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Research article

Response to copper excess in *Arabidopsis thaliana*: Impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile

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ABSTRACT

Growth, in particular reorganization of the root system architecture, mineral homeostasis and root hormone distribution were studied in *Arabidopsis thaliana* upon copper excess. Five-week-old Arabidopsis plants growing in hydroponics were exposed to different Cu^{2+} concentrations (up to 5 μM). Root biomass was more severely inhibited than shoot biomass and Cu was mainly retained in roots. Cu^{2+} excess also induced important changes in the ionome. In roots, Mg, Ca, Fe and Zn concentrations increased, whereas K and S decreased. Shoot K, Ca, P, and Mn concentrations decreased upon Cu^{2+} exposure. Further, experiments with seedlings vertically grown on agar were carried out to investigate the root architecture changes. Increasing Cu^{2+} concentrations (up to 50 μM) reduced the primary root growth and increased the density of short lateral roots. Experiment of split-root system emphasized a local toxicity of Cu^{2+} on the root system. Observations of GUS reporter lines suggested changes in auxin and cytokinin accumulations and in mitotic activity within the primary and secondary root tips treated with Cu^{2+}. At toxic Cu^{2+} concentrations (50 μM), these responses were accompanied by higher root apical meristem death. Contrary to previous reports, growth on high Cu^{2+} did not induce an ethylene production. Finally lignin deposition was detected in Cu^{2+}-treated roots, probably impacting on the translocation of nutrients. The effects on mineral profile, hormonal status, mitotic activity, cell viability and lignin deposition changes on the Cu^{2+}-induced reorganization of the root system architecture are discussed.

1. Introduction

Copper (Cu) is a transition metal which has two oxidation states under physiological conditions. As a result, Cu is the cofactor of numerous enzymes involved in electron transfer reactions. It is involved in essential biological processes such as photosynthesis, respiration, oxygen superoxide scavenging, ethylene sensing, cell wall metabolism and lignification [6,38]. However, Cu excess is highly toxic because it catalyses Fenton reactions which generate hydroxyl radicals causing damages to lipids, proteins and DNA [18]. This increase in reactive oxygen species (ROS) leads to changes in the activity of many enzymes involved in antioxidative pathways [7,43,66,79]. An overall reduction of plant biomass, an inhibition of root growth, chlorosis, bronzing and necrosis are usual reported symptoms of a Cu excess [38,41]. Cu toxicity can also result in reduction of Fe uptake [38] and in metabolic disturbances such as loss of chloroplast integrity, an alteration of plastid membrane composition and an inhibition of photosynthetic electron transport [39,52,57]. In *Arabidopsis thaliana*, the majority of the genes up-regulated by a Cu treatment are not specific to this metal and are also induced by many other stresses like exposure to ozone, salt, cold and osmotic shock [73,80]. This is likely due to the massive generation of ROS induced by Cu, which is a common response to most biotic and abiotic stresses [73]. To prevent Cu-induced damages, plants developed complex mechanisms to avoid the accumulation of free Cu ions in cells (for review see [6,56]).

Plants have evolved mechanisms in order to adapt to nutrient availability changes. Among these mechanisms, the reorganization of the root system architecture (RSA) shows a high degree of plasticity [53,54]. In many cases, exposure to stress conditions leads to a common remodelling of the RSA characterized by an inhibition of primary root (PR) growth and the simultaneous stimulation of lateral root (LR) formation [34,53,54]. For instance, this response is induced following exposure to high concentrations of heavy metals like cadmium, copper, zinc and lead [51,53,54] or low concentrations of phosphate [13,34,35,74]. However, PR growth is not affected by nitrate availability within a certain range [13,78].

Abbreviations: DW, dry weight; FW, fresh weight; LR, lateral root; PR, primary root; ROS, reactive oxygen species; RSA, root system architecture.

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Some processes, such as changes in nutrient uptake, ROS levels, hormonal homeostasis, cell elongation and cell division have been postulated to be involved in this response [22,34,51,53,54]. Plant hormones, mainly auxin, cytokinin and ethylene, control most of the characteristics of the root system, including PR growth and formation of LRs and root hairs [3,47,53]. Auxin is closely associated with LR formation and axillary branching [3,19,47]. Several studies showed the implication of auxin in the RSA remodelling following stress conditions, such as phosphate and Fe starvation or Al and Cd excess [28,35,53,54,65]. Ethylene seems also to be a player in root architecture modifications in response to stress and was shown to inhibit root elongation in phosphate-deficient and aluminium-stressed plants [34,53]. Cytokinin has an inhibitory effect on primary root growth, but in contrast to auxin, cytokinin inhibits LR formation and results in a reduction of LR density [30,47,61].

In case of exposure to high Cu, studies have reported several changes which could impact on RSA remodelling: decrease in root meristem cell proliferation [16,33], damages of cell integrity in the root transition zone [37], root cell death [77], formation of ROS [51] and increase in the activity of root peroxidases, which could decrease the cell elongation through lignin synthesis and reduction of cell wall plasticity [7,79]. While morphogenesis is known to be tightly linked to hormonal homeostasis, very few studies have detailed root hormonal changes under Cu excess.

The aim of the present work is (i) to detail the morphological changes in *Arabidopsis thaliana* in response to Cu^{2+} excess mainly at the root level and (ii) to identify some determinants in the orchestration of the RSA response, in particular changes in the hormonal distribution, lignin accumulation and mineral profile.

2. Results

2.1. Effect of Cu^{2+} excess on biomass production and mineral concentration

Experiments were conducted with plants grown in hydroponics to study the impact of copper excess on plant growth and mineral profile. Shoot and root biomass were significantly ($P < 0.01$) reduced in plants exposed to 2.5 and 5 μM Cu^{2+} for 14 days compared to control conditions (Fig. 1). At concentration lower than 2.5 μM Cu^{2+}, no significant visible effect of Cu^{2+} on growth was observed within two weeks of treatment. At moderate Cu^{2+} concentrations, root growth was more severely affected than the shoot growth. Fourteen days after the addition of 2.5 μM Cu^{2+} in the nutrient solution, a decrease of 41% and 25% in dry biomass was apparent in roots and in shoots respectively, compared to control conditions. Fig. 1C shows that the addition of 5 μM Cu^{2+} impacted on the root length.

The Cu content in shoot and root tissues increased with increasing Cu^{2+} concentrations in the nutrient solution (Table 1). Cu accumulation was always higher in the roots than in the shoots. For instance, plants treated with 5 μM Cu^{2+} for 7 days accumulated more than 600 mg Cu kg^{-1} DW in the roots and about 20 mg Cu kg^{-1} DW in the shoots. Cu^{2+} excess also modified the concentrations of other nutrients (Table 1). Significant ($P < 0.01$) changes in roots were already visible after 1 day of exposure to 5 μM Cu^{2+}. Mg and Ca concentrations increased, while P and K decreased. A prolonged exposure resulted in more drastic mineral profile changes. In roots, Mg, Ca, Fe and Zn concentrations significantly ($P < 0.01$) increased after 7-days treatment, whereas K and S decreased. In shoots, K, Ca, P, Fe, Mn concentrations decreased. After 14-days exposure a significant decrease in Mn concentration was also visible in the roots. Cu^{2+} addition in the nutrient solution significantly reduced the root to shoot translocation of Ca and Cu (Table 1).

2.2. Impact of Cu^{2+} excess on the root system architecture

The impact of Cu^{2+} on the RSA was evaluated on seedlings vertically grown on agar. In this growth system, effective copper concentrations were about 10 times higher than in hydroponics. Root architecture was considerably remodelled by the addition of Cu^{2+} (Fig. 2A). First, we observed an increase of the LR density from 10 μM to 50 μM Cu^{2+} (Fig. 2C). However, at 50 μM Cu^{2+} most of secondary roots did not elongate more than 3 mm contrary

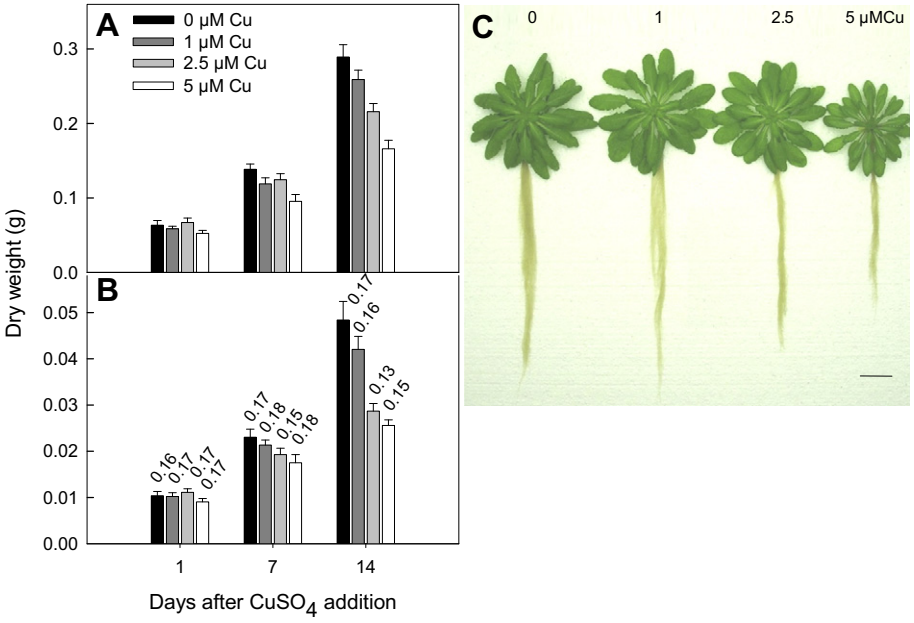

Fig. 1. Effect of Cu^{2+} excess on root and shoot biomasses of Arabidopsis plants grown in hydroponics. A-B Dry weight of shoot (A) and root (B) of plants at day 1, 7 and 14 after Cu^{2+} addition in the nutrient solution. Values are means of 6 plants ± SE. Numbers above the bars are the root/shoot ratios. C Arabidopsis plants photographed at day 14 after Cu^{2+} addition. Bar = 2 cm.

Table 1
A Mineral concentrations, expressed in mg g^{-1} DW for major elements (Mg, P, S, K, Ca) and in mg kg^{-1} DW for minor elements (Cu, Mn, Fe, Zn), of Arabidopsis plants grown hydroponically at day 1 and 7 and 14 after the Cu^{2+} addition. Values are means of 6 plants ± SE. Shoot/root ratios were calculated for each element. Asterisks indicate a significant difference between Cu^{2+} treatments and the control at $P \leq 0.05$ (*) and $P \leq 0.01$ (**), according to the Tukey test. B Statistical analyses of mineral concentrations between durations (1, 7 and 14 days) of Cu^{2+} treatments. For each mineral, different letters indicate significant differences between durations of treatment at $P \leq 0.05$ according to the Tukey test (uppercase letters for shoot, lowercase letters for root).

A	Mineral content														
	Shoot				Root				Shoot/root						
[$CuSO_4$]	0 μM	2.5 μM		5 μM		0 μM	2.5 μM		5 μM		0 μM	2.5 μM		5 μM	
Day 1															
Mg	9.4 ± 0.2	8.8 ± 0.1		8.7 ± 0.1	*	1.9 ± 0.1	1.8 ± 0.0		2.2 ± 0.1	**	4.9	4.8		3.9	
P	6.9 ± 0.1	6.8 ± 0.2		6.8 ± 0.2		9.6 ± 0.3	9.1 ± 0.1		8.2 ± 0.2	**	0.7	0.7		0.8	
S	9.5 ± 0.4	9.1 ± 0.2		9.3 ± 0.2		10.7 ± 0.4	11.9 ± 0.1	*	11.1 ± 0.3		0.9	0.8		0.8	
K	36.0 ± 0.7	36.6 ± 1.3		37.5 ± 1.5		32.3 ± 1.3	39.2 ± 0.7	**	26.6 ± 1.0	**	1.1	0.9		1.4	
Ca	50.1 ± 1.1	46.8 ± 0.5		45.6 ± 0.9	**	7.6 ± 0.2	7.3 ± 0.4		11.4 ± 0.6	**	6.6	6.4		4.0	**
Cu	7.0 ± 0.1	11.4 ± 0.4	**	10.6 ± 0.4	**	14.7 ± 0.5	205.3 ± 3.6	**	663.3 ± 29.8	**	0.5	0.1		<0.1	**
Mn	54.0 ± 2.3	49.2 ± 1.1		50.3 ± 1.1		33.4 ± 3.8	26.0 ± 2.4		162.2 ± 21.5	**	1.6	1.9		0.3	**
Fe	107.8 ± 2.6	96.7 ± 1.8		96.6 ± 1.9		1224.2 ± 70.0	1064.9 ± 25.4		1246.6 ± 48.0		0.1	0.1		0.1	
Zn	62.2 ± 7.1	64.4 ± 3.3		56.9 ± 3.3		251.7 ± 13.7	208.0 ± 3.3	*	218.6 ± 10.7		0.2	0.3		0.3	
Day 7															
Mg	9.7 ± 0.2	9.4 ± 0.2		10.1 ± 0.1		1.8 ± 0.1	1.6 ± 0.1		2.3 ± 0.1	**	5.3	5.8		4.3	
P	7.5 ± 0.2	7.1 ± 0.2	*	6.6 ± 0.1	**	10.1 ± 0.4	9.2 ± 0.4		9.2 ± 0.4		0.7	0.8		0.7	
S	9.8 ± 0.3	9.8 ± 0.3		9.2 ± 0.2		11.7 ± 0.4	9.6 ± 0.5	**	9.8 ± 0.6	*	0.8	1.0		0.9	
K	40.5 ± 1.1	41.3 ± 0.9		36.7 ± 0.4	**	36.8 ± 2.0	30.9 ± 2.1	*	22.2 ± 1.2	**	1.1	1.3		1.6	
Ca	54.0 ± 1.5	47.3 ± 0.6	**	49.5 ± 0.8	*	8.1 ± 0.4	7.9 ± 0.4		11.8 ± 0.9	**	6.7	6.0		4.2	*
Cu	7.2 ± 0.1	16.4 ± 0.3	**	19.0 ± 1.5	**	16.9 ± 1.1	313.1 ± 22.1	**	661.0 ± 24.0	**	0.4	0.1		<0.1	**
Mn	54.0 ± 1.5	47.2 ± 1.4	**	46.8 ± 1.3	**	109.1 ± 13.8	129.3 ± 26.4		135.8 ± 15.1		0.5	0.4		0.3	
Fe	117.2 ± 2.6	100.1 ± 0.3	**	106.9 ± 1.7	**	1150.7 ± 79.2	1297.1 ± 74.3		1740.0 ± 98.8	**	0.1	0.1		0.1	
Zn	62.4 ± 7.3	79.3 ± 9.2		91.9 ± 18.7		199.9 ± 13.6	319.5 ± 10.2	**	290.0 ± 13.6	**	0.3	0.2		0.3	
Day 14															
Mg	9.6 ± 0.2	9.9 ± 0.1		10.8 ± 0.1	**	2.1 ± 0.1	2.0 ± 0.0		2.4 ± 0.1	*	4.5	5.0		4.5	
P	7.5 ± 0.1	7.4 ± 0.1		6.6 ± 0.1	**	10.4 ± 0.4	9.3 ± 0.5		9.2 ± 0.4		0.7	0.8		0.7	
S	9.5 ± 0.2	11.3 ± 0.3	**	10.5 ± 0.3	*	11.8 ± 0.8	9.8 ± 0.4	*	9.1 ± 0.1	*	0.8	1.2		1.2	
K	38.2 ± 0.5	44.0 ± 0.9	**	36.4 ± 1.2		37.5 ± 2.8	30.3 ± 1.1	*	20.8 ± 0.8	*	1.0	1.5		1.7	*
Ca	54.7 ± 1.2	47.7 ± 0.4	**	48.4 ± 1.0	**	9.6 ± 0.9	8.5 ± 0.3		9.9 ± 0.6		5.7	5.6		4.9	*
Cu	6.9 ± 0.2	21.9 ± 0.7	**	29.5 ± 1.3	**	21.3 ± 1.9	344.4 ± 17.6	**	672.2 ± 24.3	**	0.3	0.1		<0.1	**
Mn	57.3 ± 1.3	47.1 ± 0.6	**	42.0 ± 1.1	**	188.7 ± 17.7	159.1 ± 31.4		89.8 ± 13.7	*	0.3	0.3		0.5	
Fe	115.4 ± 3.2	107.7 ± 1.4		122.6 ± 6.0		1244.7 ± 164.7	1612.9 ± 126.0		1970.7 ± 106.9	**	0.1	0.1		0.1	
Zn	68.7 ± 6.2	113.0 ± 17.3		116.5 ± 20.3		173.4 ± 17.0	371.7 ± 31.9	**	281.5 ± 11.0	**	0.4	0.3		0.4	

B	0 μM Cu^{2+}						2.5 μM Cu^{2+}						5 μM Cu^{2+}					
	Shoot			Root			Shoot			Root			Shoot			Root		
	1 d	7 d	14 d	1 d	7 d	14 d	1 d	7 d	14 d	1 d	7 d	14 d	1 d	7 d	14 d	1 d	7 d	14 d
Mg	A	A	A	a	a	a	C	B	A	a	b	a	C	B	A	a	a	a
P	A	A	A	a	a	a	A	A	A	a	a	a	A	A	A	a	a	a
S	A	A	A	a	a	a	B	B	A	a	b	b	B	B	A	a	ab	b
K	B	A	AB	a	a	a	B	A	A	a	b	b	A	A	A	a	b	b
Ca	A	A	A	a	a	a	A	A	A	a	a	a	B	A	AB	a	a	a
Cu	A	A	A	b	ab	a	C	B	A	b	a	a	C	B	A	a	a	a
Mn	A	A	A	c	b	a	A	A	A	b	a	a	A	A	B	a	ab	b
Fe	A	A	A	a	a	a	B	B	A	b	b	a	B	B	A	b	a	a
Zn	A	A	A	a	ab	b	B	AB	A	b	a	a	B	AB	A	b	a	a

to those growing on the control medium (Fig. 2C). Secondly, a decrease of PR growth was observed from 25 μM Cu^{2+} with a complete inhibition at 50 μM (Fig. 2B). It is worth noticing that, at 10 μM Cu^{2+}, a 35% increase in the total LR density was measured whereas no reduction of PR growth was observed.

When seeds were directly germinated on MS/2 medium supplemented with Cu^{2+}, the effect on root growth was similar to the one measured in transfer experiments. Indeed, no significant reduction in PR length was measured in seedlings exposed to 10 μM Cu^{2+} for 14 days, compared to control conditions, while a 38% reduction was measured at 25 μM Cu^{2+} (data not shown).

2.3. Changes in the hormonal balance, cell cycle and cell ability in Cu^{2+}-treated root tips

As a first step to investigate physiological processes involved in the RSA remodelling, we studied the distribution of auxin and cytokinin in Cu^{2+}-treated root tips. We analysed the activity of the β-glucuronidase (GUS) in *DR5*::*GUS* and *ARR5*::*GUS* reporter lines, considered to reflect sites of auxin and cytokinin signals, respectively [11,67]. A *35S*::*GUS* line was used as control. GUS staining in *DR5*::*GUS* line strongly decreased in PR meristems treated with 50 and 75 μM Cu^{2+} for 24 h (Fig. 3A), but also in the *35S*::*GUS* line. Interestingly, at 25 μM and more markedly at 50 μM Cu^{2+}, we observed a higher activity of the *DR5* promoter in the area just above the PR apical meristem (Fig. 3A). Compared to the LRs of control seedlings, the area of the *DR5*::*GUS* expression was reduced in the apex of most LRs treated with 50 μM Cu^{2+} for 7 days (Fig. 3B). In *ARR5*::*GUS* line, GUS staining slightly decreased in PR tips exposed to 25 μM Cu^{2+} for 24 h and strongly increased in 50 μM Cu^{2+}-treated roots. At a very toxic Cu^{2+} concentration (75 μM), the root tips were completely unstained (Fig. 3C), also in the *35S*::*GUS* control line.

Ethylene is another hormone often associated with stress-induced RSA remodelling [34,53]. We therefore evaluated the

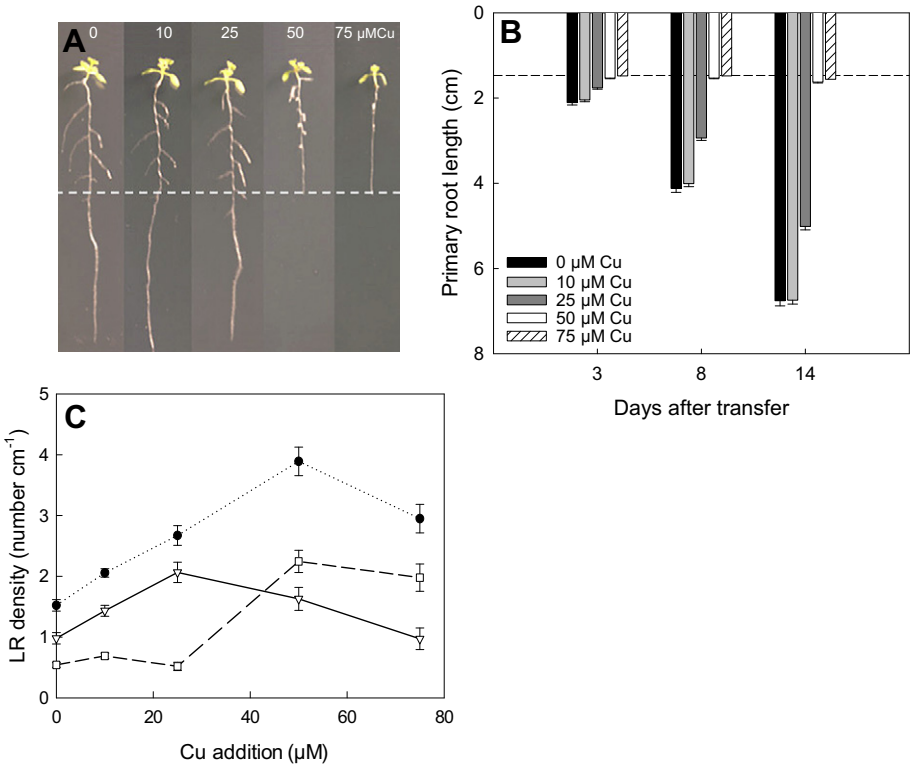

Fig. 2. Effect of Cu^{2+} excess on the root architecture of Arabidopsis seedlings grown on agar. A Root architecture of seedlings at day 7 after transfer (dotted line) to Cu^{2+} excess. Bar = 1 cm. B Primary root length of seedlings after transfer (dotted line) to plates containing different Cu^{2+} concentrations. C Lateral root density, calculated as the LR number divided by the distance from the hypocotyl to the last emerged LR, at day 8 after transfer to plates containing increasing Cu concentrations; ● total lateral root density, □ density of lateral roots < 0.3 cm, ▽ density of lateral roots >0.3 cm. Values are means of ~50–80 seedlings ± SE.

copper ability to induce ethylene production in Arabidopsis. Seedlings challenged by Cu stress (25 or 50 µM Cu^{2+}) for 9 days slightly produced more ethylene than the control ones but these differences were not statistically significant ($P < 0.01$) (Table 2).

To investigate in more details the mechanisms by which Cu^{2+} excess inhibits the PR growth, we examined the pattern of mitotic activity in the root meristems. *CYCB1;1* is a marker of cells competent for mitosis [15] and *CYCB1*::*GUS* fusion is a way to visualize the pattern of potential mitotic activity in the PR meristem and during LR initiation events [9]. Similarly to *DR5*::*GUS*, the staining had disappeared in the PR apex and in the oldest LRs exposed to 50 µM Cu^{2+} (Fig. 3E, F). No change in *CYCB1*::*GUS* expression was observed after 10 and 25 µM Cu^{2+} treatments.

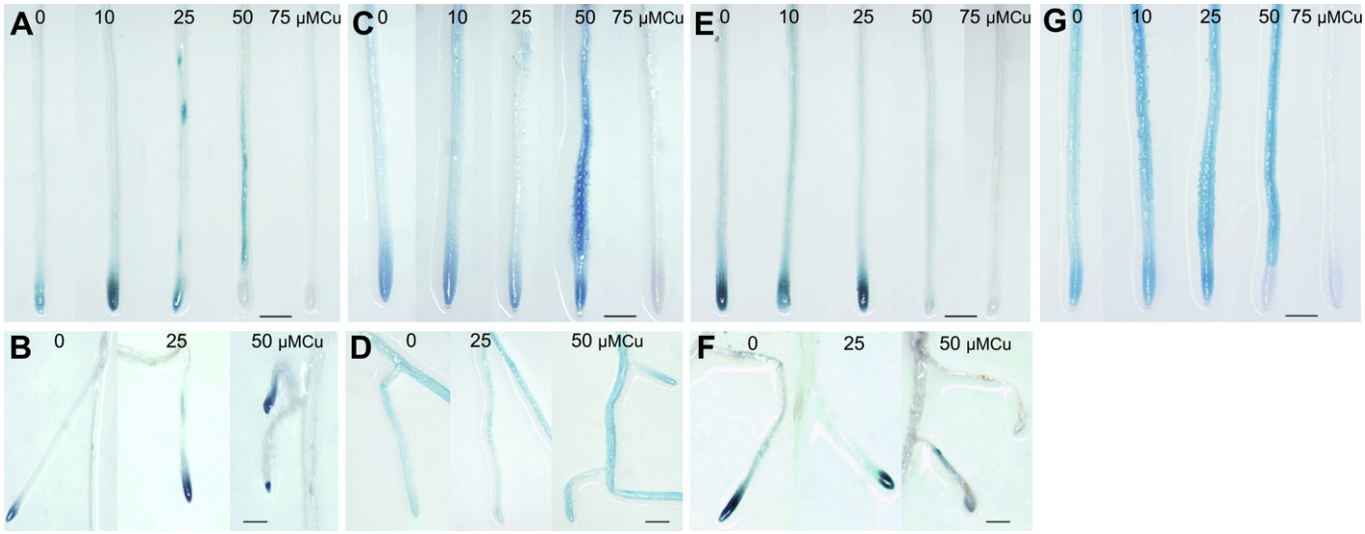

Fig. 3. Effect of Cu^{2+} excess on the hormone distribution and the mitotic activity in the root apex of seedlings grown on agar. GUS-stained root apex of *DR5*::*GUS* (A, B), *ARR5*::*GUS* (C, D), *CYCB1*::*GUS* (E, F) and 35S::GUS (G) reporter lines, 24 h (A, C, E, G) and 7 days (B, D, F) after the transfer to plates containing increasing Cu^{2+} concentrations. A, C, E, G Primary root tips; B, D, F Lateral root tips. Bars = 500 µm. The blue precipitation observed in the apex of *ARR5*::*GUS* roots treated with 50 µM Cu^{2+} may be due to diffusion because the cells are not metabolically active (see the apex of *35S*::*GUS* roots in part G).

Table 2
Ethylene production, expressed in nl g^{-1} (FW) h^{-1}, by 9-day-old Arabidopsis plantlets grown on agar in vertical plates supplemented with 0, 25 or 50 μM Cu^{2+}. No significant difference was measured at $P \leq 0.05$ according to the Tukey test.

[$CuSO_4$]	C_2H_4 production (nl g^{-1} h^{-1})	
0 μM	1.72	±0.43
25 μM	2.52	±0.49
50 μM	2.17	±0.23

The cells in the root apex of GUS reporter lines showed signs of senescence after the transfer to toxic Cu^{2+} concentrations (50 and 75 μM). Cell viability was therefore assessed in root tips using propidium iodide staining. That chemical compound is able to penetrate damaged cell membranes and to intercalate with DNA and RNA to form a bright-red fluorescent complex seen in dead cells [36]. Results showed that the cell viability in the PR tips treated with 50 or 75 μM Cu^{2+} for 24 h was strongly decreased compared to control plants (Fig. 4B). In addition, we also observed a strong red staining in the cells of short LRs formed in plants exposed to 50 μM Cu^{2+} for 7 days (Fig. 4D).

2.4. Local toxicity of Cu in roots

In order to determine whether Cu^{2+} excess acted as a local or a systemic signal, we carried out a split-root experiment in which half of the root system was experiencing the control Cu^{2+} concentration (= no addition of $CuSO_4$ to the MS medium) and the other half a high Cu^{2+} concentration (addition of 50 μM $CuSO_4$). Fig. 5A shows that the root portion contacting high Cu^{2+} was inhibited, unlike the other portion growing in the uncontaminated medium. A split-root system was set up in hydroponics in order to obtain enough material to quantify the Cu content in split roots. Though one part of the root was growing in a solution supplemented with 5 μM Cu^{2+}, no significant increase in Cu content was measured in the part of the root growing in the control medium (data not shown). In addition, a split-root system experiment was carried out with *DR5*::*GUS* and *ARR5*::*GUS* transgenic lines growing on agar. Although auxin and cytokinin distributions, as reflected by reporter lines, were affected in the root portion contacting 50 μM Cu^{2+}, there was no change in the expression of the *DR5*::*GUS* and *ARR5*::*GUS* reporters in the root portion contacting the control medium (data not shown).

2.5. Accumulation of lignin in Cu^{2+}-treated roots

To investigate the possible change in lignin accumulation of Cu^{2+}-treated roots, the roots were incubated in phloroglucinol–HCl, which stains lignin in red. Seven days after the transfer to Cu^{2+} concentrations (≥25 μM Cu^{2+}), the roots were partially red-stained compared to the control roots, in which lignin deposition was not detected (Fig. 6). The accumulation of lignin was not uniform in roots exposed to 25 μM Cu^{2+} but was more homogeneously distributed at 50 and 75 μM.

3. Discussion

3.1. Effect of Cu^{2+} excess on plant growth and mineral status

Biomass reduction due to Cu^{2+} excess is a common feature in most plant species [1,48,76]. Cu^{2+} excess applied at a moderate concentration (2.5 μM) reduces more drastically the root biomass than the shoot biomass, which is related to the very large proportion of the absorbed Cu retained in the roots (Table 1).

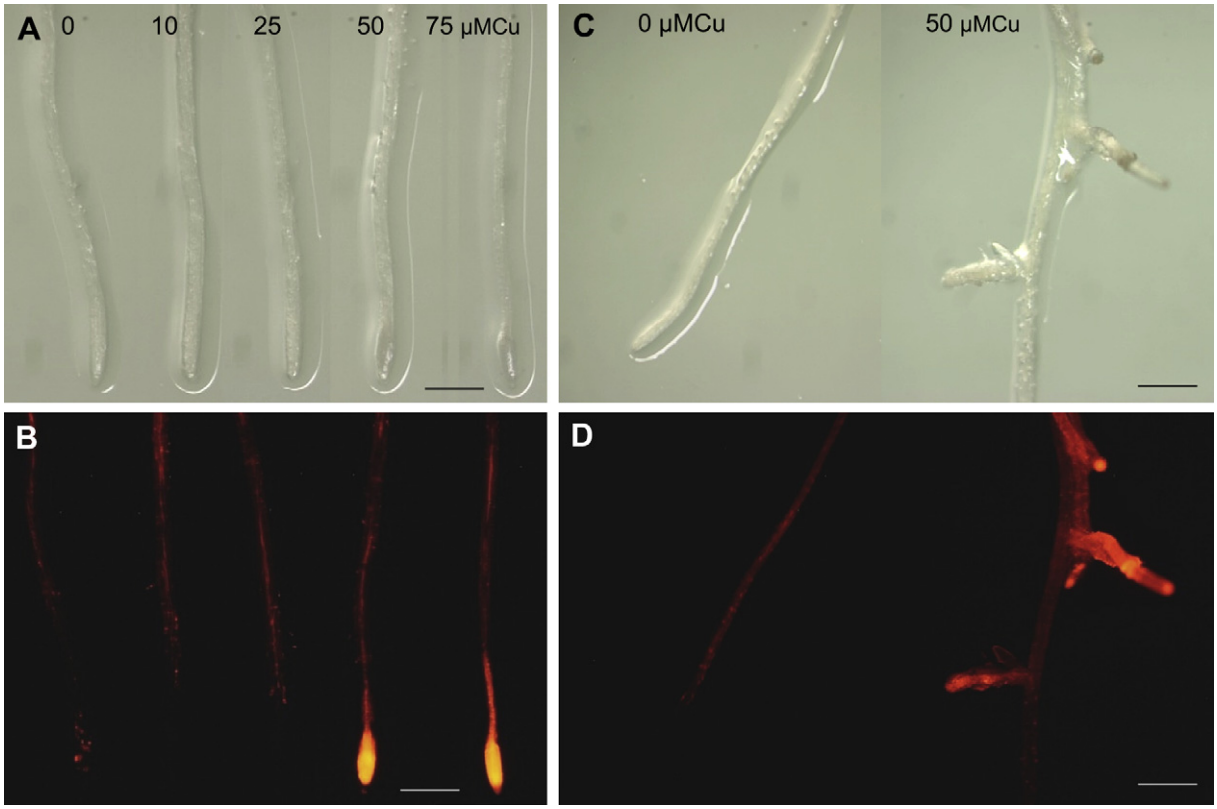

Fig. 4. Effect of Cu^{2+} excess on the cell viability of primary and lateral root tips of seedlings grown on agar. B, D Propidium iodide staining of primary (B) and lateral (D) root tips 24 h (B) and 7 days (D) after transfer to plates containing increasing Cu^{2+} concentrations. A, C Bright-field views of primary (A) and lateral (C) root tips. Bars = 500 μm.

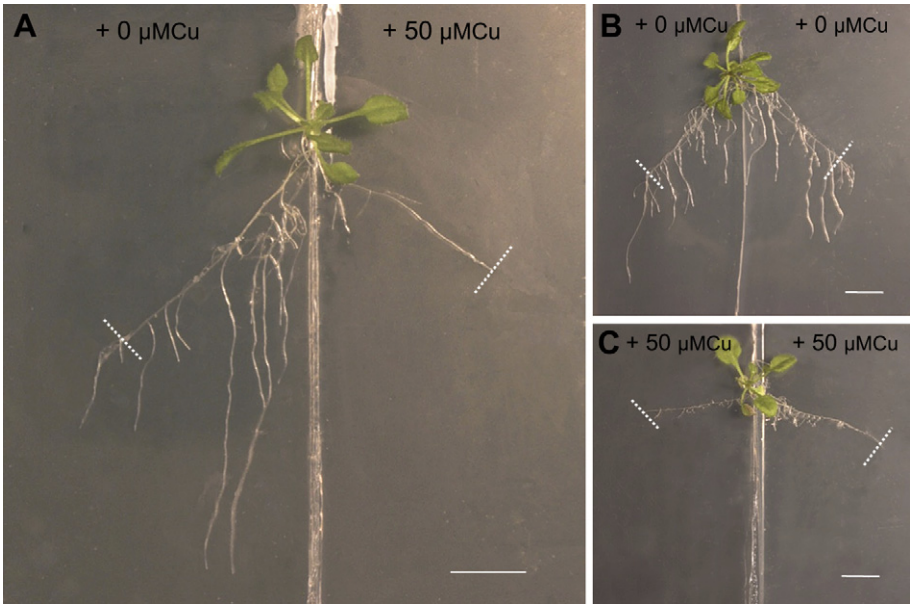

Fig. 5. Morphological adaptation of the Arabidopsis root system to an heterogeneous Cu^{2+} supply. A The two first emerged lateral roots of a 15 day-old seedling were split and put (dotted line) for 5 days into two compartments: one compartment supplemented with 50 µM Cu^{2+} and one compartment uncontaminated with Cu^{2+}. B, C Control plates where the two compartments had the same Cu^{2+} supply: 0 µM (B), 50 µM Cu (C). Bars = 1 cm.

This relationship is not always verified as Cd usually affects more shoot than root growth while accumulating mainly in roots [69,72]. The difference may be related to the capacity of vacuolar transport. Up to now it has not been demonstrated that Cu ions could be sequestrated into the vacuole. Our observations are consistent with previous reports showing that Cu is translocated at a very low rate to the shoot [1,48,76]. A possible reason is that Cu is retained in the root apoplasm at the cell wall [20]. As revealed by a recent QTL analysis, the capacity of Arabidopsis to export Cu from the root to the shoot is an important determinant of Cu^{2+} tolerance in that species [27]. This trait seems to be governed by *HEAVY METAL ATPASE 5* (*HMA5*).

Increasing Cu^{2+} concentrations in the nutrient solution markedly modifies the mineral profile in Arabidopsis (Table 1). Among these changes, the decrease of K has previously been reported by Murphy et al. [45]. The efflux of K^+ serves as a counterion during the Cu^{2+}-induced citrate efflux. This citrate efflux is necessary because Cu may inhibit a cytosolic form of aconitase leading to an accumulation of citrate in the cell [45]. Another effect of Cu on ion partitioning which has often been observed is the decrease of Ca in the shoot accompanied with an increase in the root. It was also reported that calcium translocation from root to shoot is disrupted in the presence of Cu^{2+} excess [40]. On the contrary, there is no common trend in Mg, S, Fe and Zn variations in response to Cu^{2+} excess. The concentrations of these nutrients are differently affected depending on the plant species [25,48,50]. Natural variation in S, Fe and Mn contents was also observed between accessions of *Arabidopsis thaliana* exposed to Cu^{2+} excess [64]. Besides, Schiavon et al. [64] found that accessions with higher Cu tolerance index (measured in relative root growth) had also a lower reduction in S, Fe and Mn contents, suggesting that Cu tolerance may be correlated to the maintenance of nutrient homeostasis. In our study, the decrease in root S and K content and in shoot K, Ca, P, Fe and Mn contents probably impacts negatively on plant growth. The

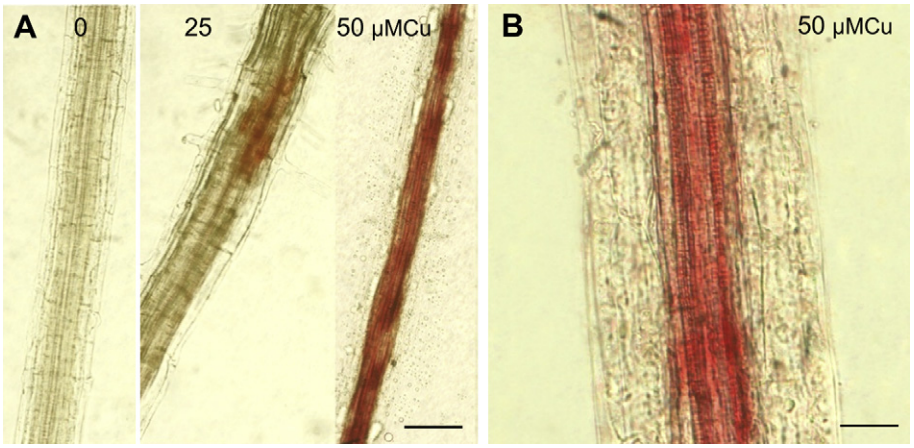

Fig. 6. Effect of Cu^{2+} excess on the lignin deposition in roots of seedlings grown on agar. A Phloroglucinol staining of the primary roots at day 7 after the transfer to plates containing increasing Cu^{2+} concentrations. Bar = 100 µm. B Magnification of the root treated with 50 µM Cu^{2+} for 7 days and stained with phloroglucinol. Bar = 30 µm.

transporters which mediate the influx of transition metals into the cytoplasm have generally a lower metal selectivity than other characterized metal transporters [26]. Therefore, in the presence of high Cu^{2+} concentrations in the plant environment, a competition of Cu ions is probable at the active site of other metal influx transporters. Hence, the increase in Fe and Zn concentrations in roots of Cu^{2+}-treated plants is surprising. Zn accumulation in roots is particularly unexpected since the pathways of Zn and Cu acquisition could be partially linked. Arabidopsis ZINC-REGULATED TRANSPORTER, IRON-REGULATED TRANSPORTER PROTEIN (ZIP) genes *ZIP2* and *ZIP4* complement growth defects of yeast Zn and Cu transport mutants and the expression of both genes is up-regulated by Zn^{2+} and Cu^{2+} in plants, suggesting their involvement in both Zn and Cu uptake [6,75]. In the same way, the Fe uptake by roots seems to be dependent of Cu ions. Although the ZIP member IRON-REGULATED TRANSPORTER 1 (IRT1), the main Fe uptake system, was not shown to transport Cu [29,55], both elements require FERRIC REDUCTION OXIDASE 2 (FRO2) to be reduced before import [56]. However, the presence of Fe–EDTA in the nutrient solution could explain a higher accumulation of Fe in Cu^{2+}-treated plants since Cu ions can displace Fe from Fe–EDTA complexes, making Fe ions more available for plants. In addition, the observed increases in Fe and Zn metals content in root tissues have to be carefully examined. The induction of peroxidase activities in Cu^{2+}-treated roots, documented in the literature [7,43,79], could be responsible for the precipitation of metals at the root surface. Indeed, peroxidases may polymerize phenolic compounds which can themselves chelate metal cations at the root surface [24,31,42].

3.2. Root system remodelling during Cu^{2+} excess

Experiments with the split-root system (Fig. 5) emphasized a local toxicity of Cu^{2+} ions on the root system and this is in agreement with the low Cu translocation into the shoot. The local signal induced by Cu^{2+} excess triggers an important reorganization of the RSA. In our vertical growth system, Cu^{2+} concentrations from 25 μM negatively affect the PR elongation (Fig. 2). Similarly to this result, a previous study also showed that Cu^{2+} concentrations higher than 20 μM were needed to cause visible phytotoxic symptoms in roots of Arabidopsis plants grown on agar medium [46]. It is worth noticing that the Cu^{2+}-induced phytotoxic effect in the hydroponic system is observed with approximately 10-fold lower Cu^{2+} concentrations than in the MS/2 agar medium. Several reasons could explain this observation. First, in vertical Petri plates, seedlings only contact the surface of the medium contrary to the hydroponic system where the roots are bathing in the nutrient solution. Secondly, the root structures may be different between growth systems. Furthermore, because of its cation exchange capacity, agar can interact with Cu^{2+} ions making them less available for the plant [68]. Lower transpiration rates contained in the environment of Petri dishes compared to the growth chamber can also partly explain the lower toxicity of Cu excess on agar. Finally, the compositions of the MS/2 medium and the hydroponic solution were different and it was already demonstrated that there is a clear influence of the growth medium composition on the metal-related phenotypes [68].

Cu^{2+} excess is also responsible for an increase of the density of short LRs (Fig. 2). At 10 μM Cu^{2+}, LRs increase in the density while PR growth is not affected, which clearly indicates an effect of copper on RSA. At toxic Cu^{2+} concentrations (50 μM), the increase in LRs can be explained by the shorter PR.

Although the root architecture reprogramming induced by a Cu^{2+} excess has been largely described in various plants [51,54,76], the molecular mechanisms underlying this response are still poorly understood.

3.3. Influence of Cu^{2+} excess on cell viability, hormonal status, cell divisions and lignin deposition within root tips

The effects of some physiological changes on the RSA remodelling after treatment with excess Cu^{2+} are summarized in Fig. 7. When Cu^{2+} is added at moderate concentrations (25 μM) to the medium, cell viability and mitotic activity are maintained in the root apex, allowing PR growth. However, when 50 μM Cu^{2+} are added, cells of the PR and LRs apex die (Fig. 4). Yeh et al. [77] also found that rice roots underwent a rapid cell death upon Cu^{2+} treatment. Exposure to Cu^{2+} has been widely reported to result in increasing ROS levels [18], inducing specific MAP kinase activities [23]. This high ROS production might explain the cell death observed in Cu^{2+}-treated root tips, as reported by Pan et al. [49] in barley roots exposed to an Al^{3+} excess. In agreement with this hypothesis, Pasternak et al. [51] reported that ROS play a key role in controlling the architectural changes in Cu^{2+}-stressed *Arabidopsis* seedlings. The cell death can explain the total growth inhibition of the primary and secondary roots exposed to Cu^{2+} concentrations above 50 μM.

At 25 μM Cu^{2+}, there was no significant change in *CYCB1;1* promoter driven GUS expression, suggesting that the observed reduction in root growth may be due to a lower rate of cellular elongation. At 50 μM Cu^{2+}, the decrease in *CYCB1::GUS* expression

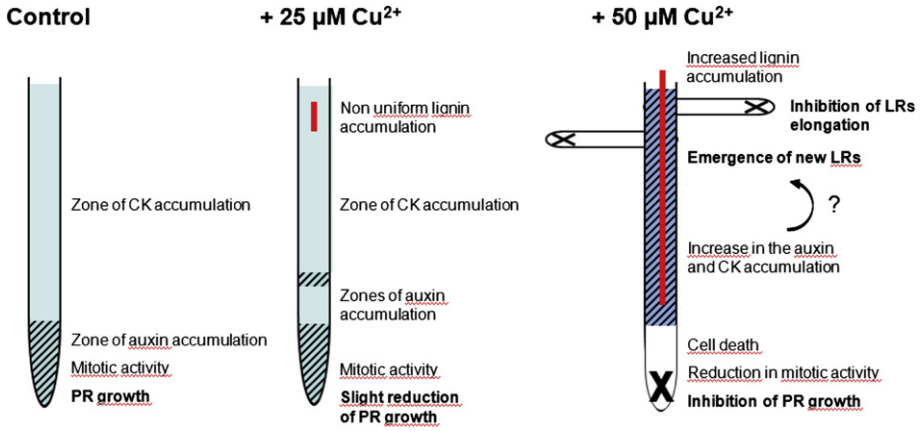

Fig. 7. Schematic diagram of the root tip showing the impact of Cu^{2+} excess on the root system architecture remodelling through changes in the hormonal status, mitotic activity, cell viability and lignin deposition within PR and LR tips.

was concomitant to a general decrease in gene expression as revealed by the 35S::GUS control and cell viability test. Therefore, at high Cu^{2+} concentrations, the arrest of cell divisions seems to be a consequence of the cell death. In agreement, Jiang et al. [22] showed that the mitotic index in root tips of *Zea mays* decreased progressively with increased Cu^{2+} concentrations. In the same way, previous studies reported that metal stresses such as Cd^{2+} or Al^{3+} excess led to a reduction in the cell division rates within the meristem of the PR [17,60].

Furthermore, Cu^{2+} exposure is responsible for changes in the hormonal status (Fig. 3A–D). Auxin, cytokinin and ethylene are key hormones involved in the root architecture [3,47,53]. Auxin is mainly produced in the young leaves and is transported via the phloem towards the root apical meristem. Auxin promotes the initiation, emergence and elongation of roots hairs and LRs [3,19,47]. The accumulation of auxin observed at 25 μM Cu^{2+} and more markedly at 50 μM Cu^{2+} in the root section just above the apex can account for the formation of LRs. It might also be involved in the formation of root hairs which has previously been shown to be induced in the area adjacent to the Cu^{2+}-treated root tips of *Arabidopsis thaliana* [51]. A role for auxin in the reorganization of the root architecture has often been reported for other metal stresses such as Al^{3+} and Cd^{2+} excess or phosphate and Fe^{2+} deficiency [28,35,53,54,65]. Cytokinin is mainly produced in the cap of the PR and has an inhibitory effect on PR growth and LR formation [30,47,61]. Růžička et al. [61] showed that exogenous application of cytokinin reduced the zone of the *CYCB1*::*GUS* expression, suggesting that the cytokinin treatment inhibits the root growth by interfering with mitotic activity. Therefore, the increase in cytokinin pool observed in 50 μM Cu^{2+}-treated roots might account for the root growth inhibition. However, cytokinin treatment inhibits LR initiation which is not the case upon Cu^{2+} treatment [30,47,61]. Ethylene is mainly associated with growth retardation in plants such as an inhibition of root growth [34,53]. Contrary to previous reports, a higher production of ethylene was not observed in plants growing in Cu^{2+} excess for 9 days, even at toxic concentrations (Table 2). Our results suggest that ethylene is not involved in the long-term root architecture reorganization. However, further investigations should study the kinetics of ethylene evolution by Cu^{2+}-treated Arabidopsis at short term. The work of Arteca and Arteca [4] showing an increase of ethylene production following a Cu^{2+} excess in Arabidopsis was carried out on plants grown hydroponically and treated with high Cu^{2+} concentrations (from 50 to 400 μM) for a short period (24 h).

Increasing Cu^{2+} concentrations in the medium also leads to a lignin deposition in roots (Fig. 6) An increase in lignin content has already been reported in Cu^{2+}-treated plants species, e.g. in *Capsicum annuum* [14] *Raphanus sativus* [8], *Glycine max* [32] and *Panax ginseng* [2]. The polymerization of lignin precursors is currently known to be catalysed by peroxidases and by laccases, which are Cu-containing glycoproteins [5,14,32,58]. The activity of both enzymes has been shown to increase in parallel with the lignification process in Cu^{2+}-treated plants [8,32]. Phloroglucinol staining of roots did not allow us to determine the precise localization of lignin deposition. However, the lignin staining seems to be localized in the middle part of the root (Fig. 6B), probably at the endodermis, as shown upon Cd^{2+} excess by van de Mortel et al. [70], or at the xylem cell walls. Consequently, the lignin deposition might limit the efflux of metals from the vascular cylinder into the shoot [71] and have an impact on the mineral profile, such as the reduction of Ca root to shoot translocation. In addition, as suggested by Sasaki et al. [62], the lignification could also limit the cell growth.

Taken together, these results show that many metabolic processes induced by Cu^{2+} excess, in particular changes in the mineral homeostasis, hormonal status, cell viability, mitotic activity and lignin deposition, can be involved in the RSA remodelling. A genetic approach is currently undertaken to tackle the components of this response.

4. Material and methods

4.1. Hydroponic culture

Seeds of *Arabidopis thaliana* (L.) Heynh Columbia (Col-0) were germinated and allowed to grow in a peat-based compost for 3 weeks in a short day regime (8 h light: 100 μmole photons m^{-2} s^{-1}/16 h darkness) prior to transferring to hydroponics. Upon transfer, the roots of the plantlets were rinsed in distilled water and immediately placed on the cover of plastic black-painted containers (capacity of 4.5 l) filled with a nutrient solution as described in Hermans and Verbruggen [21]. The pH of the solution was adjusted to 5.8 ± 0.1 with 1 M KOH. The treatment was applied after two weeks of hydroponic culture. The treatment consisted of either maintaining the initial copper concentration (= 0.1 μM) or adding $CuSO_4$ at concentrations of 1, 2.5 or 5 μM. Nutrient solutions were replaced every 4 days. The growth conditions in the culture room were: temperature of 22 ± 2 °C and relative humidity of 50 ± 5%.

4.2. In vitro culture

Seeds of *Arabidopis thaliana* (Col-0) were surface-sterilized for 5 min in ethanol 70% (v/v), 5 min in 5% (v/v) sodium hypochlorite and rinsed twice with sterile water prior to suspending them in 0.1% (v/v) agar. Surface-sterilized seeds were sown in square dishes on half the strength of Murashige and Skoog (MS/2) medium [44] with 1% (w/v) sucrose and 0.8% (w/v) plant agar. The dishes were stratified for 2 days at 4 °C and then placed vertically at a temperature of 20 ± 0.5 °C and under long day regime (16 h light: 50 μmole photons m^{-2} s^{-1}/8 h darkness). For transfer experiments, seedlings were transferred 5 days after germination to MS/2 medium supplemented with $CuSO_4$ concentrations from 0 to 75 μM.

In the split-root experiment, the primary root tip of a 15-day-old seedling was cut so that only the two first lateral roots were saved. Five days later, the plantlet was transferred on a MS/2 plate divided in two parts: one part containing no additional $CuSO_4$ and one part supplemented with 50 μM $CuSO_4$. Each saved lateral root was placed on one of the two parts and allowed to grow.

4.3. Mineral analysis

Leaves and roots of plants growing in hydroponic solution were harvested at day 1, 7 and 14 after the $CuSO_4$ addition in the nutrient solution. Roots were rinsed with deionized water for 1 min. Fresh material was then dried at 60 °C during 48 h before being crushed into a powder. Dried material was digested with 6 M nitric acid for 2 h at 60 °C and 6 h at 120 °C. Digested samples were assayed by ICP-MS (Purdue Ionomics Information Management Systems, IN, USA). Three protocols of root washing were tested to study their impact on the Cu content in plants treated with 2.5 μM Cu^{2+} for 7 days (Table IS). The Cu contents after root washing with $Pb(NO_3)_2$, which is a way to remove extra-cellular copper [12,63,69], were statistically similar to those washed with water.

4.4. Histochemical staining

β-glucuronidase activity in transgenic marker lines was visualized by incubating tissues for 1 h (*35S*::*GUS* lines), 2 h (*ARR5*::*GUS* lines) or 18 h (*DR5*::*GUS* and *CYCB1*::*GUS* lines) in darkness at 37 °C in a buffer containing 0.5 mg mL^{-1} X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) dissolved

in *N*-dimethyl-formamide, 0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.5 M EDTA (pH = 8) and 100% Triton X-100.

Cell viability assay was performed as described by Ma et al. [36]. Roots were placed in distilled water for 5 min and then stained using 3 mg l^{-1} propidium iodide solution for 15 min.

For lignin staining, samples were rinsed in distilled water for 5 min and then incubated in a 1% (w/v) phloroglucinol–HCl 6 N solution for 5 min [59].

In all cases, the histochemical staining was observed and captured with a microscope Eclipse E800M Nikon equipped with a camera DXM1200 Nikon. For the cell viability assay, samples were visualized under an epifluorescence illumination (excitation: 540–552 nm, broad band filter: 590 nm). All figures are representative of the staining detected in roots of at least four plants.

4.5. Ethylene measurements

Thirty seeds were sown and allowed to grow for 9 days in vertical plates containing MS/2 medium supplemented with 0, 25 or 50 μM $CuSO_4$. Before subjecting plantlets to ethylene measurements, the plates were kept in the growth chamber used for measurements for 1 day to acclimatize the plants (150 μmol m^{-2} s^{-1} continuous light and constant temperature of 21 °C). For ethylene measurements, the bottom part of the dish with the agar was covered with a glass plate with an inlet and outlet for gas flow. The system was tided together with two metal pieces and was connected to a sensitive laser-based ethylene detector in combination with a gas flow-through system developed at the Department of Molecular and Laser Physics, University of Nijmegen, the Netherlands. The cuvettes, fitted with inlet and outlet ports, were alternatively flushed with compressed air as carrier gas at a flow rate of 3 l h^{-1} for 12 h. The flow from each cuvette was directed into a photoacoustic cell where ethylene was quantified. A detailed description of the system has been given previously [10]. For each Cu^{2+} concentration tested (25 and 50 μM), measurements were done in parallel with control plates and were repeated at least 3 times. The ethylene levels from a cuvette containing an agar plate without seedlings were also measured and subtracted from the emission rates obtained.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plaphy.2010.05.005.

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