selenium-induced phytotoxicity is accompanied by developmental alterations such as primary root (PR) shortening. Growth changes are provoked by the modulation of hormone status and signalling. Cytokinin (CK) cooperates with the nitric oxide (NO) in many aspects of plant development; however, their interaction under abiotic stress has not been examined. Selenite inhibited the growth of *Arabidopsis* seedlings and reduced root meristem size through cell division arrest. The CK-dependent *pARR5::GUS* activity revealed the intensification of CK signalling in the PR tip, which may be partly responsible for the root meristem shortening. The selenite-induced alterations in the in situ expressions of cytokinin oxidases (*AtCKX4::GUS*, *AtCKX5::GUS*) are associated with selenite-triggered changes of CK signalling. In *wild-type* (WT) and NO-deficient *nia1nia2* root, selenite led to the diminution of NO content, but CK overproducer *ipt-161* and -deficient *35S:CKX2* roots did not show NO decrease. Exogenous NO (S-nitroso-N-acetyl-DL-penicillamine, SNAP) reduced the *pARR5::GFP* and *pTCS::GFP* expressions. Roots of the *35S:CKX* and *cyr1* plants suffered more severe selenite-triggered viability loss than the WT, while in *ipt-161* and *gsnor1-3* no obvious viability decrease was observed. Exogenous NO ameliorated viability loss, but benzyladenine intensified it. Based on the results, selenite impacts development by oppositely modifying CK signalling and NO level. In the root system, CK signalling intensifies which possibly contributes to the nitrate reductase-independent NO diminution. A mutually negative CK-NO interplay exists in selenite-exposed roots; however, overproduction of both molecules worsens selenite sensing. Hereby, we suggest novel regulatory interplay and role for NO and CK in abiotic stress signalling.

Keywords Cytokinin · Nitric oxide · Root growth · Selenite

Introduction

Selenium (Se) is a metalloid element, which essentiality in higher plants has not been evidenced so far (El-Ramady et al. 2015). The two major Se forms found in the environment, selenate and selenite are chemically similar to sulphur (S), therefore in plants they are metabolized via S pathways (Pilon-Smits and Quinn 2010). Elevated selenium levels are found naturally in soils derived from Cretaceous shale rock and selenium may also accumulate in the environment as the result of different mining or oil producing processes (Pilon-Smits and Quinn 2010; Lemly 2004). On the other hand, there are Se-deficient areas

Communicated by C.-H. Dong.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-016-2028-5) contains supplementary material, which is available to authorized users.

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worldwide where optimal Se supplementation is crucial for both animals and human (Rayman 2012). Soil fertilization with different Se salts is one possibility to increase the Se content of soils and food plants (Varo et al. 1988). High levels of selenium are toxic for most organisms, hence for non-hyperaccumulator plant species like *Arabidopsis thaliana*, as well (White et al. 2004). This justifies research focusing on the effects of Se on the physiological processes of plants. At cellular level, the formation of selenoproteins and disruption of redox balance contribute to selenium phytotoxicity (Van Hoewyk 2013). Selenite is able to induce mitochondrial oxidative stress and alters primary metabolism in *Brassica* roots (Dimkovikj and Van Hoewyk 2014). Additionally, selenium-triggered reactive oxygen species (ROS) production and oxidative stress were detected in *Brassica rapa* roots (Chen et al. 2014). Besides oxidative damage, selenite exposure resulted in protein nitration, and consequently, nitrosative stress in the organs of *Pisum sativum* as well implying that selenium phytotoxicity materializes partly through secondary nitro-oxidative stress (Lehotai et al. 2016). At the whole plant level chlorotic or necrotic lesions, withering and drying of leaves, reduced photosynthetic activity and cell death are characteristic symptoms of high Se content (Terry et al. 2000). Excess selenium reduces shoot biomass by decreasing fresh weight, hypocotyl length and cotyledon diameter of *Arabidopsis* (Grant et al. 2011; Ohno et al. 2012; Lehotai et al. 2011a). The growth of the root system is also affected, since elongation of the primary root (PR) is markedly inhibited by Se excess (Grant et al. 2011; Lehotai et al. 2011a).

Morphogens, such as cytokinins (CK), are major regulators of plant developmental processes. The rate of *de novo* synthesis, catabolism, import and export, and the generation and breakdown of conjugates are all regulatory processes of CK metabolism (Osugi and Sakakibara 2015). This group of plant hormones regulates several developmental and physiological processes, including cell division in meristems, leaf senescence, nutrient mobilization and seed germination (Hare and Van Staden 1997). Cytokinin inhibits cell proliferation in the root, but triggers shoot growth (Kuderová et al. 2008; Werner et al. 2003, 2010). In *Arabidopsis*, numerous genes encoding CK signalling-associated proteins are affected by abiotic stresses (Argueso et al. 2009) reflecting a potential role for CKs in the responses of plants to suboptimal environmental conditions (Zwack and Rashotte 2015).

During diverse physiological processes cytokinins cooperate with nitric oxide (NO). Nitric oxide is a small lipophilic redox active gaseous molecule produced by enzymatic, nonenzymatic, reductive and oxidative routes in plants (Sanz et al. 2015). In root cells, the major enzymatic source of NO production is considered to be nitrate reductase (NR, Yamasaki and Sakihama 2000). Depending on the physiological response and plant species, the nature of CK-NO interactions can be synergistic or antagonistic (Freschi 2013; Sanz et al. 2015). There is a positive link between CK and NO, e.g., during leaf senescence, programmed cell death, cell division and differentiation (Mishina et al. 2007; Carimi et al. 2005; Shen et al. 2013). Some cytokinin functions are executed primarily through the control of cell division, which is one of the main cellular processes determining plant growth and development. During the regulation of cell division NO may act downstream of cytokinin (and auxin) through the activation of *CYCD3;1* expression (Correa-Aragunde et al. 2006; Shen et al. 2013). Oppositely, an antagonistic relationship between these two molecules was evidenced in tobacco containing modified CK levels (Wilhelmová et al. 2006). Additionally, nitration of the adenine group of cytokinins by NO was observed, which reduces its own endogenous level, reflecting that cytokinins have a protecting role during nitrosative stress by scavenging NO (Liu et al. 2013). Nitric oxide also negatively impacts cytokinin signalling through the S-nitrosylation of histidine phosphotransfer protein 1 (AHP1), a key element in the multistep phosphorelay of cytokinin signal transduction in *Arabidopsis* (Feng et al. 2013). Exogenous cytokinin treatments were shown to concentration-dependently induce NO production in cell cultures and intact seedlings (Tun et al. 2001, 2008; Carimi et al. 2004; Shen et al. 2013). However, NO reducing effects were also obtained using mutant or transgenic plants with modified CK contents or using exogenous CK treatments (Xiao-Ping and Xi-Gui 2006; Romanov et al. 2008; Liu et al. 2013).

Based on the above, the hormonal and signal background mechanisms of selenium phytotoxicity are not well understood and the link between CK and NO in the signal transduction of abiotic stress responses has not yet been examined in detail. Therefore, our results aim to contribute to the further understanding of the effects of selenium exposure on CK and NO metabolism, the relationship between these molecules and their roles in the regulatory network of selenium phytotoxicity in *Arabidopsis*.

Materials and methods

Plant material and growth conditions

The following *Arabidopsis* lines were used in this work: the *Col-0* wild-type (WT), the isopentenyl transferase-overexpressing *ipt-161* containing increased zeatin and zeatin riboside content (van der Graaff et al. 2001), the cytokinin oxidase 2-overexpressing *35S:CKX2* line possessing ~40 % zeatin content of the wild-type (Werner

et al. 2003), the cytokinin resistant *cyr1* (Deikman and Ulrich 1995), the NO-deficient nitrate reductase double mutant *nia1nia2* (Wilkinson and Crawford 1993), the GSNO reductase-deficient *gsnor1-3* (Feechan et al. 2005), the cytokinin-inducible *pARR5::GUS, pARR5::GFP* (D'Agostino et al. 2000) and *pTCS::GFP* (Zürcher et al. 2013), different *AtCKX::GUS* (*AtCKX4, AtCKX5, AtCKX6*) transgenic lines (Werner et al. 2003, 2010) and the *cyclinB1;1::GFP* (*CYCB1;1::GFP*, Doerner and Potuschak 2001) *Arabidopsis* line. The seeds of the following plant lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Loughborough, UK): *ipt-161* (NASC ID: N117), *cyr1* (N8032), *pARR5::GUS* (N25261), *pARR5::GFP* (N23893) and *pTCS::GFP* (N66322). Most of the *Arabidopsis thaliana* L. lines used in the study are in the Columbia (*Col-0*) background except of *ipt-161* (C24), *pTCS::GFP* (CS8066), *pARR5::GFP* and *pARR5::GUS* (Ws).

Seeds were surface sterilized with 70 % (v/v) EtOH for 1 min and 5 % (v/v) sodium hypochlorite for 5 min and rinsed five times with sterile distilled water. Seeds were then transferred to half strength Murashige and Skoog medium (Duchefa Biochemie, 1 % sucrose and 0.8 % agar, w/v) supplemented with 0, 10, 20 and 40 μM sodium selenite (Na_2SeO_3). The NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) was used at 100 μM and 6-benzylaminopurine (BA) as exogenous cytokinin was applied at 0.1 μM concentration. Petri dishes were kept horizontally under greenhouse conditions at a photo flux density of 150 μmol m^{-2} s^{-1} (12/12 light/dark cycle) at a relative humidity of 55–60 % and 25 ± 2 °C. The experimental period was 4 days long after 4 days of germination (DAG). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Element content analysis by inductively coupled plasma mass spectrometer (ICP-MS)

Concentrations of sulphur, selenium and several microelements (Fe, Mn, B, Cu, Zn, Mo, Ni) were determined in the tissues of 14-day-old wild-type *Arabidopsis*. Root and shoot material of control, 10, 20 or 40 μM selenite-treated *Arabidopsis* were harvested separately and rinsed with distilled water. Three replicates, each from 200–250 seedlings were used. After the drying (72 h, 70 °C) and digestion of the plant material (digestion process: 65 % (w/v), nitric acid was added to the samples followed by 2 h of incubation; then 30 % (w/v) hydrogen peroxide was added then the samples were subjected to 200 °C and 1600 W for 15 min) the values of selenium and sulphur concentrations were determined by inductively coupled plasma mass spectrometer (Thermo Scientific XSeries II, Asheville, USA). Selenium concentrations are given in μg/g dry weight (DW) and the concentration data of sulphur are given in mg/g DW. From the concentration data shoot:root ratios were also calculated. Concentrations of microelements are given in μg/g dry weight and the ratios are presented in circle graphs (see Suppl Fig. 1).

Morphological measurements

Cotyledon area (mm^2), hypocotyl (mm) and primary root length (mm) of the 4-day-old seedlings were determined on digital images supplied with a millimetre scale using Fiji software (http://fiji.sc/Fiji; Schindelin et al. 2012). Fresh and dry weights (mg of ten seedlings) were measured using a balance. Stomatal apertures in the epidermis of *Arabidopsis* cotyledons were visualized according to Sugano et al. (2010) with modifications. The whole seedlings were fixed in precooled 90 % (v/v) acetone at −20 °C for 1 h and cleared in chloral hydrate solution (chloral hydrate:water:glycerol (8:2:1, w/v/w)) for 10 min. After washing the samples with distilled water, stomata were stained with 1 μg ml^{-1} Safranin-O for 20 min. Leaf epidermis was examined under microscope (Zeiss Axiovert 200 M, Carl Zeiss, Jena, Germany) using 40× objective. In case of every treatment, the pore diameter of ~100 stomata was measured. Seedling morphology of the wild-type and mutant or transgenic *Arabidopsis* was observed under Zeiss Axioskope 200-C stereomicroscope (Carl Zeiss, Jena, Germany). At least 15 seedlings were measured in each experiment.

Histochemical β-glucuronidase (GUS) staining

In GUS-tagged *Arabidopsis* lines (*pARR5::GUS*, *AtCKX4::GUS, AtCKX5::GUS* and *AtCKX6::GUS*), the β-glucuronidase activity was visualized according to Zhong et al. (2014). Whole seedlings were incubated in 90 % (v/v) acetone at −20 °C for 30 min and then rinsed three times with 1× PBS buffer (pH 7.4). The seedlings were then incubated in the staining solution (1 mM K_3 Fe(III)$(CN)_6$, 0.5 mM K_4Fe(II)$(CN)_6$, 1 mM EDTA, 1 % Triton X-100, 1 mg/ml X-Gluc in 1× PBS) for 8 h at 37 °C. After incubation, samples were washed with 30, 70 and 95 % (v/v) ethanol and were prepared on microscopic slides. Samples were visualized with Zeiss Axiovert 200 M-type microscope. In each experiment, at least ten seedlings were stained and representative images were selected.

Fluorescent microscopy

Nitric oxide levels in *Arabidopsis* cotyledon and root were detected by 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) according to Pető et al.

(2011) with modifications. Whole seedlings were incubated for 30 min in 10 μM dye solution (prepared in 10 mM TRIS–HCl, pH 7.4) and were washed twice within 30 min with TRIS–HCl.

Fluorescein diacetate was used for the determination of cell viability according to Lehotai et al. (2011b). Whole seedlings were incubated in 2 mL of 10 μM fluorescein diacetate (FDA) staining solution (prepared in 10/50 mM MES/KCl buffer, pH 6.15) for 30 min, then washed four times with MES/KCl.

Fluorescent microscopic studies were carried out using Zeiss Axiovert 200 M-type microscope (Carl Zeiss, Jena, Germany) and filter set 10 (excitation 535–585 nm, emission 600–655 nm). Fluorescence emission (pixel intensity) was measured on digital images with the help of Axiovision Rel. 4.8 software. Cell viability is given as percentage of the pixel intensity of control fluorescein-labelled samples.

Confocal laser scanning microscopy

Prior to imaging, 4-day-old seedlings (*Col-0, CYCB1;1::GFP, pARR5::GFP* and *pTCS::GFP*) were stained with 10 μg/ml propidium iodide for 1 min, then were washed once with distilled water and were prepared on microscopic slides.

Meristem length (μm) of wild-type seedlings was determined by measuring the distance between the quiescent centre and the first elongated cortex cell. Meristem cell number was counted and expressed as the number of cells in the cortex files extending from the quiescent centre to the first elongated cortex cell. Optical sections of roots were collected with Zeiss LSM 880 (Carl Zeiss, Jena, Germany) and Olympus LSM 700 (Olympus, Tokyo, Japan) confocal laser scanning microscopes. Propidium iodide was excited by a 488-nm diode laser and the emission was detected between 620 and 700 nm wavelengths. GFP fluorescence was excited by a 488-nm diode laser and was measured below 555 nm. The localization pattern and frequency of *CYCB1;1::GFP* containing cells were examined. Additionally, relative fluorescent intensities of *pARR5::GFP* and *pTCS::GFP* were also determined in the root tips using Zeiss Zen2010 and Olympus Fluoview FV100 software.

Statistical analysis

Results are expressed as mean ± SE. Statistically significant differences from the wild-type control were determined with Microsoft Excel 2010 and Student's *t* test ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P \leq 0.05$) and Duncan's test. All experiments were carried out at least twice and in each treatment 6–15 samples were measured.

Results

Arabidopsis accumulates selenium into the root and shoot system, resulting in modified sulphur content

The total selenium and sulphur contents of the shoot and root system of selenite-exposed *Col-0 Arabidopsis* were determined (Table 1). The significant enhancement of total Se concentrations was observed in both organs, which was directly proportional to the external selenite concentrations in the growth medium. The rate of the accumulation proved to be similar in both organs. Shoot:root ratios reveal changes in the distribution of Se within the plant body. Under control conditions, the shoot:root ratio for selenium was <1 indicating a higher Se content in the root system compared to the shoot. However, Se exposure led to the marked increment of shoot:root ratios, which reflects a more pronounced Se accumulation in the shoot system. The 10 and 20 μM selenite treatment doubled the sulphur contents in the roots, although this effect did not prove to

Table 1 The effect of selenite on total selenium and sulphur contents of wild-type *Arabidopsis*

Selenite cc. (μM)	Se concentration (μg g^{-1} DW)		Shoot: root ratio of Se	S concentration (mg g^{-1} DW)		Shoot:root ratio of S	$[Se]_{shoot}/[S]_{shoot}$	$[Se]_{root}/[S]_{root}$
	Shoot	Root		Shoot	Root			
0	0.69 ± 0.08^{d}	1.14 ± 0.24^{d}	0.60	6.12 ± 11.95^{b}	12.93 ± 1.33^{c}	0.47	0.00011	0.0000881
10	31.29 ± 3.01^{c}	34.92 ± 2.50^{c}	0.89	5.44 ± 11.08^{c}	24.68 ± 3.77^{a}	0.22	0.0057	0.0014
20	68.99 ± 3.51^{b}	60.5 ± 8.49^{b}	1.14	5.99 ± 5.70^{b}	24.35 ± 4.27^{a}	0.24	0.011	0.0024
40	118.0 ± 18.91^{a}	104.4 ± 5.25^{a}	1.13	8.09 ± 1.84^{a}	22.64 ± 2.41^{b}	0.35	0.014	0.0046

Concentrations of total selenium (μg/g dry weight, DW) and sulphur (mg/g dry weight, DW) in the shoot and root system of 14-day-old wild-type (*Col-0*) *Arabidopsis* grown in the presence of 0, 10, 20 or 40 μM sodium selenite. Shoot:root ratios and selenium/sulphur ratios ($[Se]_{shoot}/[S]_{shoot}$ and $[Se]_{root}/[S]_{root}$) were also calculated from the concentration values. Different letters indicate significant differences according to Duncan's test ($n = 6$, $P \leq 0.05$)

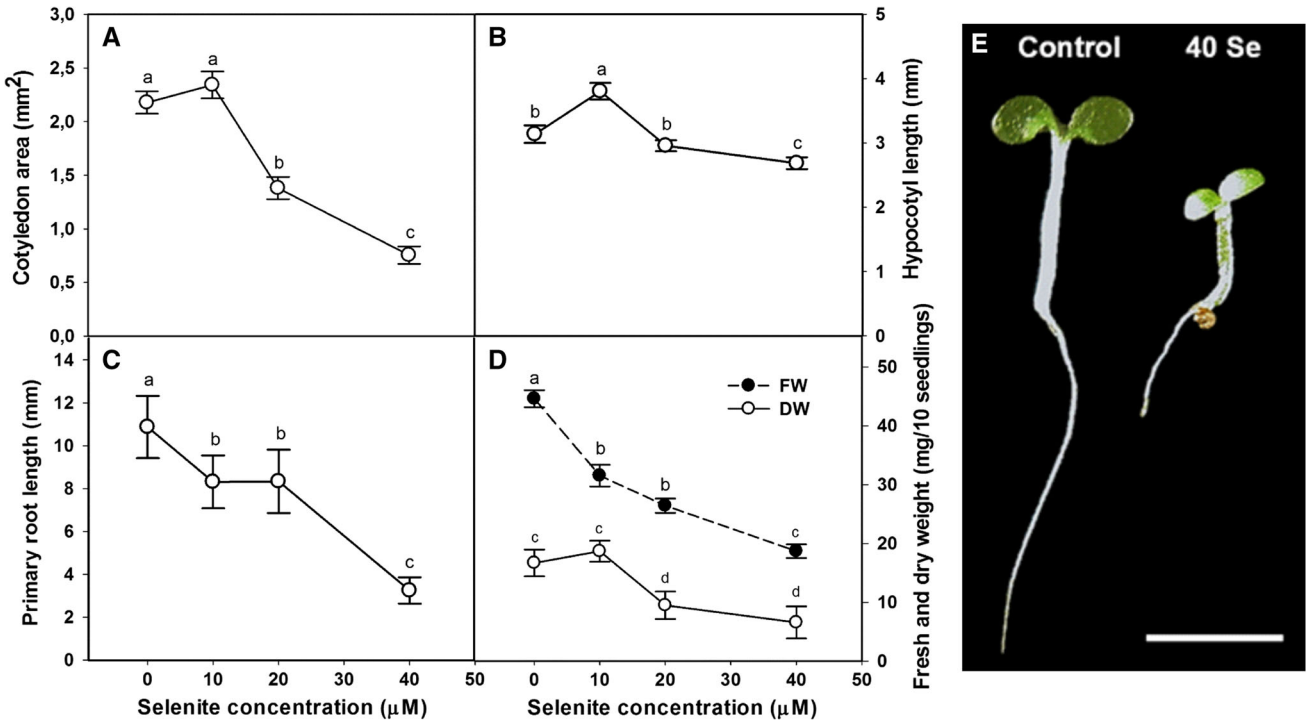

Fig. 1 Selenite influences seedling development. Cotyledon area (mm^2, **a**), hypocotyl length (mm, **b**), primary root length (mm, **c**) fresh and dry weight (mg/10 seedlings, **d**) of 4-day-old *Col-0 Arabidopsis* grown on agar medium supplemented with 0, 10, 20 or 40 μM sodium selenite. *Different letters* indicate significant differences according to Duncan's test ($n = 15$, $P \leq 0.05$). **e** Representative stereomicroscopic images of 4-day-old control and 40 μM selenite-treated wild-type *Arabidopsis*. *Bar* 2 mm

be dependent on the external selenite concentration. Moreover, in the shoot system the highest selenite dose increased the S content (Table 1), while the lowest applied selenite concentration significantly decreased it. Therefore, in contrast to selenium, the shoot:root ratio for S was diminished by all treatments. The relationship between Se and S can be described by the ratio of their concentrations in the plant organs. As the effect of selenite increment in the nutrient medium, the Se:S concentration ratios enhanced in both organs. This elevation in element ratios proved to be more pronounced within the shoot system (~125-fold in case of 40 μM selenite) compared to the root (~50-fold in case of 40 μM selenite). Regarding the microelement homeostasis, selenite had no obvious effect on the ratios of Fe, Mn, B, Cu, Zn, Mo, Ni concentrations either in the root or in the shoot (Suppl Fig. 1).

Selenite concentration-dependently modifies the development of *Arabidopsis* seedlings

Selenite significantly affected cotyledon growth of the 4-day-old seedlings. At 20 and 40 μM concentration, selenite resulted in the heavy reduction of cotyledon area (Fig. 1a, e). However, the lowest applied selenite dose (10 μM) slightly enhanced the size of cotyledons (Fig. 1a). The beneficial effect of 10 μM and the inhibitory effect of 40 μM selenite were evident also for hypocotyl elongation (Fig. 1b, e). Regarding the primary root growth, a clear and strong inhibition was observed with all selenite concentrations (Fig. 1c, e). The 40 μM selenite reduced the PR length by ~70 % compared to the untreated control. The fresh weight of *Col-0* seedlings drastically diminished, since the lowest applied selenite dose caused 45 % reduction and the 40 μM selenite caused 70 % reduction of it (Fig. 1d, e). In contrary, the lowest applied selenite concentration slightly enhanced the seedling dry weight, while 20 and 40 μM selenite notably decreased it (Fig. 1d). Additionally, 10 μM selenite significantly induced stomatal opening (Suppl Fig. 2). Despite the remarkable growth inhibition, selenite-exposed plants did not show visible signs of damages (e.g., necrotic lesions); although chlorosis was apparent on their cotyledons (Fig. 1e).

Selenite affects root meristem size

Studying root growth under selenite stress conditions, there was an obvious alteration of tissue structure within the primary root (Fig. 2a). Length of the primary root meristem as well as the number of meristem cells suffered significant decrease as the effect of 40 μM selenite (Fig. 2a–c).

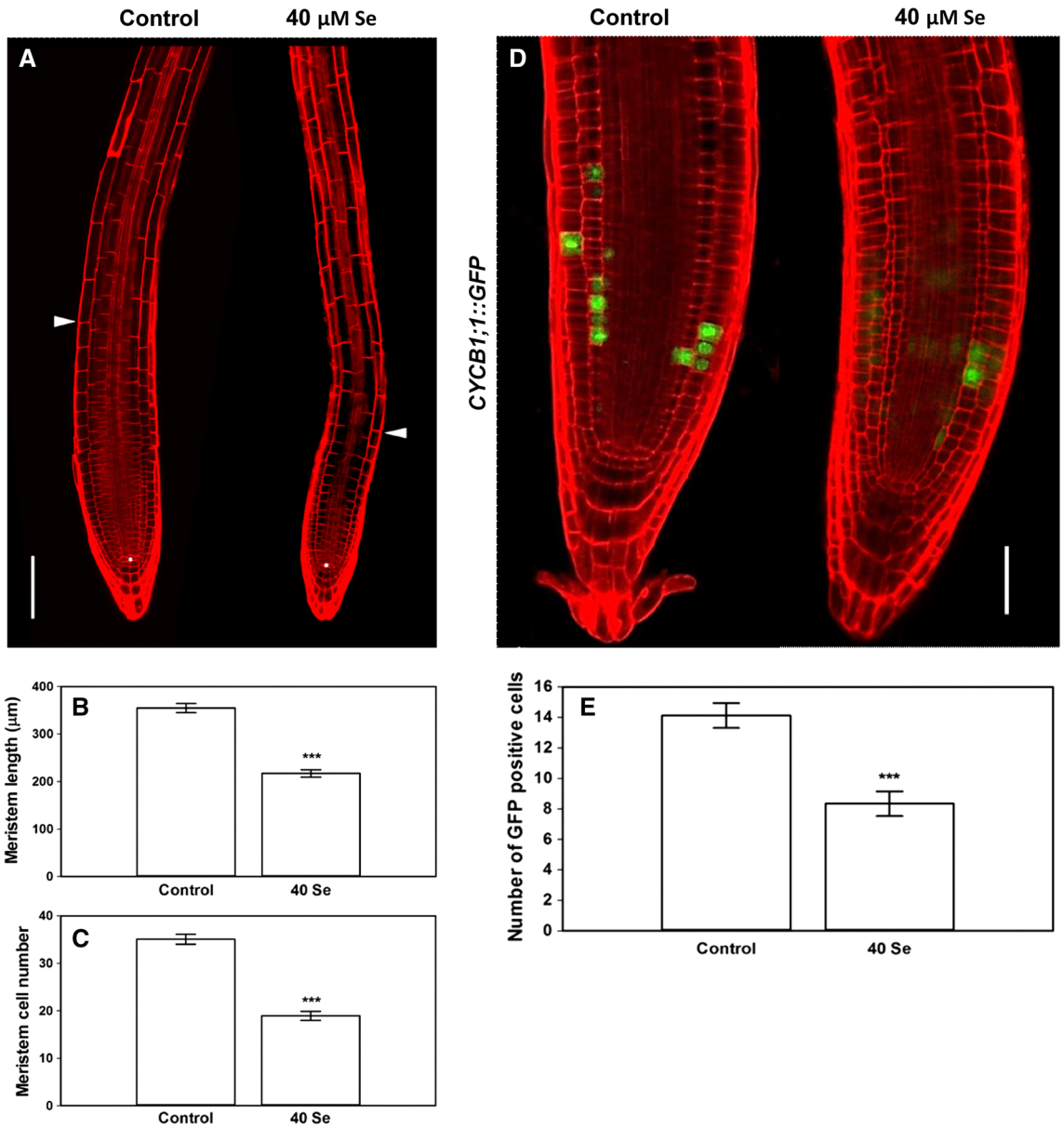

Fig. 2 The effect of selenite on root meristem size. **a** Primary root morphology of 4-day-old control and 40 µM selenite-exposed *Col-0 Arabidopsis* plants visualized by 10 µg/ml propidium iodide staining. The quiescent centres are indicated by *asterisks* and the first elongated cortex cells are shown by *white arrowheads*. *Bar* 500 µm. **b** Meristem length (µm) was determined by measuring the distance between the quiescent centre and the first elongated cortex cell. Data are shown as mean ± SE ($n = 15$). Student's *t* test, $***P < 0.001$. **c** Meristem cell number was expressed as the number of cells in the cortex files extending from the quiescent centre to the first elongated cortex cell. Data are shown as mean ± SE ($n = 15$). Student's *t* test, $***P < 0.001$. **d** Primary root tips of 4-day-old *Arabidopsis* expressing *CYCB1;1::GFP* grown on agar medium supplemented with 0 or 40 µM sodium selenite. Roots were counterstained with 10 µg/ml propidium iodide. *Bar* 40 µm. **e** Quantification of GFP expressing cells from experiment depicted in **d**. Values represent mean ± SE from at least 15 roots. Student's *t* test, $***P < 0.001$

In case of both parameters ~40 % decrease was caused by the highest applied selenite dose. The rate of cell division was analysed in a line carrying *CYCB1;1::GFP* marker for G2/M phase of the cell cycle. The number of cells expressing GFP was reduced in selenite-treated samples (Fig. 2d, e).

Selenite oppositely influences cytokinin-dependent *pARR5::GUS* activity in the shoot and root system

To study the selenite-induced alterations in cytokinin-dependent gene expression directed by the ARR5 promoter, the root and shoot system of *pARR5::GUS Arabidopsis*

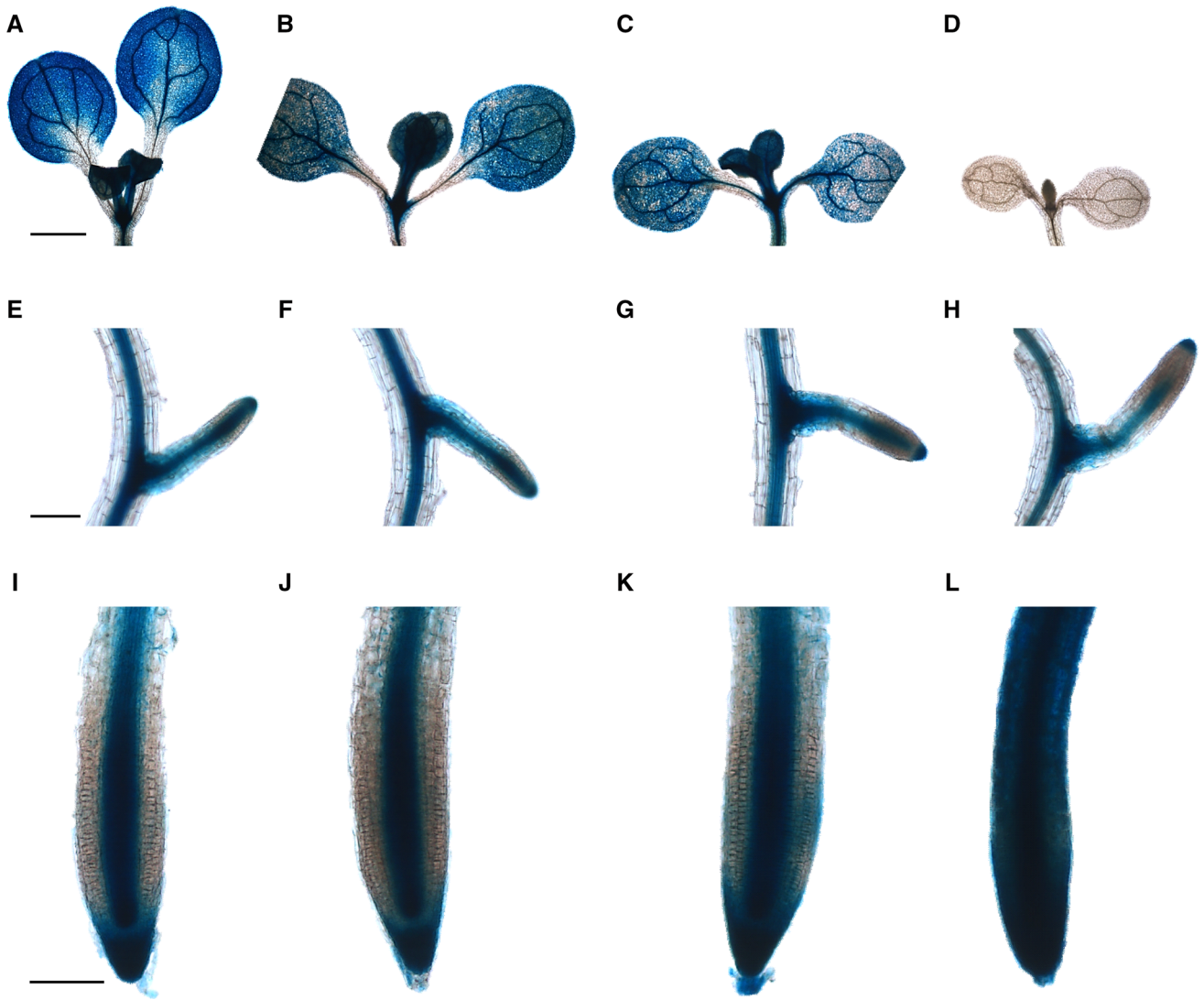

Fig. 3 Selenite differentially influences *pARR5::GUS* activity in *Arabidopsis* organs. **a–d** Shoot system of 4-day-old X-Gluc-stained *pARR5::GUS Arabidopsis* grown on agar medium supplemented with 0 (**a**), 10 µM (**b**), 20 µM (**c**), 40 µM (**d**) selenite. *Bar* 1 mm. **e–h** Lateral root (older than stage VII) of 4-day-old X-Gluc-stained *pARR5::GUS Arabidopsis* grown on agar medium supplemented with 0 (**e**), 10 µM (**f**), 20 µM (**g**), 40 µM (**h**) selenite. *Bar* 100 µm. **i–l** Primary root tip of 4-day-old X-Gluc-stained *pARR5::GUS Arabidopsis* grown on agar medium supplemented with 0 (**i**), 10 µM (**j**), 20 µM (**k**), 40 µM (**l**) selenite. *Bar* 100 µm

seedlings were visualized by histochemical GUS staining (Fig. 3). Control cotyledons showed intense GUS activity, which reduced depending on the selenite concentrations (Fig. 3a–d), and the 40 µM selenite-exposed plants showed no *pARR5::GUS* activity in their cotyledons (Fig. 3d). In the upper root parts and in older lateral roots, the *pARR5::GUS* activity was typically present in the stele and this pattern was not influenced by lower selenite doses (Fig. 3e–g). Moreover, the highest selenite concentration resulted in a slight reduction of *pARR5::GUS* staining, especially, in the lateral root meristem (Fig. 3h). In control primary root tips, the *ARR5* expression was restricted to the root cap and the central cylinder of the meristem (Fig. 3i). Selenite at 10 µM did not alter this GUS activity pattern (Fig. 3j), but in 40 µM selenite-treated plants, the blue colorization reflecting the cytokinin-dependent GUS activity showed a remarkable extension to all tissues of the primary root tip (Fig. 3l).

To examine the possible involvement of the enzymatic cytokinin degradation in the observed selenite-triggered disturbances of CK-dependent GUS activities, the in situ expressions of cytokinin oxidases (*CKX4*, *CKX5* and *CKX6*) were analysed using *AtCKX::GUS* seedlings (Fig. 4). During control circumstances, *CKX4* was expressed mainly in guard cells (Fig. 4a) and in the root cap (Fig. 4g), while *CKX5* was present in young leaves and

in the vascular cylinder of the PR meristem (Fig. 4d, j). The *CKX6* expression was visible in the vascular tissue of the developing cotyledon (Fig. 4e) and the root vascular cylinder with the exception of the root meristem (Fig. 4k). As the effect of selenite exposure, the expression of *CKX4* and *CKX5* intensified in the cotyledons (Fig. 4b, d), while in the root tip, only the *CKX4* proved to be down-regulated by selenite (Fig. 4h). In our experimental system, *CKX6* responded to selenite neither in the cotyledons nor in the roots (Fig. 4f, l).

Selenite influences nitric oxide levels in *Arabidopsis* roots

The endogenous NO levels were determined in the cotyledons and in the meristematic zone of the primary root by fluorescent microscopy (Fig. 5). Nitric oxide levels in the cotyledons did not show any remarkable changes; although a significant selenite-induced decrease was observed in the root meristem (Fig. 5a). The diminution of nitric oxide level did not prove to be proportional to the external selenite concentrations, and a reduction of 50 % was observed in 40 μM selenite-exposed root tips. Furthermore, selenite at 20 and 40 μM concentrations led to the decrease of the NO levels in *nia1nia2* root tips (Fig. 5b), with a similar extent to *Col-0* roots.

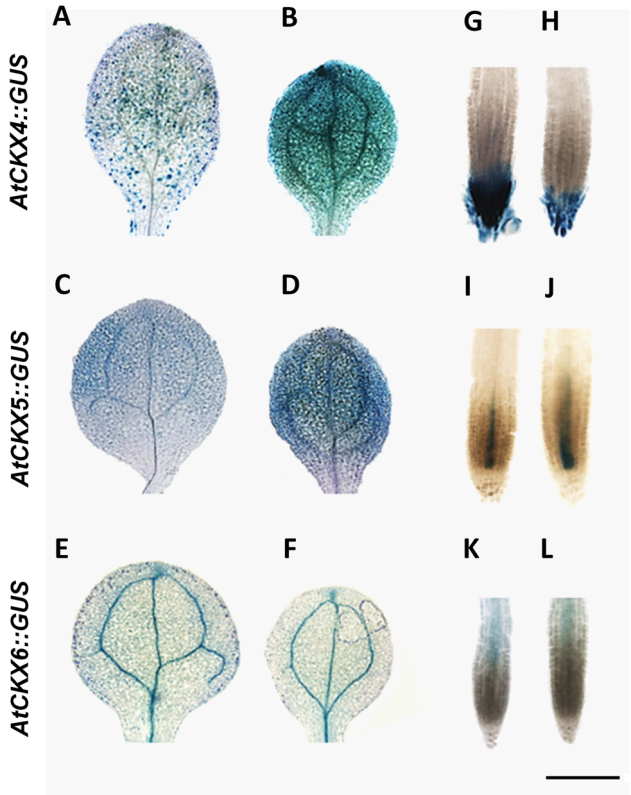

Fig. 4 The effect of selenite on *CKX::GUS* activities in Arabidopsis organs. **a**–**f** Cotyledons of 4-day-old *CKX::GUS Arabidopsis* lines treated with 0 (**a**, **c**, **e**) or 40 μM (**b**, **d**, **f**) selenite. *Bar* 1 mm. **g**–**l** Primary root tips of 4-day-old *CKX::GUS Arabidopsis* lines treated with 0 (**g**, **i**, **k**) or 40 μM (**h**, **j**, **l**) selenite. *Bar* 1 mm

The relationship between cytokinin and nitric oxide during selenite stress

The effect of cytokinin and nitric oxide on each other's signalling/level was analysed first in unstressed *Arabidopsis* roots (Fig. 6). The exogenous application of a synthetic cytokinin benzyladenine at the concentration of 0.1 μM enhanced *pARR5::GUS* expression (Suppl. Figure 3) and significantly decreased (by 45 %) the NO content of the *Col-0* root tip (Fig. 6a). Similar to the biochemical cytokinin supplementation, the genetically enhanced cytokinin content of *ipt-161* mutant resulted in

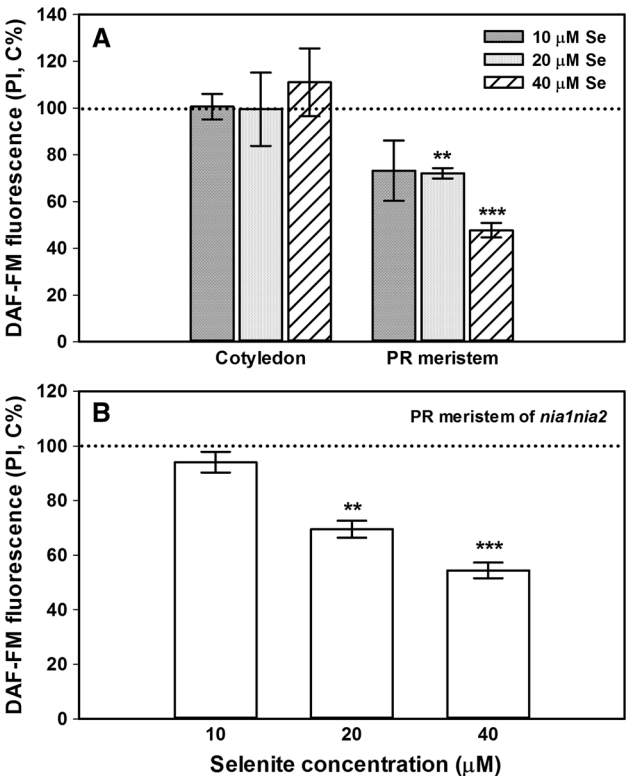

Fig. 5 Nitric oxide levels of *Arabidopsis* organs are differentially affected by selenite. **a** Values of NO-specific fluorescence [pixel intensity (PI) of DAF-FM] in primary root meristems of 4-day-old wild-type *Arabidopsis* grown in the absence (control) or in the presence of 10, 20, 40 μM sodium selenite. Data are shown as percentage of control ($n = 10$) Student's t test, $^{**}P < 0.01$ and $^{***}P < 0.001$. **b** Values of NO-specific fluorescence [pixel intensity (PI) of DAF-FM] in primary root meristems of 4-day-old nitrate reductase-deficient *nia1nia2 Arabidopsis* grown in the absence (control) or in the presence of 10, 20, 40 μM sodium selenite. Data are shown as percentage of control ($n = 10$) Student's t test, $^{**}P < 0.01$ and $^{***}P < 0.001$

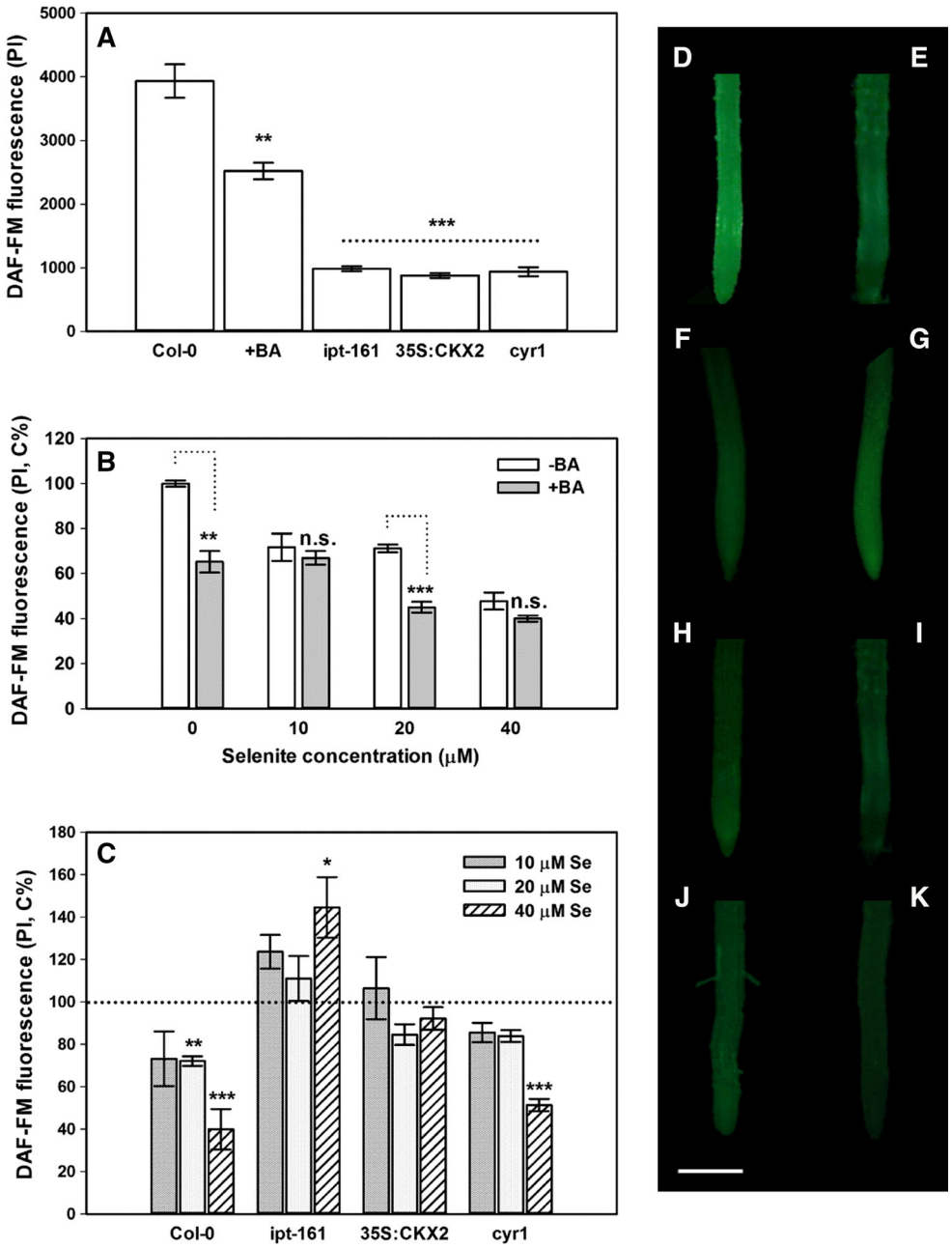

Fig. 6 The effect of cytokinin on nitric oxide levels in control and selenite-exposed *Arabidopsis* roots. **a** Values of NO-specific fluorescence [pixel intensity (PI) of DAF-FM] in primary root meristems of 4-day-old wild-type *Arabidopsis* with (+BA) or without 0.1 μM benzyladenine (−BA) (*Col-0*) and 4-day-old *ipt-161*, *35S:CKX2* and *cyr1 Arabidopsis* lines. Values represent mean ± SE from at least 15 roots. Student's *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$. **b** Values of NO-specific fluorescence [pixel intensity (PI) of DAF-FM] in primary root meristems of 4-day-old 0, 10, 20 or 40 μM selenite-exposed wild-type *Arabidopsis* with (+BA) or without (−BA) 0.1 μM benzyladenine treatment. Data are shown as percentage of control ($n = 15$) Student's *t* test, $^{**}P < 0.01$ and $^{***}P < 0.001$. **c** Values of NO-specific fluorescence [pixel intensity (PI) of DAF-FM] in primary root meristems of 4-day-old wild-type (*Col-0*), *ipt-161, 35S:CKX2* and *cyr1 Arabidopsis* grown in the presence of 0, 10, 20 or 40 μM sodium selenite. Data are shown as percentage of control ($n = 15$) Student's *t* test, $^{*}P \leq 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (D-K) Representative fluorescent microscopic images of DAF-FM DA-stained primary root tips of 4-day-old control (**d**, **f**, **h**, **j**) and 40 μM selenite-treated (**e**, **g**, **i**, **k**) wild-type (*Col-0*, **d**, **e**), *ipt-161* (**f**, **g**), *35S:CKX2* (**h**, **i**) and *cyr1* (**j**, **k**) plants. *Bar* 500 μm

decreased NO content; however, the reduction of the NO level proved to be more pronounced (75 %) (Fig. 6a, f). Similarly, low NO levels were detected in CK-deficient *35S:CKX2* and CK insensitive *cyr1* roots (Fig. 6a, h, j). Moreover, the addition of BA caused NO content diminution in 0 and 20 μM selenite-treated roots (Fig. 6b).

In case of 40 µM selenite treatment, the NO level of *Col-0* roots diminished (Figs. 5a, 6c, e) while in *ipt-161* root tips, the NO content increased (Fig. 6c, g). In the *35S:CKX2* transgenic line, selenite did not influence NO level (Fig. 6c, i), but *cyr1* plants showed reduced NO content of the root tip comparable to the wild-type (Fig. 6c, k).

The effect of exogenous NO supplementation on cytokinin-related signal transduction was evaluated in root tips expressing either *pARR5::GFP* or the synthetic promoter *pTCS::GFP* (Fig. 7). Selenite at 40 µM concentration caused an intensified *pARR5::GFP* expression in the root cap cells (Fig. 7c, i), which was significantly reduced by NO donor addition (Fig. 7d, i). In case of unstressed roots, the chemical NO donor SNAP did not affect *pARR5::GFP* expression (Fig. 7b, i). In control root tips, the expression of *pTCS::GFP* was restricted to the root cap columella cells (Fig. 7e), which was significantly decreased by SNAP addition (Fig. 7f, j). Moreover, selenite caused a threefold increase in *pTCS::GFP* expression in root columella (Fig. 7g, j). When NO donor was added to selenite-treated roots, the *pTCS::GFP* expression decreased under the control level (Fig. 7h, j).

The involvement of cytokinin and NO in selenium sensing

To reveal the involvement of CK and NO in Se sensing, the selenite sensitivity of root cells of mutant plants with modified CK and NO contents was examined (Fig. 8a). The sensitivity was determined based on the detection of root cell viability by fluorescent microscopy. Wild-type root cells showed a significant viability loss as the effect of all applied selenite concentrations, while in the *ipt-161* root tips, cell viability did not decrease as the effect of selenite treatments. In case of the CK-deficient transgenic line (*35S:CKX2*), viability of the root tip cells was seriously (by ~80 %) reduced by all selenite concentrations. In *cyr1* plants, deficient in cytokinin signal transduction, selenium sensitivity was pronounced, since all selenite concentrations significantly decreased the root cell viability. Similar to *ipt-161*, the nitric oxide-overproducer *gsnor1-3* did not show Se-induced viability diminution in the root tips. The 10 and 20 µM selenite had no effect on the cell viability of *nia1nia2* root cells, but this plant line containing reduced endogenous NO, suffered a serious viability loss in case of 40 µM selenite (Fig. 8a). The genetic studies were

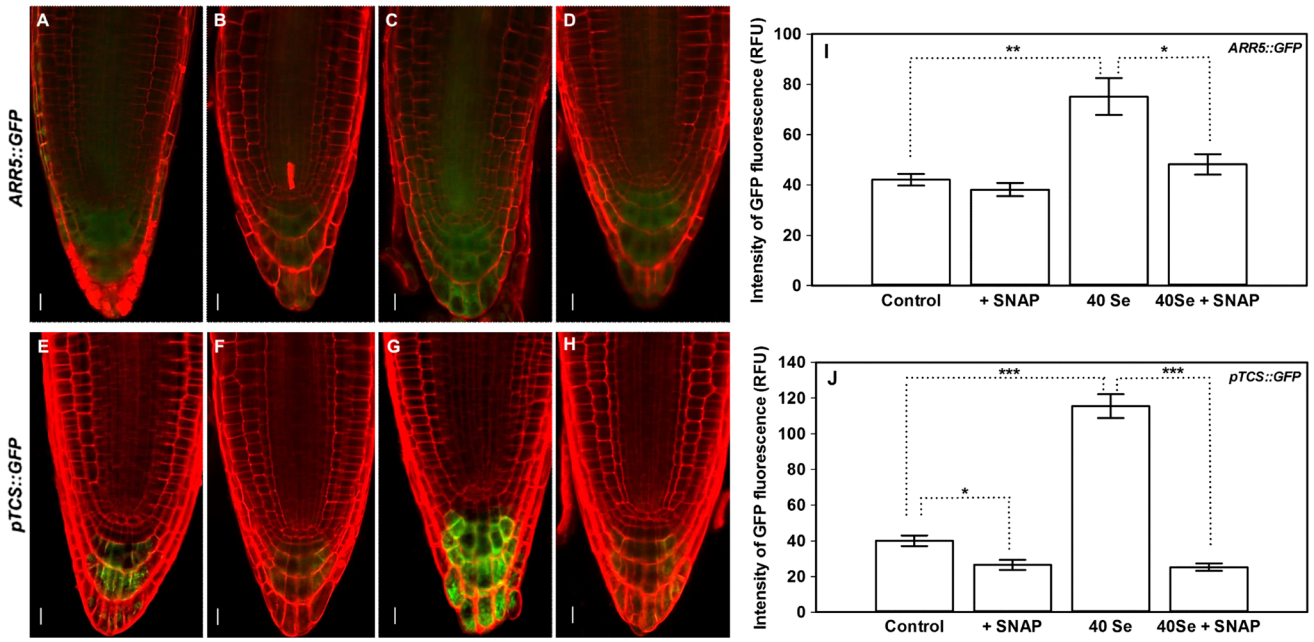

Fig. 7 Exogenous NO reduces cytokinin-dependent *pARR5::GFP* and *pTCS::GFP* expressions. **a–d** Four-day-old *Arabidopsis* roots expressing *pARR5::GFP*. **a** control, **b** 100 µM SNAP, **c** 40 µM selenite, **d** 40 µM selenite + 100 µM SNAP. Roots were counterstained with 10 µg/ml propidium iodide. *Bar* 10 µm. **e–h** Four-day-old *Arabidopsis* roots expressing *pTCS::GFP*. **e** Control, **f** 100 µM SNAP, **g** 40 µM selenite, **h** 40 µM selenite + 100 µM SNAP. Roots were counterstained with 10 µg/ml propidium iodide. *Bar* 10 µm. **i** Intensity of *pARR5::GFP* fluorescence (relative fluorescent unit, RFU) in the primary root meristem of 4-day-old *Arabidopsis* plants treated with 0 or 40 µM selenite with or without 100 µM SNAP. Values represent mean ± SE from at least 15 roots. Student's *t* test, $*P \leq 0.05$, $**P < 0.01$. **j** Intensity of *pTCS::GFP* fluorescence (relative fluorescent unit, RFU) in the primary root meristem of 4-day-old *Arabidopsis* plants treated with 0 or 40 µM selenite with or without 100 µM SNAP. Values represent mean ± SE from at least 15 roots. Student's *t* test, $*P \leq 0.05$, $***P < 0.001$

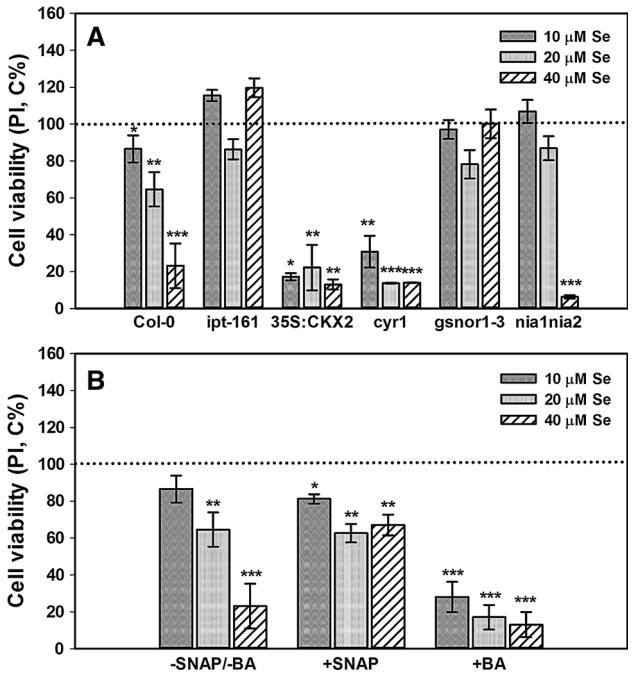

Fig. 8 Cytokinin and nitric oxide overproduction causes selenite insensitivity. **a** Cell viability [as pixel intensity (PI) of fluorescein in control %] in the primary root meristem of 10, 20 or 40 µM selenite-treated wild-type (*Col-0*), *ipt-161*, *35S:CKX2*, *cyr1*, *gsnor1-3* and *nia1nia2 Arabidopsis*. Significant differences according to Student's *t* test ($n = 15$, $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) are indicated. **b** Cell viability [as pixel intensity (PI) of fluorescein in control %] in the primary root meristem of wild-type *Arabidopsis* treated with 10, 20 or 40 µM selenite with or without 100 µM SNAP or 0.1 µM benzyladenine (BA). Significant differences according to Student's *t* test ($n = 15$, $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) are indicated

completed with biochemical experiments using wild-type plants (Fig. 8b). The application of the NO donor SNAP resulted in the significant enhancement of viability in case of the highest selenite concentration compared to the roots treated with selenite alone. In contrast, the application of benzyladenine at the dose of 0.1 µM notably intensified the selenite-induced viability loss of the PR meristem cells (Fig. 8b).

Discussion

Although *Arabidopsis thaliana* is a non-accumulator of selenium (Pilon-Smits and Quinn 2010), our results pointed out that it was capable to increase endogenous Se content 500-fold from the elevated selenite concentration in the external growth media (Table 1). The similar accumulation rate in the shoot and root system suggests that *Arabidopsis* is not able to exclude Se from the shoot tissues. However, this result contradicts earlier observations which revealed that selenite is poorly translocated to the shoot system; most of it is retained in the roots, where it is converted into organic forms (reviewed by El-Ramady et al. 2015). Resulting from their chemical similarity, selenium competes with sulphur during uptake and assimilation (Hopper and Parker 1999). Moreover, the plant uptake of selenite seems to be independent from the sulphate uptake system since it is rather connected to phosphate uptake in rice (Zhang et al. 2014) and in wheat (Li et al. 2008), or it was suggested to be realized through metabolically independent passive transport (El-Ramady et al. 2015). Anyhow, in our experimental system, a connection between selenite and sulphur was found, since sulphur uptake was upregulated by selenite addition (Table 1). This can be explained by the putative up-regulation of sulphate transporters, as it was observed in 15 µM selenite-treated *Arabidopsis*, where the expression of *SULTR2;2* and *SULTR3;1* and *SULTR3;5* were enhanced; although resulting sulphur accumulation was not detected (Van Hoewyk et al. 2008). Moreover, in *Brassica napus* fed with selenite, the shoot sulphur concentrations decreased, while that of the root increased (Dimkovikj and Van Hoewyk 2014) just like in our study. Further evaluation of the data revealed that the interorgan distribution of both Se and S changed as the effect of selenite exposure. Similarly, the proportion of the two elements was altered. It was enhanced by selenite in favour of selenium, especially in the shoot system. These results indicate a relationship between selenite and sulphate metabolism. At the same time, selenite supplementation did not influence the microelement contents of *Arabidopsis* plants (Suppl. Figure 1) suggesting that the observed growth inhibition (Fig. 1) is independent from any disturbances of the microelement homeostasis.

Exposure to elevated Se levels resulted in an overall growth inhibition of *Arabidopsis* seedlings (Fig. 1). Root elongation proved to be more sensitive to selenium, since it was inhibited already by 10 µM selenite, while shoot growth was promoted by this concentration. The higher sensitivity of the root system can be explained by the fact that this organ is directly exposed to selenite. The 10 µM selenite-induced vigorous reduction of seedling fresh weight seems to be not justified by the enhancement of cotyledon area and hypocotyl length. Non-hyperaccumulators, like *Arabidopsis thaliana,* are able to cope with the enhanced selenium content of their tissues via the production and vaporization of the volatile dimethyl-selenide (LeDuc et al. 2004). The results supported our hypothesis that the 10 µM selenite-triggered fresh weight diminution can be explained by intensified water loss through stomatal opening (Fig. 1d; Suppl. Figure 2). The enhanced evaporation may serve selenium volatilization, and the signal leading to stomatal opening may be the accumulated selenium in the cotyledons.

Regarding root growth, the reduction of meristem cell number could be caused by either a decreased rate of cell

division in the meristematic cells or a more rapid elongation-differentiation of the cells. Analysis of *CYCB1;1::GFP* line revealed that a possible reason for selenite-induced meristem reduction is the arresting of cell division (Fig. 2), suggesting that the inhibitory effect of selenite on meristem size and on cell division (Fig. 2) is eminently responsible for primary root shortening (Fig. 1c). This is supported also by that selenite did not significantly influence meristem cell length (data not shown).

Plant hormones are candidates for acting in the transformation of stress-related signals into gene expression changes being required for the effective adaptation to suboptimal environmental conditions. Among them, cytokinins regulate several aspects of plant development (Kieber and Schaller 2014) and are known to play a relevant and complex role in response to abiotic stress stimuli, such as drought, salt, low temperature or photooxidative damage (Zwack and Rashotte 2015). The activity pattern of the cytokinin-inducible *pARR5::GUS* promoter reflecting CK-dependent signalling was markedly altered by selenite excess (Fig. 3). Since the root tip is the primary site of CK synthesis (Torrey 1976), the accumulation of CK in the root system and its complete elimination from the shoot both suggest inhibited root-to-shoot translocation as the effect of selenite. Indeed, cytokinin translocation via the xylem is regulated by environmental signals (Kudo et al. 2010). Drought stress, for example, was shown to decrease the level of cytokinin in the xylem sap (Bano et al. 1993; Shashidhar et al. 1996) suggesting slight root-to-shoot translocation of cytokinin. These changes in CK-dependent signalling could explain the induced size reduction of both organs, since cytokinin represses root development and has a stimulatory effect on shoot development (Werner et al. 2003). According to Dello Ioio et al. (2007), exogenous CK reduces root meristem size and meristem cell number by acting on the rate of cell differentiation but not on cell division. Considering the decreased expression of *CYCB1;1::GFP* in selenite-treated root meristem (Fig. 2d, e), CK accumulation may only be partly responsible for the root meristem shortening induced by selenite, the contribution of other hormonal mechanisms are suggested. Selenite decreased the auxin-responsive *pDR5::GUS* activity in the primary root tips of *Arabidopsis* (Lehotai et al. 2012), which can be responsible for the observed reduction of cell division rate. Moreover, cytokinin was shown to repress PIN-mediated auxin transport in the root meristem (reviewed by Chapman and Estelle 2009) thus it is conceivable that the complex effect of selenite on root meristem is materialized through an auxin-cytokinin antagonism.

To evaluate the contribution of cytokinin oxidase-dependent degradation to the altered CK signal distribution, the in situ expressions of *CKX4*, *CKX5* and *CKX6*, three from the seven cytokinin oxidase genes of *Arabidopsis* using GUS reporter lines (Schmülling et al. 2003; Werner et al. 2003) were analysed (Fig. 4). The expression pattern of CKXs in unstressed plants (Fig. 4a, c, e, g, i, k) proved to be equal to that published by Werner et al. (2003). The up-regulation of *CKX4* and *CKX5* may contribute to the Se-induced dramatic reduction of the CK levels in the cotyledon; while in the primary root, the down-regulation of *CKX4* may partly be responsible for the Se-induced cytokinin accumulation. In contrast to Tamaoki et al. (2008), *CKX6* did not prove to be Se-responsive in our experimental system.

Cytokinin acts together with NO in many aspects of plant development (Freschi 2013). The level of this signal molecule was negatively affected by selenite stress in the root meristem of both the WT and the NR-deficient *nia1-nia2* (Fig. 5a, b), indicating that the nitrate reductase activity is not involved in selenite-induced NO level changes. It was published that NO is able to nitrate the adenine group of cytokinin, leading to the reduction of its own endogenous level (Liu et al. 2013), which can be considered as a possible background mechanism of selenite-induced NO diminution.

In case of unstressed plants, both biochemically and genetically enhanced cytokinin content negatively affected NO production and the effect was more pronounced in case of *ipt-161* mutation compared to benzyladenine treatment (Fig. 6a). Also, the *Arabidopsis* line overexpressing cytokinin oxidase is NO-deficient, implying the negative effect of reduced CK content on NO production. Additionally, the deficiency of CK sensing (*cyr1* mutant) resulted in low NO content. These suppose a complex interaction between cytokinin and NO signalling in *Arabidopsis* plants during even control circumstances, which is supported by the often contradictory literature data (see "Introduction").

As it was already mentioned, selenite caused the reduction of NO levels in *Col-0* root meristem. In comparison, the modified CK contents in *ipt-161* and *35S:CKX2* plants resulted in the absence of Se-induced NO diminution, what is more, the overproduction of CK favours for selenite-induced NO generation suggesting that CK may influence NO metabolism under selenite stress. In the roots of *cyr1* plant deficient in CK sensing, NO levels decreased similar degree to the wild-type. This implies that possibly there may not be connection between the CYR gene-related cytokinin sensing and the NO signalling during selenite stress.

The prevention of Se-induced NO decrease by NO donor addition resulted in a significant diminution of CK-dependent *pARR5::GFP* and *pTCS::GFP* expression, monitored by GFP fluorescence (Fig. 7), implicating the requirement of NO level decrease in selenite-induced CK

signal accumulation. This also points out the negative NO-CK relationship under selenite exposure. The hypothesis concerning the nitration of CK by NO (Liu et al. 2013) is further supported by the fact that in cotyledons of selenite-exposed plants, CK-dependent gene expression was completely inhibited and NO level did not change, while in the root tips, the intensification of CK signal permitted the NO level decrease (see Fig. 3). To our knowledge, this is the first report providing results about CK-NO interplay during abiotic stress.

Selenium sensing in the root tip cells of *Arabidopsis* seedlings was evaluated by detecting the viability of the root meristem. Based on the lack of viability loss (Fig. 8a), the CK-overproducing *ipt-161* could be characterized by notable selenium insensitivity. In contrast, the exogenous CK (0.1 μM BA) aggravated the viability loss induced by Se (Fig. 8b). These contradictory results can be explained by the fact that benzyladenine, at relatively high concentrations, is able to induce cell death (Carimi et al. 2004), thus intensifying viability loss in *Arabidopsis* roots. More importantly, the selenium insensitivity of the *ipt-161* mutant raises the possibility that the overproduction of isopentenyl transferase enzyme is associated with reduced selenium sensing and concomitantly better selenium tolerance. According to Merewitz et al. (2012), the enhanced cytokinin content in *ipt* transgenic creeping bentgrass promotes drought tolerance. Similarly, the *ipt* transgenic tobacco was found to possess improved drought tolerance (Rivero et al. 2010). The overproduction of NO in *gsnor1-3* (Pető et al. 2011) completely prevented Se-induced viability loss (Fig. 8a), which reflects that high NO concentrations worsen selenite stress sensing. The reduced NO content in *nia1nia2* promoted selenite sensitivity, which implies the possibility that low NO levels favour for better selenite sensing, further supporting the involvement of NO signal in selenium stress response. Moreover, chemical NO supplementation (SNAP) improved viability under selenite stress (Fig. 8b), which provides additional support for the contribution of NO to selenium insensitivity. The stress mitigating effect of NO is also well characterized during other unfavourable environmental conditions, such as drought, salinity, heat or ozone (refs. in Fancy et al. 2016). The main contributors to plant selenium toxicity are the malformation of selenoproteins and the evolved oxidative stress (van Hoewyk 2013). There are experimental evidences that under abiotic stress conditions such as arsenic exposure or cold, NO positively regulates the activities of antioxidant enzymes and it reacts directly with different ROS, acting as an antioxidant itself (Ismail 2012; Sehrawat and Deswal 2014). However, it has to be mentioned that selenite exposure resulted in intensified protein tyrosine nitration in pea plants, suggesting selenite-triggered secondary nitrosative stress (Lehotai et al. 2016). Depending on several factors (e.g., plant species, stress, plant age, etc.) nitric oxide can have diverse role in selenium stress responses, after all.

Conclusions

As the effect of selenite exposure, *Arabidopsis* plants effectively accumulate selenium in both of their organs. Selenium accumulation influences interorgan distribution of sulphur, but has no significant effect on microelement homeostasis. The inhibitory effect of selenite on root meristem size and cell division is responsible for primary root shortening. Selenite modifies the cytokinin metabolism and signal transduction in *Arabidopsis* seedlings, possibly through the inhibition of the root-to-shoot cytokinin translocation and the regulation of cytokinin oxidase (*CKX4* and *AtCKX)* expression. The intensified cytokinin signalling together with other hormonal alterations (e.g., auxin) may result in meristem shortening. Also, the metabolism of NO is affected by selenite in a nitrate reductase-independent process. In selenite-treated root meristems, CK and NO act as suppressors of each other's signalling/levels possibly through a direct interaction between them. Moreover, both cytokinin and nitric oxide play a role in selenium sensing in primary root tip. The results provide new evidence for CK and NO action and for their regulatory interplay in abiotic stress signalling.

Author contribution statement Nóra Lehotai: experimental design, carrying out the experiments, microscopy (light, fluorescent, confocal), preparation of ICP samples, data analysis, and writing the manuscript. Gábor Feigl: participation in carrying out the experiments, light microscopy and preparation of ICP samples. Ágnes Koós: participation in carrying out the experiments, fluorescent microscopy and analysing data. Attila Ördög: ICP measurements and data analysis. Andrea Pető: data analysis. Árpád Molnár: participation in carrying out the experiments. Prof. László Erdei: theoretical support and useful advices. Dr. Zsuzsanna Kolbert: design and supervision of the research project, carrying out supplementary experiments, theoretical support and useful advices, and writing the manuscript.

Acknowledgments The NR double mutant *nia1nia2* plants were kindly provided by Prof. G. F. E. Scherer (University Hannover, Germany), the *gsnor1-3* seeds were donated by Prof. Christian Lindermayr (Helmholtz Zentrum München, Neuherberg, Germany). Seeds of *35S:CKX2* and *AtCKX::GUS* lines were kindly provided by Prof. Thomas Schmülling and Prof. Thomáš Werner (Freie Universität Berlin, Germany). We thank the SALK Institute for Biological Studies for providing the *CYCB1;1::GFP* seeds (Dr. Peter Doerner). Special thanks to Dr. Wolfgang Busch for his help and useful advices by carrying out confocal imaging, Bonnie Wohlrab for the valuable

technical help, Christian Göschl for the computing work (Gregor Mendel Institute of Molecular Plant Biology, Vienna, Austria) and Dr. Ferhan Ayaydin (Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary) for his support in part of the confocal microscopy work. Prof. Péter Hegyi (I. Department of Internal Medicine, University of Szeged) provided the opportunity for supplemental confocal microscopic experiments and Dr. József Maléth provided excellent technical assistance. Both of their kind help is highly appreciated. Special thanks to Dr. Barnabás Wodala for the proofreading.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding The research was funded by the Hungarian Scientific Research Fund (Grant no. OTKA PD100504) and by the Hungarian Academy of Sciences János Bolyai Fellowship.

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