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RESEARCH PAPER

Selenite-induced hormonal and signalling mechanisms during root growth of *Arabidopsis thaliana* L.

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Abstract

Selenium excess can cause toxicity symptoms, e.g. root growth inhibition in non-hyperaccumulator plants such as *Arabidopsis*. Selenite-induced hormonal and signalling mechanisms in the course of development are poorly understood; therefore this study set out to investigate the possible hormonal and signalling processes using transgenic and mutant *Arabidopsis* plants. Significant alterations were observed in the root architecture of the selenite-treated plants, due to the loss of cell viability in the root apex. During mild selenite excess, the plants showed symptoms of the morphogenic response: primary root (PR) shortening and increased initiation of laterals, ensuring better nutrient and water uptake and stress acclimation. As well as lower meristem cell activity, the second reason for the Se-induced growth hindrance is the hormonal imbalance, since the *in situ* expression of the auxin-responsive *DR5::GUS*, and consequently the auxin levels, significantly decreased, while that of the cytokinin-inducible *ARR5::GUS* and the ethylene biosynthetic *ACS8::GUS* increased. It is assumed that auxin and ethylene might positively regulate selenium tolerance, since reduced levels of them resulted in sensitivity. Moreover, high cytokinin levels caused notable selenite tolerance. During early seedling development, nitric oxide (NO) contents decreased but hydrogen peroxide levels increased reflecting the antagonism between the two signal molecules during Se excess. High levels of NO in *gsnor1-3*, lead to selenite tolerance, while low NO production in *nia1nia2* resulted in selenite sensitivity. Consequently, NO derived from the root nitrate reductase activity is responsible for the large-scale selenite tolerance in *Arabidopsis*.

Key words: *Arabidopsis thaliana* L., hydrogen peroxide, hormones, nitric oxide, root growth, selenite.

Introduction

Selenium (Se) is a non-metal element, naturally occurring in the soil or accumulating as a result of anthropogenic activities such as agriculture or mining (Sors *et al.*, 2005). Principally, plants are able to take up selenate or selenite from the soil solution, as these forms show several chemical similarities with sulphur; therefore they can be taken up by sulphate transporters and metabolized by sulphur metabolic pathways (Tamaoki *et al.*, 2008). Selenium excess causes important changes in root anatomy. Hartikainen *et al.* (2001) observed decreased dry weight, width, and length, and surface area and volume of the root system in selenite-treated lettuce and ryegrass. Selenium content in lettuce roots was found to be positively correlated with the intensity of root morphological alterations (Simojoki, 2003). Peng *et al.* (2000) reported that low selenite concentrations induce the development of hydroponically grown wheat, whereas serious Se excess inhibits its growth in a non-linear \dose–response relationship.

For root architecture formation, auxin concentration gradients and local maxima are crucial, which are partly regulated by membrane transporters (e.g. AUX1 efflux carrier) involved in polar auxin transport (Peer *et al.*, 2011). As well as auxin,

cytokinin is also an important factor in the regulatory system of root development, as confirmed by Kuderová *et al*. (2008), who found that increased cytokinin levels in bacterial isopentenyl transferase (IPT) -overexpressing plants brought about a reduction in the meristem size and root length. On the contrary, reduced cytokinin levels in mutants lead to increased meristem size and primary root (PR) elongation; compared to the wild type, the mutants are characterized by more lateral roots (LRs) and higher total root biomass (Werner *et al.*, 2010). The negative regulator ethylene induces auxin synthesis, transport, and signal transduction in the root tip, leading to the inhibition of root cell elongation. Increased expression of auxin influx (AUX1) and efflux (PIN proteins) transporters directs ethylene-induced auxin movement during root growth (Růžička *et al*., 2007). Changes in auxin, cytokinin, and ethylene metabolism and/or sensitivity induced by various stress factors (e.g. cadmium, salinity) can be partly responsible for the observed morphological alterations (Wang *et al.*, 2009; Maksymiec, 2011).

Nitric oxide (NO) is a multifunctional gaseous signalling molecule, playing a regulatory role in developmental processes. This molecule positively regulates auxin signalling during LR development, since the NO donor sodium nitroprusside induced the expression of tomato D-type cyclin *CYCD3;1* (Correa-Aragunde *et al.*, 2006), which was found to be elevated also in *Arabidopsis* mutants with increased cytokinin levels and was induced by exogenous cytokinin treatment in cell cultures and whole plants (Riou-Khamlichi *et al.*, 1999). It is well known that NO and ethylene are antagonists during plant senescence and fruit ripening; however, very little is known about their relationship during other physiological processes.

Hydrogen peroxide (H_2O_2) is able to modulate cell division, elongation, somatic embryogenesis, and formation of adventitious roots or root hairs (see references in Potters *et al.*, 2009). In plant cells, there are various ways that NO and H_2O_2 interact. NO can eliminate superoxide radical (O_2^-) in a chemical reaction yielding peroxynitrite ($ONOO^-$) and it can induce the expression of genes of several antioxidant enzymes or enhance the synthesis of non-enzymic antioxidants, leading to detoxification of H_2O_2 (Mazid *et al.*, 2011).

The present study focuses on the hormonal and signalling background mechanisms during selenite-induced root growth responses. *Arabidopsis* mutants and microscopic methods were used to gain a better understanding of the possible roles and relationships between the hormonal (auxin, cytokinin, ethylene) and signalling (NO and H_2O_2) components of the complex regulatory network during selenite-induced stress.

Materials and methods

Plant material and growth conditions

The experiments were carried out using wild-type (WT, Col-0) *Arabidopsis* seedlings 2, 4, 7, and 14 days after germination (DAG). The hormonal status was examined in PRs of different β-glucuronidase (GUS) transgenic lines, most of them obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, Loughborough, UK): the highly auxin-inducible *DR5::GUS* (Ulmasov *et al.*, 1997), the cytokinin-responsive *ARR5::GUS* (N25261; D'Agostino *et al*., 2000) and the *ACS8::GUS/GFP* (expressing 1-amino-cyclopropane-1-carboxylate (ACC) synthase, which produces the precursor of ethylene biosynthesis; N31385; Tsuchisaka and Theologis, 2004). The auxin-resistant and -deficient *aux1-7* (AT2G38120, N16704; Maher and Martindale, 1980), the cytokinin-overexpressing *ipt6-1* (the isopentenyl transferase gene product plays a role in cytokinin biosynthesis resulting in 10-fold increase in the zeatin content of the WT; AT1G25410.1, N117; van der Graaff *et al*., 2001), the ethylene-deficient hookless (*hls1-1*, AT4G37580, N3073; Guzmán and Ecker, 1990), and the *etr1-1 Arabidopsis* lacking ethylene-dependent signal transduction (AT1G66340, N237; Chang *et al.*, 1993) were also used 4 DAG. In order to study the putative role of NO, the *nia1nia2* double mutant was used, which exhibits only 1% nitrate reductase (NR) activity of the WT (Wilkinson and Crawford, 1993) as well as reduced NO content in the PRs (Kolbert *et al.*, 2010), along with the S-nitrosoglutathione reductase (GSNOR)-deficient *gsnor1-3*, in which lower enzyme activities and higher total S-nitrosothiol contents were measured (Feechan *et al.*, 2005). Mutant *Arabidopsis* plants with low (*vtc2-1*, containing 25–30% of WT ascorbic acid; Conklin *et al.*, 2000) or high (*miox4*, showing 2–3-fold ascorbic acid accumulation; Lorence *et al.*, 2004) ascorbate contents were also used. All *Arabidopsis* lines were of the ecotype Columbia (Col) background except *ipt6-1*, which is derived from the C24 background.

The seeds of all plant lines were surface sterilized with 5% (v/v) sodium hypochlorite and transferred to half-strength Murashige and Skoog medium (1% sucrose and 0.8% agar, w/v) supplemented with 0, 10, 20, or 40 μM Na_2SeO_3. Selenite was added to the nutrient medium before sterilization. The Petri dishes were kept in a greenhouse 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.

Element analysis by inductively coupled plasma MS

Root and shoot material of 14-day-old control and 40 μM selenite-treated WT *Arabidopsis* were harvested separately and rinsed with distilled water. Three replicates, consisting of 200–250 seedlings each were used. After drying (70 °C, 72 h), nitric acid (65%, w/v) and H_2O_2 (30%, w/v) was added. The samples were destroyed by microwave-assisted digestion (MarsXpress CEM, Matthews, USA) at 200 °C and 1600 W for 15 min. Cooled samples were diluted with distilled water and the element contents were determined by inductively coupled plasma MS (Thermo Scientific XSeries II, Asheville, USA). Selenium concentrations are given in μg (g dry weight)$^{-1}$.

Root morphological measurements

In the case of WT plants, PR length (mm) was measured at 2, 4, 7, and 14 DAG manually or under a Axiowert 200M microscope (Carl Zeiss, Jena, Germany). The developmental stages of LRs (smaller or larger than stage VII) were determined in *DR5::GUS Arabidopsis* stained with 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid, according to Malamy and Benfey (1997). Primary root length (mm) of hormone and NO or reactive oxygen species (ROS) mutant plants were determined at 4 DAG.

GUS histochemical staining

The β-GUS activity in transgenic *Arabidopsis* lines (*DR5::GUS*, *ARR5::GUS*, *ACS8::GUS/GFP*) was visualized according to Jefferson *et al.* (1987) using a Axiowert 200M-type inverted microscope with ×10 magnification.

Fluorescent microscopy

NO levels in *Arabidopsis* roots were detected by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate 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. For *in situ* H_2O_2 detection, 10-acetyl-3,7-dihydroxyphenoxazine (ADHP or Ampiflu) fluorescent dye was used. Seedlings were incubated in small Petri dishes with 2 ml of 50 μM ADHP solution (prepared in 50 mM sodium phosphate

buffer, pH 7.5) for 30 min and washed once with buffer (Gomes *et al.*, 2005). Fluorescein diacetate was used for determination of cell viability according to Lehotai *et al.* (2011). Microscopic studies were carried out using a Axiowert 200M-type inverted fluorescent microscope equipped with a high-resolution digital camera (Axiocam HR, HQ CCD) and filter set 10 (excitation 450–490 nm, emission 515–565 nm) or filter set 20HE (excitation 535–585 nm, emission 600–655 nm). Fluorescence emission (pixel intensity) was measured on digital images within circles of 60-µm or 150-µm radii using Axiovision Rel. 4.8 software.

Statistical analysis

Results are expressed as mean ± SD. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P < 0.05$) and Duncan's test. In several cases, Microsoft Excel 2010 and Student's t-test were used ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). All experiments were carried out at least twice and in each treatment at least 10 samples were measured.

Results and discussion

Selenium uptake and translocation by Arabidopsis *grown in agar medium*

Using inductively coupled plasma MS technology, this study was able to measure the selenium concentrations in the control and 40 µM selenite-treated *Arabidopsis* roots and shoots. Plants grown in control conditions showed higher Se concentrations in their roots compared to the shoots; however, selenite-treated plants accumulated more Se in their shoots (Table 1). This observation is contrast to that of Zhang *et al.* (2007), who found that selenite treatment resulted in higher root Se concentrations of *Arabidopsis* and the observed accumulation pattern appeared in selenate-treated plants. One likely explanation of this can be that the oxidation of selenite to selenate may happen during the experimental procedure. Although, both selenate and selenite are reduced to selenide, and from this step they have a common pathway in the metabolism of selenium (Suzuki, 2005).

Effects of selenite on the morphology and viability of Arabidopsis thaliana *root system*

At 2 DAG, the PR length of the seedlings was not affected by lower selenite concentrations (10 and 20 µM); however 40 µM resulted in significant decrease in PR length. During the later phase of development, the effect of selenite proved to be more intensive, since all the applied concentrations significantly decreased the root length (Fig. 1A). Although one reason for the PR length reduction may be the Se-induced cell death and consequently the lack of cell divisions in the root apical meristem (Lequeux *et al.*, 2010), downregulation of several cell-cycle genes (e.g. cyclins) by selenium might also be directly responsible for growth hindrance (Van Hoewyk *et al.*, 2008). In *Arabidopsis*, eight stages (stages I–VII and emergence) of LR development can be distinguished, according to Malamy and Benfey (1997). During stage I–VII, LR primordia are mainly generated by cell division, while LR emergence is driven by cell expansion and elongation. Selenite had no effect on the division (based on the number of LR primordia smaller than at stage VII) and expansion/elongation (based on the number of LR larger than at stage VII) processes of the laterals during the early development, while at 4 DAG 40 µM Se caused a reduction. The most significant effect of Se treatment was observed at 7 DAG, where almost all applied selenite concentrations notably inhibited LR development. Interestingly, in 2-week-old *Arabidopsis*, 10 µM Se caused a significant induction of both LR initiation and expansion/elongation (Fig. 1B), which is a characteristic symptom of the stress-induced morphogenic response (Potters *et al.*, 2009). A similar stress-induced morphogenic response phenotype was observed in copper-treated *Arabidopsis* (Pasternak *et al.*, 2005). It is assumed that the 10 µM selenite-induced growth reorientation may be a basic element of the acclimation process, since the enhanced number of LRs can contribute to better water and nutrient supplies, and thus to the survival of the plant. Cell viability in root meristem was affected only by high selenite concentrations during the first developmental period; however, later on all Se treatments had concentration-dependent inhibitory effects of meristem cells (Fig. 1C and 1D). During the whole developmental period, the Se-induced PR reduction strongly correlated with the significant loss of viability of the meristem cells (Fig. 1A and 1C). Se-induced cell death can be explained by disturbances of the protein synthesis, as well as structural and functional defects triggered by selenocystein and selenomethionine formation (Tamaoki *et al.*, 2008).

Table 1. Tissue Se concentrations in 14-day-old wild-type *Arabidopsis* seedlings treated with and without selenite on agar

Shoot and root tissues were separated and the element analyses were carried out by inductively coupled plasma MS. Different superscript letters indicate significant differences according to Duncan test ($P \leq 0.05$).

Selenite treatment (µM)	Se concentration (µg (g dry weight)$^{-1}$]	
	Root	Shoot
0	15 ± 0.2^{a}	3 ± 0.05^{b}
40	1289 ± 2.5^{c}	1814 ± 21.9^{d}

Selenite alters endogenous hormonal status of Arabidopsis *roots*

The hormonal background of selenite-induced root growth inhibition was examined using *DR5::GUS* (indicator of auxin levels), *ARR5::GUS* (indicator of cytokinin levels), and *ACS8::GUS/GFP* (for ethylene synthesis) transgenic *Arabidopsis* plants. DR5 is a highly active synthetic auxin response element, whose expression reflects the endogenous auxin levels (Ulmasov *et al.*, 1997). In 2-day-old roots, mild selenium exposure (10 µM) slightly increased the expression pattern of *DR5* (Fig. 2B and 2C), however, high Se concentration (40 µM) reduced it in the 1-week-old roots (Fig. 2K and 2L). It may seem worthy to note that 10 µM Se caused no decrease in growth of PR in the auxin-deficient *aux1-7* mutant, accompanied by a maintained cell viability, or even higher cell viability at 40 µM Se as compared to the treated WT (Fig. 3A–C). These observations suggest that the control WT plants possess the optimum auxin concentration for the root growth, while *aux1-7* has a suboptimal level of it. Auxin in physiological concentrations is a regulator of PR elongation

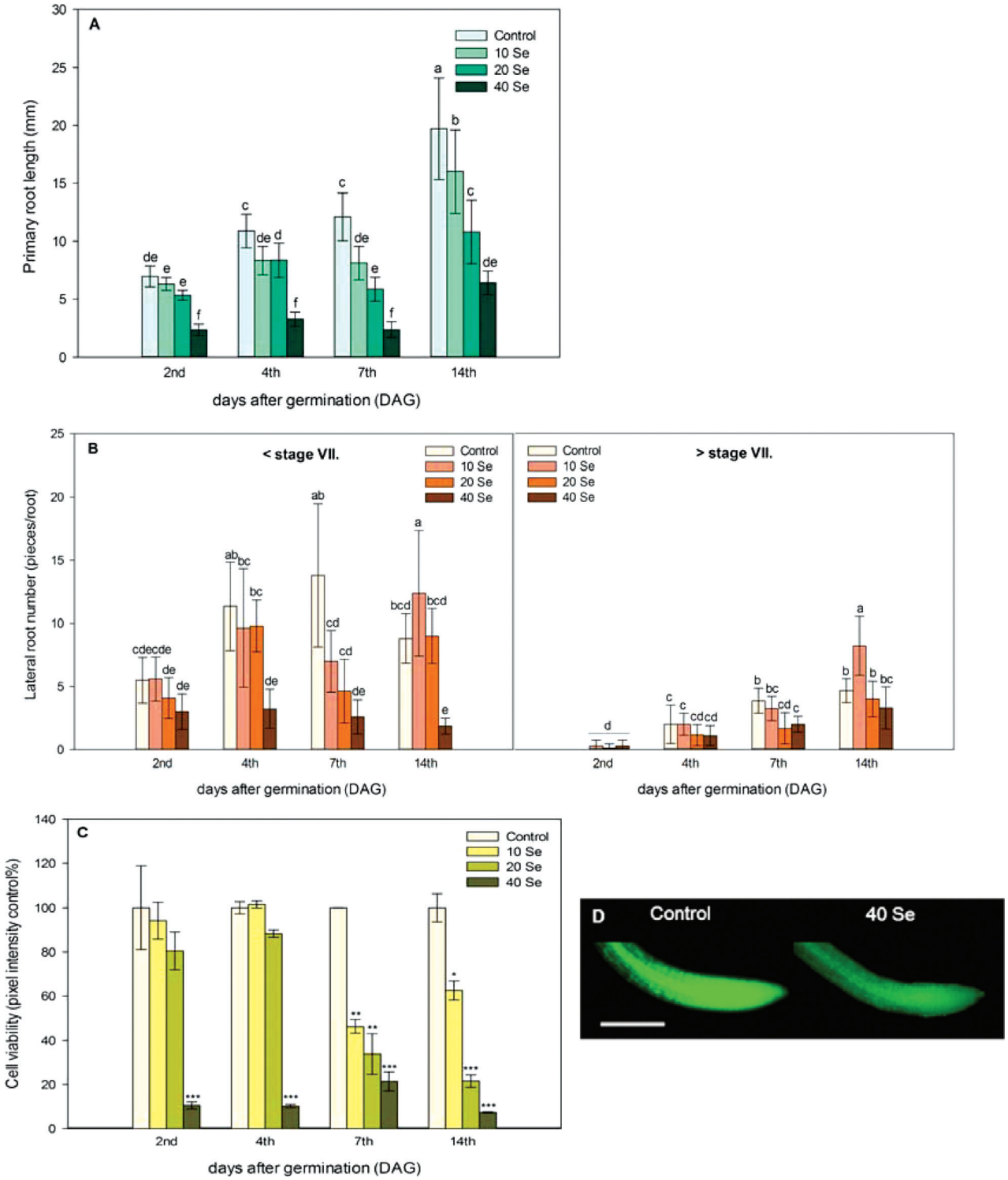

Fig. 1. Primary root length (A), lateral root number (smaller and larger than at stage VII, B), and cell viability (C) in primary root meristems of wild-type *Arabidopsis* treated with 0, 10, 20, or 40 μM selenite. Different letters indicate significant difference according to Duncan's test ($n = 10$, $P \leq 0.05$). Asterisks indicate significant difference to control according to Student's t-test ($n = 10$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). (D) Cell viability in primary root tips of 7-day-old plants: bar, 0.5 mm.

and selenite caused the decrease of *DR5* expression within the root tips (Fig. 2A–L), indicating a reduction in auxin levels. These results are supported by those of Wang *et al.* (1992), who found that sodium selenite decreased the levels of endogenous indole-3-acetic acid in tobacco. A reduced expression of *DR5* was also found in PR meristems of copper- or cadmium-treated *Arabidopsis* (Potters *et al.*, 2009; Lequeux *et al.*, 2010), further confirming the connection between PR shortening and the inhibition of meristem cell divisions. Selenium treatment had an effect on auxin transport and conjugation, too, since it

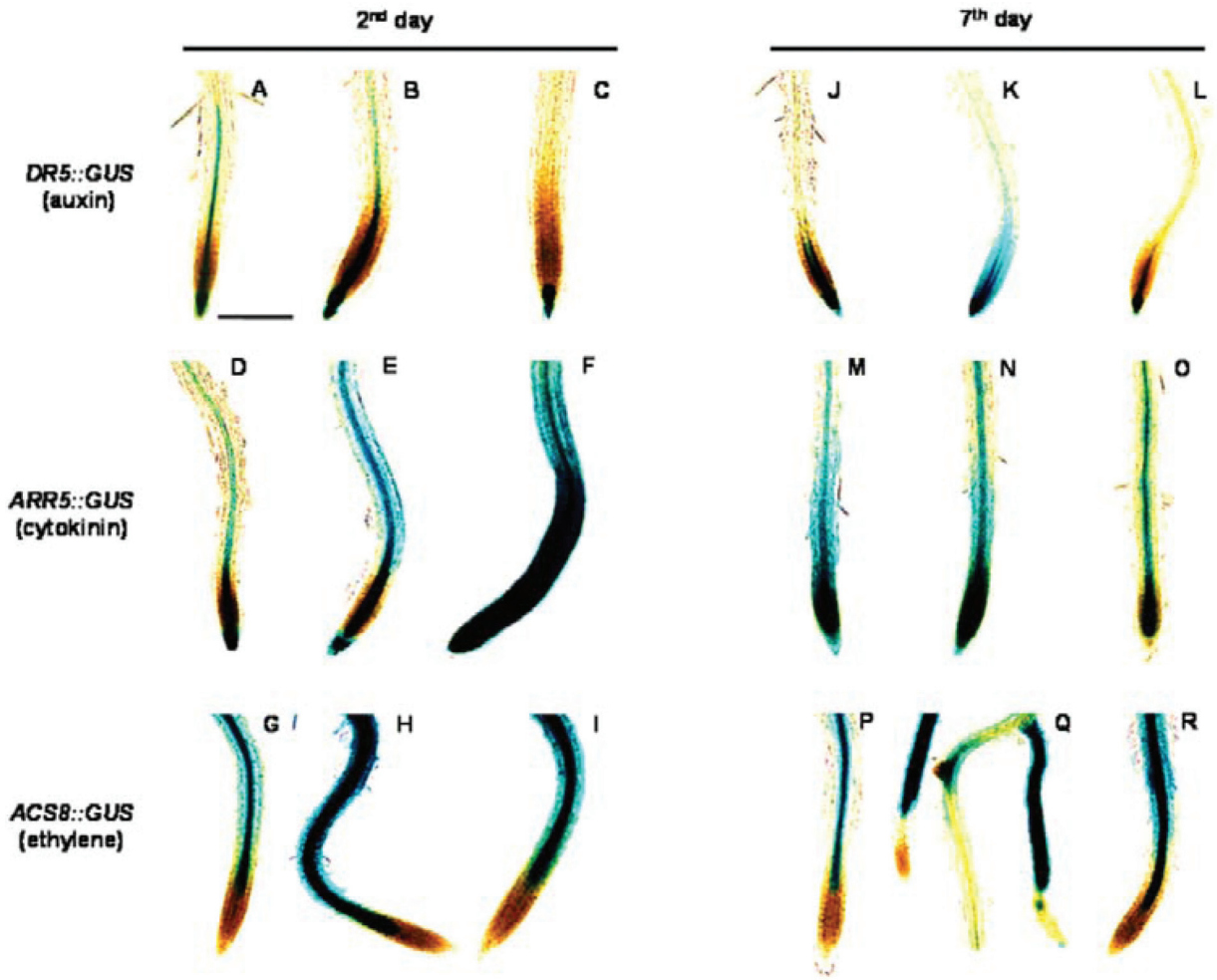

Fig. 2. *In situ* expression of hormone-associated genes at 2 and 7 days after germination. (A–C and J–L) *DR5::GUS* for auxin levels. (D–F and M–O) *ARR5::GUS* for cytokinin levels. (G–I and P–R) ACS8::GUS for ethylene synthesis. (A, D, G, J, M, P) Control 0 μM Se; (B, N, H, K, N, Q) 10 μM Se; (C, F, I, L, O, R) 40 μM Se. Bar, 0.5 mm.

downregulated the gene of auxin efflux carrier (PIN1) protein and upregulated the indol-3-acetate β-glucosyltransferase gene, which produces inactive auxin conjugates. Moreover, in selenium-treated *Arabidopsis*, the expression levels of several genes encoding auxin-regulated signal components were lower (Van Hoewyk *et al.*, 2008).

The *in situ* expression of the cytokinin-inducible primary response gene (*ARR5*) was heavily increased in response to 10 and 40 μM Se (Fig. 2D–F) during early development, which indicates an elevation in the cytokinin levels and can partly explain the growth inhibition, since cytokinin is known to be a negative regulator of PR elongation (Medford *et al.*, 1989). According to the transcriptome analysis by Van Hoewyk *et al.* (2008), the cytokinin oxidase gene (*ATCKX6*) was strongly downregulated in selenate-treated *Arabidopsis,* which suggests an increase in the cytokinin levels induced by Se. Additionally, selenate also inhibited the expression of a negative regulator of cytokinin-mediated signals (At1g74890).

Compared to control, the ACC synthase gene (*ACS8*), involved in ethylene biosynthesis, was expressed significantly in the PRs of Se-treated plants at 2 DAG (Fig. 2G–I) and 7 DAG (Fig. 2P–R), suggesting an increase in ethylene generation. Earlier, Konze *et al.* (1978) published that selenomethionine treatment enhanced ethylene production in the senescing flower tissues of *Ipomoea tricolor* Cay. in auxin-treated pea stem sections. Similarly, selenate treatment lead to increased ethylene levels in *Stylosanthes humilis* seedlings (Ribeiro *et al.*, 2011). The background mechanism of Se-induced ethylene production is the expression of genes associated with ethylene synthesis and ethylene-regulated signal transduction, which can be increased by selenate and selenite (Tamaoki *et al.*, 2008; Van Hoewyk *et al.*, 2008).

Primary roots of hormone mutants show changes in growth and selenium tolerance

In *aux1-7* plants, the mutation of the auxin influx carrier protein results in defective shoot-to-root auxin transport, decreased auxin concentrations, and lower sensitivity within the root tip, as compared to the WT (Pickett *et al.*, 1990). Only higher selenite concentrations (20 and/or 40 μM) caused PR shortening in *aux1-7* and in the two ethylene mutants (Fig. 3A). Compared

to the WT, auxin-resistant plants possessed increased sensitivity, since 40 μM and 20 μM selenite reduced the viability of the root tip cells. The ethylene-deficient mutant (*hls1-1*) proved to be the most sensitive, since all selenite concentrations significantly reduced the cell viability in it. In root meristems of the other ethylene mutant (ethylene resistant *etr1-1*), practically no loss in cell viability was detected (Fig. 3B and 3C). Based on these findings, it can be stated that, in the course of auxin and ethylene deficiencies, selenite is able to exert its strong effects on PR shortening and meristem cell death, which reflects selenium sensitivity. However, differences were found between the ethylene-deficient plants in Se tolerance, since *hls1-1* showing low ethylene concentrations possesses heavy selenite sensitivity, whereas *etr1-1 Arabidopsis* lacking normal ethylene signalling (and having WT-like ethylene levels) was resistant to selenite exposure. This indicates that changes in ethylene concentration within the *Arabidopsis* root tissues determine rather the tolerance than ethylene sensitivity under selenite excess. These results are partly confirmed by the work of Tamaoki *et al.* (2008), who found that endogenous ethylene concentrations were significantly increased by 15 μM selenite and that ethylene proved to be necessary for the induction of sulphur assimilation genes as an effect of selenite excess. Interestingly, the cytokinin (zeatin)-overproducing *ipt6-1* plants treated with selenite showed no reduction in PR length and cell viability compared to the WT, suggesting the involvement of this hormone in the large-scale Se tolerance. High levels of cytokinin induced the transcription of the adenosine-phosphosulphate-reductase 1 gene (*APR1*) gene, which promotes selenium metabolism (Ohkama *et al.*, 2002), contributing to tolerance. NR is also cytokinin-inducible (Samuelson *et al.*, 1995) and this may lead to a more efficient nitrogen metabolism and selenium endurance of the plant. It is worth mentioning that NR is one of the major enzymic NO sources in the roots (Xu and Zhao, 2003), therefore, its activation by cytokinin may result in NO production, as well, which can also induce defence mechanisms against selenite.

Selenium alters NO and H_2O_2 status of WT Arabidopsis *roots*

As far as is known, this is the first report investigating NO metabolism during selenium exposure in higher plants. NO

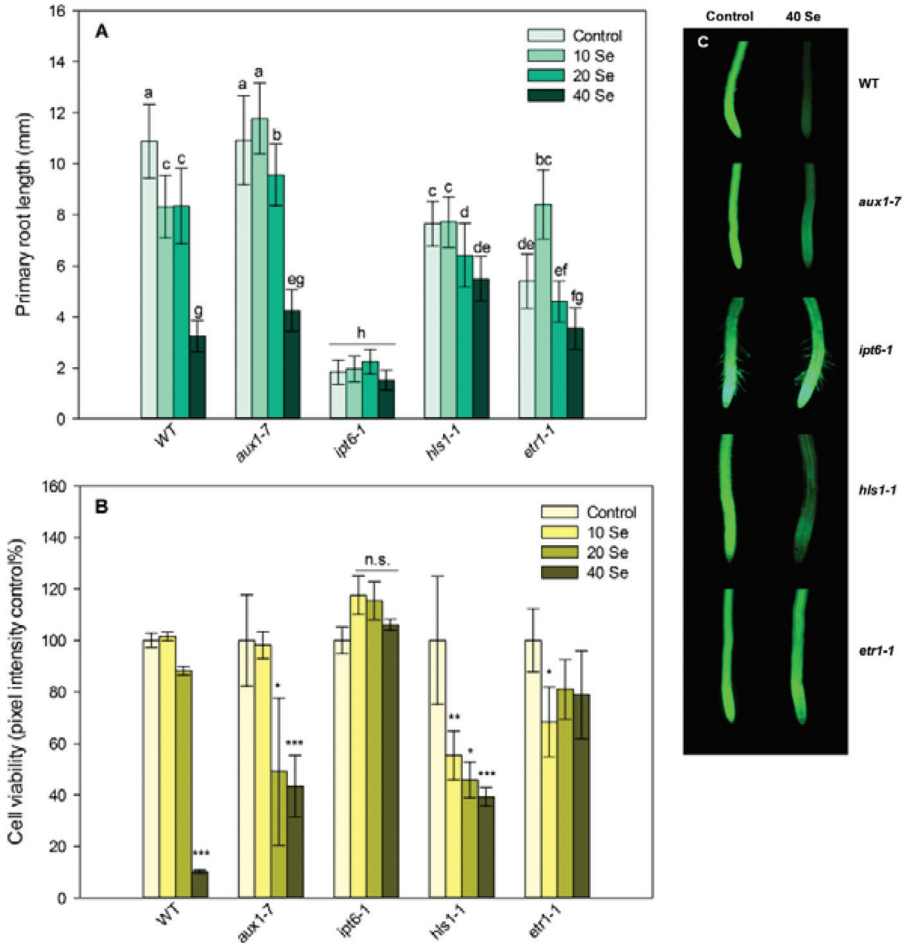

Fig. 3. Primary root length (A) and meristem cell viability (B) of control and Se-treated WT, *aux1-7* (auxin-resistant), *ipt6-1* (cytokinin-overproducing), *hls1-1* (ethylene-deficient) and *etr1-1* (ethylene-resistant) *Arabidopsis* 4 days after germination. Different letters indicate significant difference according to Duncan's test ($n = 10$, $P \leq 0.05$). Asterisks indicate significant difference to control according to Student's t-test ($n = 10$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). (C) Representative fluorescence microscopy images of control and 40 μM selenite-treated wild-type and mutant root tips stained with fluorescein diacetate. Bar, 0.5 mm.

concentration of control PR meristems was very high during the early growth, whereas later on the level of NO decreased and remained at the base level. This suggests the involvement of NO in the normal, early seedling development (Gniazdowska *et al.*, 2010). Interestingly, 2 and 4 DAG, selenite decreased NO level in a concentration-dependent manner; however, during the later plant growth phases, NO levels increased in response to Se, especially at 14 DAG (Fig. 4A). Being a functional signal molecule, the actual NO concentration of a tissue has to be strictly regulated by its synthesis and removal. Possibly, during the early development NO can be removed by its reaction with oxygen, glutathione, plant haemoglobins, or different ROS forms (H_2O_2 and/or O_2^-) (Misra *et al.*, 2011). In the present study, the last possibility seems to be confirmed by the high H_2O_2 levels detected in selenium-treated young root tips (Fig. 4B). The selenite-induced NO generation in older roots may be the result of, for example, enzymic NO generation by NR. Selenite exposure was found to intensify NR activity in lettuce (Rios *et al.*, 2010), either directly or indirectly via a molybdenum increase induced by sulphur deficiency (Shinmachi *et al.*, 2010; Yu *et al.*, 2010). However, other possible mechanisms can contribute to NO level changes in this experimental system.

The H_2O_2-dependent resorufin fluorescence intensity was low in control plants, and selenite excess enhanced it in a concentration-dependent way during the early development (2 and 4 DAG) directly or indirectly as a result of Se-induced glutathione depletion (Grant *et al.*, 2011). In the present experimental system, high H_2O_2 levels were not obviously connected to cell death in the young PR meristems (Fig. 1C and Fig. 4B). In the second developmental phase, H_2O_2 concentrations decreased or did not change in response to selenite, which may be the result of the activation of antioxidant systems. The selenium-induced antagonism between NO and H_2O_2 observed during *Arabidopsis* PR development, can originate from chemical reactions (e.g. H_2O_2 or $O_2^- + NO \rightarrow ONOO^-$) and enzymic or non-enzymic background mechanisms (e.g. NR, antioxidants), which need to be further analysed in the future.

Arabidopsis *mutants possess altered NO and H_2O_2 homeostasis and differences in selenium tolerance*

During studies on the GSNO reductase-deficient *gsnor3-1* mutant, the NO-lacking double mutant *nia1nia2*, and the ascorbate-deficient *vtc2-1* and ascorbate-overproducing (via *myo*-inositol oxygenase) *miox4* plants, this study observed a significantly higher NO concentration in PRs of *gsnor1-3*, while the *nia1nia2* roots showed lower NO levels than the WT. In the PR of the ascorbic acid-deficient *vtc2-1*, lower, but in the *miox4* mutant higher, H_2O_2 and total intracellular ROS contents were detected, although these differences were not significant compared to the WT (data not shown).

Similarly to the WT, all selenium treatments inhibited root elongation in the case of NO excess (*gsnor1-3*) but the viability of meristem cells was not affected (Fig. 5), which suggests the contribution of this molecule to the Se-induced PR shortening and simultaneous Se tolerance. The high GSNO levels of *gsnor1-3* were demonstrated to be important also during disease resistance or thermotolerance (Feechan *et al.*, 2005; Lee *et al.*, 2008). Recently, an NO-overproducing tomato mutant (*shr*) was isolated in which the observed short root phenotype and the disease resistance were associated with the enhanced NO production (Negi *et al.*, 2010). In *nia1nia2* plants, the reduced NO level resulted in Se sensitivity showing the possible involvement of NO produced by the root NR (Fig. 5B). The reduced NO level also helped to maintain a better root growth under suboptimal conditions (Fig. 5A). This double mutant proved to be less tolerant to other stressors such as water deficit (Lozano-Juste and León, 2010). The high H_2O_2 content resulted from the ascorbic acid deficiency in *vtc2-1* roots contributed to selenite sensitivity, but the inhibited

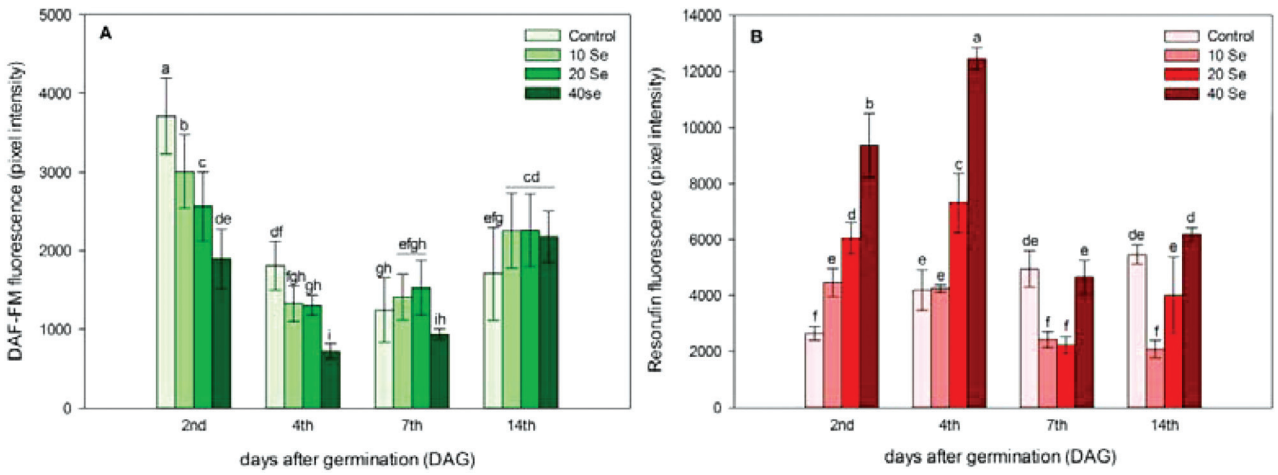

Fig. 4. Nitric oxide-dependent fluorescence (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate, DAF-FM, A) and hydrogen peroxide-dependent fluorescence (resorufin, B) in root meristems of control and selenite-treated wild-type *Arabidopsis* at 2, 4, 7, and 14 days after germination. Different letters indicate significant differences according to Duncan's test ($n = 10$, $P \leq 0.05$).

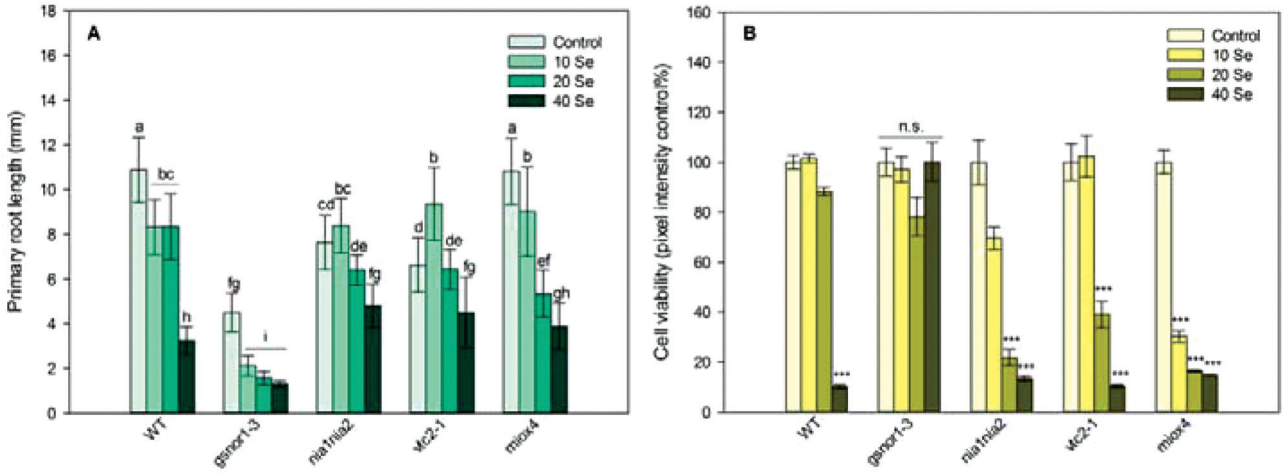

Fig. 5. Primary root length (A) and cell viability (B) in root meristems of control and selenite-treated wild-type, *gsnor1-3* (GSNOR-deficient), *nia1nia2* (NR-deficient), *vtc2-1* (ascorbic acid-deficient), and *miox4* (ascorbic acid-overproducing) *Arabidopsis* at 4 days after germination. Different letters indicate significant difference according to Duncan's test ($n = 10$, $P \leq 0.05$). Asterisks indicate significant difference to control according to Student's t-test ($n = 10$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

root growth was alleviated by H_2O_2 (Fig. 5). This indicates that plants, which can decrease their (root) growth processes significantly, are able to rearrange their means from development to defence mechanisms, resulting in a better survival. Nitrate reductase-dependent NO seems to be a relevant molecule to coordinate this acclimation process, at least in terms of PR growth.

The relationship between hormonal and signal regulatory components during selenite excess

High NO levels were detected in control *aux1-7* plants (Fig. 6A and 6C), which reflects a negative correlation between auxin and NO in the PR. During the growth of copper-treated *Arabidopsis*, a negative regulatory link was discovered between auxin and NO (Pető *et al*., 2011). Recently, Fernández-Marcos *et al*. (2011) published that high levels of NO reduced *DR5::GUS* expression and PIN1-mediated auxin transport in *Arabidopsis* PRs. In *aux1-7*, the low H_2O_2 levels could explain the slight sensitivity of this mutant to oxidative stress (Blomster *et al.*, 2011) and a putative positive regulation between them (Fig. 6B and 6D). ROS can act downstream of auxin signalling in processes such as gravitropism, and auxins are able to modulate H_2O_2 production in guard cells (Potters *et al.*, 2009). Similarly to the WT, 40 μM Se reduced NO and enhanced H_2O_2 generation in *aux1-7*, but these effects were not statistically significant (Fig. 6). This study's hypothesis is that, during the early seedling development, the H_2O_2-dependent mitogen-activated protein kinase cascade negatively affects auxin sensitivity by downregulating the auxin-inducible gene expression (Nakagami *et al*., 2006), resulting in growth inhibition. In the later growth phase (14 DAG), the Se-induced NO reduces PIN1-mediated auxin transport, resulting in low auxin levels and PR growth inhibition.

In the case of control *ipt6-1 Arabidopsis* possessing high cytokinin content, more NO was produced than in the WT (Fig. 6A and 6C), a finding which is supported by the work of Tun *et al.* (2001), where exogenously applied cytokinin rapidly induced NO production. Moreover, in some physiological processes, such as hypocotyl elongation, NO exerts cytokinin-like effects supporting the positive regulatory relationship between this hormone and the signal molecule (Beligni and Lamattina, 2001). In response to Se, NO levels were not reduced, but showed a slight, nonsignificant increase. The *ipt6-1* mutation also resulted in lower ROS production (Fig. 6B and 6D), which supports a possibly negative regulation between these components.

In control *hls1-1* and *etr1-1* mutants showing lower ethylene concentrations and deficiency in signalling, the level of NO was extremely high compared to the WT (Fig. 6A and 6C). This suggests the relevant antagonism between this plant morphogen and NO during *Arabidopsis* root growth. Another evidence for NO–ethylene antagonism is provided by the review of Besson-Bard *et al.* (2009), where the downregulation of the ethylene biosynthetic ACC oxidase gene (*ACO4*) by NO was reported. However, selenite induced significant decreases in NO contents such as in the WT, which suggests that there is no regulatory relationship between these molecules during PR growth under Se excess. Hydrogen peroxide is a possible downstream element of ethylene signalling, since the level of it was low in control *hls1-1* and *etr1-1*, and Se was not able to increase its content in the mutant roots (Fig. 6B and 6D). These results are supported by the findings that histidine kinases are strongly H_2O_2-responsive and they also modulate cellular responses to, for example, ethylene (Desikan *et al.*, 2001).

Taken together, higher Se concentrations (20 and 40 μM) reduces PR development, which can be considered an adaptation process of the plant, since the reorientation of means from development for protection mechanisms ensures better survival. Selenium exposure disturbs protein synthesis through the formation of selenomethionine and selenocystein directly leading to cell death in the PR meristem and growth inhibition. The hormonal balance of the PR is also affected by selenium. During the early development, Se-induced H_2O_2 can reduce auxin-responsive gene expression, while NO inhibits auxin transport in older roots and the decrease of root auxin level results in growth inhibition. Selenium enhances cytokinin-responsive gene expression (consequently cytokinin levels), which leads to PR

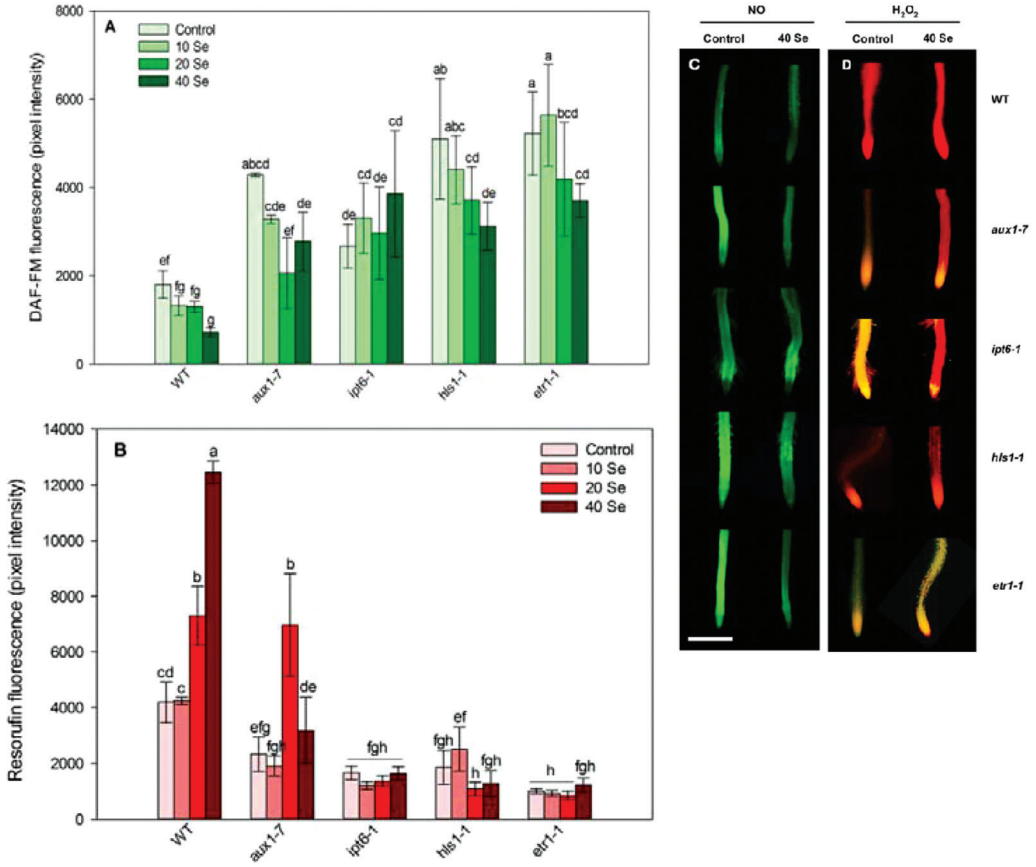

Fig. 6. Nitric oxide-dependent fluorescence (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate, DAF-FM, A) and hydrogen peroxide-dependent fluorescence (resorufin, B) in root meristems of 0, 10, 20, and 40 μM selenite-treated wild-type, *aux1-7* (auxin-resistant), *ipt6-1* (cytokinin-overproducing), *hls1-1* (ethylene-deficient), and *etr1-1* (ethylene-resistant) plants at 4 days after germination. Different letters indicate significant differences according to Duncan's test ($n = 10$, $P \leq 0.05$). Representative microscopic images of control and 40 μM selenite-treated wild-type and mutant root tips stained with DAF-FM (C) or Ampiflu (D). Bar, 0.5 mm.

growth inhibition possibly through NR-dependent NO synthesis and/or through the reduction of H_2O_2 level. The selenite-induced enhancement of ethylene biosynthesis may cause cell death resulting growth hindrance and H_2O_2 is a downstream element of its signalling, while there is no regulatory link between ethylene and NO under Se excess. The optimal level of H_2O_2 is necessary for Se tolerance and NO overproduction in *Arabidopsis* roots ensures Se tolerance.

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