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Copper Regulates Primary Root Elongation Through PIN1-Mediated Auxin Redistribution

Hong-Mei Yuan, Heng-Hao Xu, Wen-Cheng Liu and Ying-Tang Lu*

College of Life Sciences, Wuhan University, Wuhan 430072, China
*Corresponding author: Email, yingtlu@whu.edu.cn; Fax, +86-27-68753551.
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The heavy metal copper (Cu) is an essential microelement required for normal plant growth and development, but it inhibits primary root growth when in excess. The mechanism underlying how excess Cu functions in this process remains to be further elucidated. Here, we report that a higher concentration of $CuSO_4$ inhibited primary root elongation of Arabidopsis seedlings by affecting both the elongation and meristem zones. In the meristem zone, meristematic cell division potential was reduced by excess Cu. Further experiments showed that Cu can modulate auxin distribution, resulting in higher auxin activities in both the elongation and meristem zones of Cu-treated roots based on *DR5::GUS* expression patterns. This Cu-mediated auxin redistribution was shown to be responsible for Cu-mediated inhibition of primary root elongation. Additional genetic and physiological data demonstrated that it was PINFORMED1 (PIN1), but not PIN2 or AUXIN1 (AUX1), that regulated this process. However, Cu-induced hydrogen peroxide accumulation did not contribute to Cu-induced auxin redistribution for inhibition of root elongation. When the possible role of ethylene in this process was analyzed, Cu had a similar impact on the root elongation of both the wild type and the *ein2-1* mutant, implying that Cu-mediated inhibition of primary root elongation was not due to the ethylene signaling pathway.

Keywords: *Arabidopsis thaliana* • Auxin • Cu toxicity • Ethylene • Root elongation • ROS.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AUXIN1 (AUX1); GFP, green fluorescent protein; GUS, β-glucuronidase; HPF, 3′-(*p*-hydroxyphenyl) fluorescein; KI, potassium iodide; MS, Murashige and Skoog; NO, nitric oxide; PAT, polar auxin transport; PIN, PINFORMED; QC, quiesescent center; ROS, reactive oxygen species; YFP, yellow fluorescent protein.

Introduction

The heavy metal copper (Cu) is an essential microelement required for normal plant growth and development (Maksymiec 1998). Because of its chemical redox potential, the element Cu has been selected as a cofactor in the active centers of numerous enzymes involved in electron transfer reactions (Clarkson and Hanson 1980, Da Silva and Williams 2001, Burkhead et al. 2009, Bernal et al. 2012). Furthermore, Cu plays roles in many essential physiological processes such as photosynthesis, respiration, oxygen superoxide scavenging, ethylene perception, cell wall remodeling and lignification (Rodrıguez et al. 1999, Himelblau and Amasino 2000, Burkhead et al. 2009). However, the presence of excess trace metals including Cu causes a serious environmental and financial problem (Rengel 2003, Kopittke et al. 2010). Cu as an essential microelement is highly toxic when in excess. Excess Cu pollution is of major concern because crops with Cu accumulation have potential hazards to human health, and thus their commercial value is decreased. It has been documented that excess Cu can hamper plant growth. This is because a higher Cu concentration easily interferes with numerous metabolic and physiological processes such as chloroplast integrity, plastid membrane composition and photosynthetic electron transport (Pätsikkä et al. 2002, Demirevska-Kepova et al. 2004, Wang et al. 2012), thus directly causing damage to lipids, proteins and DNA, and ultimately cell death (Drążkiewicz et al. 2004, Shao et al. 2010). Consequently, to avoid Cu-induced damage, plants have evolved different strategies and finely tuned mechanisms to decrease the accumulation of free Cu ions in cells, such as regulation of Cu uptake, chelation and efflux (Puig and Thiele 2002, Ducic and Polle 2005, Grotz and Guerinot 2006, Prohaska 2008, Burkhead et al. 2009, Palmer and Guerinot 2009, Puig and Peñarrubia 2009). Inhibition of primary root elongation is one of the earliest and most distinct symptoms exhibited by plants exposed to excess Cu (Alaoui-Sossé et al. 2004, Navari-Izzo et al. 2006, Pető et al. 2011). Recently, both auxin and nitric oxide (NO) have also been reported to regulate each other's level during organ development under Cu excess (Pető et al. 2011). Although current data have suggested that endogenous phytohormonal signal transduction is important for root architecture, the mechanisms underlying how excess Cu modulates the changes in phytohormonal concentration and distribution for root growth remain elusive.

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For root system architecture, the roles of both phytohormones and reactive oxygen species (ROS) have been documented (Torrey 1976, Le et al. 2001). Ethylene is often associated with stress, such as heat, drought, ozone, aluminum (Al) excess and phosphate starvation (Larkindale and Knight 2002, Massot et al. 2002, Rao et al. 2002, Sun et al. 2010). If plants are exogenously supplied with either ethylene or 1-aminocyclopropane-1-carboxylic acid (ACC), root elongation is reduced and root hair growth is promoted (Le et al. 2001, Ruzicka et al. 2007). Auxin, another key regulator for plant growth and development, has also been found to affect directly plant responses to environmental stresses, such as phosphate starvation, salt stress and excess metals [Al, cadmium (Cd) and Cu] (Kollmeier et al. 2000, Potters et al. 2007, Pérez-Torres et al. 2008, Wang et al. 2009, Li et al. 2011). Auxin homeostasis is involved in plant development and environmental responses and modulated by its synthesis, distribution and polar transport (Dharmasiri et al. 2005, Tanaka et al. 2006, Ruzicka et al. 2007, Petrášek and Friml 2009, Wang et al. 2009, Li et al. 2011). This polar auxin transport (PAT) is mediated by influx carriers of the AUXIN1/LIKE AUX1 (AUX/LAX) family and efflux carriers of the PINFORMED (PIN) family (Vieten et al. 2007, Křeček et al. 2009, Péret et al. 2012). While both AUX1 and PIN2 are required specifically for the basipetal transport of auxin through the outer root cell layers (Marchant et al. 1999, Rashotte et al. 2000), PIN1 localized at the basal end of the vascular cells is responsible for direct acropetal auxin flow in the root stele (Blilou et al. 2005, Kleine-Vehn and Friml 2008). Auxin transport has been proposed to play a role in stress-induced changes. For example, Al inhibits root length by affecting auxin redistribution via modulation of AUX1 and PIN2 (Kollmeier et al. 2001, Sun et al. 2010).

In addition to phytohormones, the increased ROS production is often associated with many types of stresses (Potters et al. 2007). ROS act as an important second messenger in the perception of stress and plant responses following exposure to stress. However, following exposure to unfavorable environmental conditions, ROS are formed in excess, leading to oxidative stress (Schützendübel and Polle 2002, Apel and Hirt 2004). It has been documented that oxidative stress in Arabidopsis exposed to Cd is due to hydrogen peroxide (H_2O_2) accumulation, resulting in the activation of MPK3 and MPK6 (Cho and Seo 2005, Liu et al. 2010). The increased ROS levels are also evidenced in Cu-exposed plants (Drążkiewicz et al. 2004). Furthermore, ROS accumulation can modulate root elongation in Arabidopsis (Tsukagoshi et al. 2010).

Here, we report that higher concentrations of $CuSO_4$ inhibited primary root elongation of Arabidopsis seedlings by affecting the meristem zone via reducing meristematic cell division potential. Further physiological and genetic data showed that Cu-mediated inhibition of primary root elongation was due to its ability to modulate auxin distribution via PIN1, but not PIN2 or AUX1. Moreover, Cu-induced H_2O_2 accumulation did not contribute to Cu-induced auxin redistribution for the inhibition of root elongation.

Results

Cu inhibited primary root growth by affecting the root meristem zone and elongation zone

It has been documented that application of excess Cu can inhibit primary root growth (Pasternak et al. 2005, Chen et al. 2011, Bernal et al. 2012). In our experiments, we would like to explore further the possible mechanisms for Cu-modulated inhibition of root elongation. Thus, different concentrations of Cu were tested to determine a suitable concentration for our further work. To do so, 5-day-old Arabidopsis seedlings were exposed to 0, 25, 40, 50 and 60 μM $CuSO_4$ for 9 h. Then the treated seedlings were transferred to half-strength Murashige and Skoog (1/2 MS) medium for continued growth for 2 d, and the newly grown root lengths in 1/2 MS medium were measured. Our results indicated that excess Cu rapidly inhibited primary root elongation, and the inhibition of root elongation was positively dependent on $CuSO_4$ concentrations (**Fig. 1**). Primary root elongation was inhibited by 10% when exposed to 25 μM $CuSO_4$ and by up to 68% in 60 μM $CuSO_4$-treated roots (**Fig. 1A**). The inhibition of root growth by higher Cu concentrations is also reported in a previous study in which primary root length was significantly decreased in 1-week-old seedlings treated with excess Cu for 3 d (Chen et al. 2011). Our results also indicated that the roots were still alive for continued growth in 1/2 MS medium after Cu treatment in our conditions because it was the newly grown root length that was measured in our experiments. To examine this inhibition in detail, the lengths of both the root meristem and elongation zones were assayed in the $CuSO_4$-treated roots. Our experiments showed that both the root meristem and elongation zones were reduced in response to higher $CuSO_4$ concentrations (from 40 to 60 μM) for 9 h compared with untreated roots (**Fig. 1B, C**). Thus, 40–60 μM $CuSO_4$ was used to treat the materials, with statistical analyses of the newly grown root lengths in our further experiments.

It is reported that reduced stem cell niche activity can lead to a decrease in meristem size (Sabatini et al. 2003). To test whether the reduction in meristem size was caused by reduced stem cell niche activity, *QC25::GUS* (β-glucuronidase), *QC46::GUS* and *SCR::GFP* (green fluorescent protein) lines were employed in our Cu-treated experiments. Both *QC25::GUS* and *QC46::GUS* are quiescent center (QC)-expressed promoter traps and *SCR::GFP* can be used to assay *SCR* (*SCARECROW*) promoter activity. Our GUS staining showed that all were expressed in the roots of these three lines treated with 5 μM $CuSO_4$ for 9 h (**Fig. 1D**), implying that Cu did not affect stem cell niche potential. An alternative factor for the decreased meristem size could be the loss of meristematic cell division potential. This can be analyzed with *CYCB1;1::GUS*, an excellent marker for cells undergoing mitosis to monitor cell cycle progression (Colón-Carmona et al. 1999). Our results with GUS staining of this report line treated with excess Cu revealed

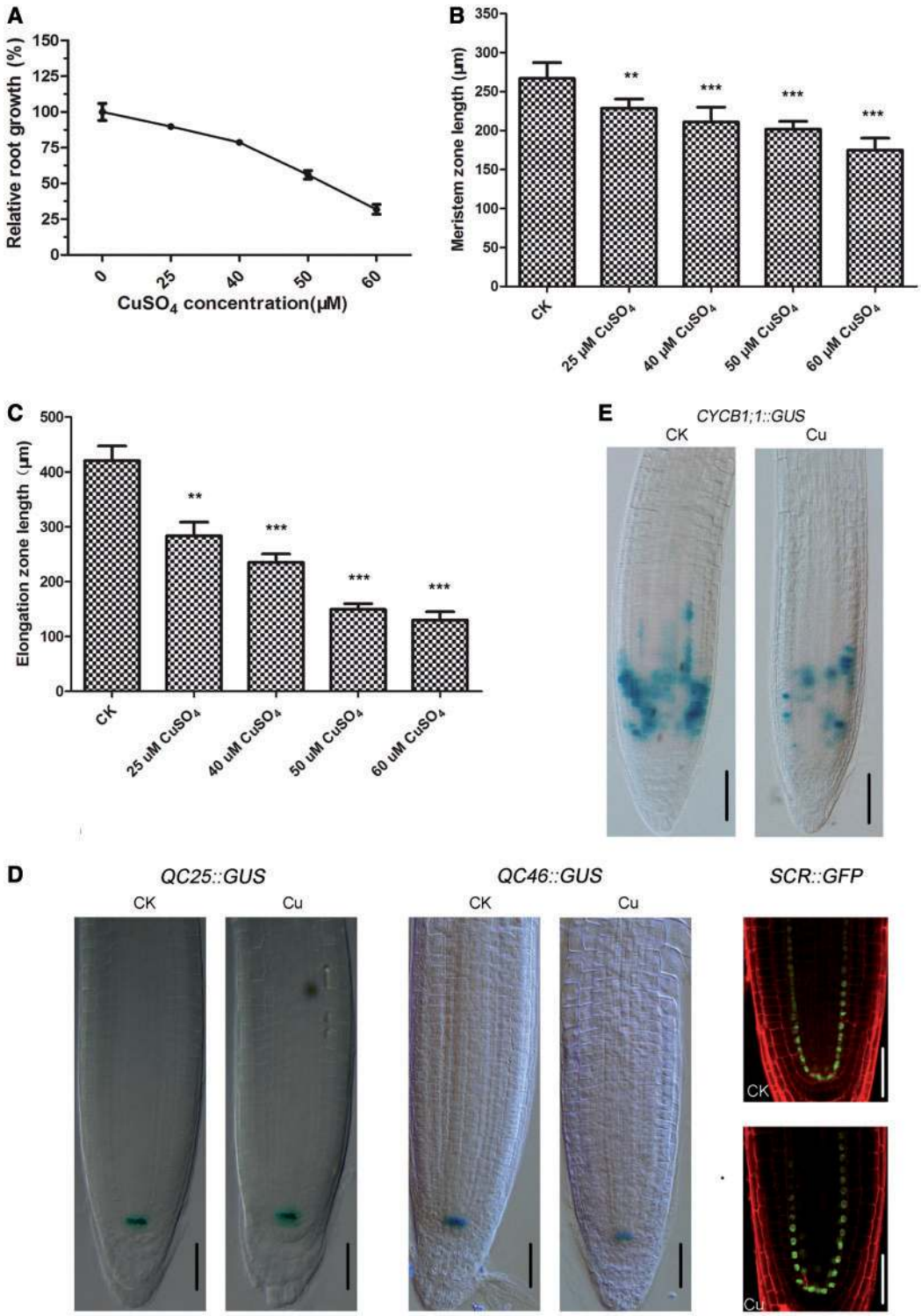

Fig. 1 $CuSO_4$ inhibits primary root growth through its regulation of the root meristem and elongation zones. (A) The effect of varying concentrations of $CuSO_4$ on primary root elongation in Arabidopsis wild-type seedlings. Five-day-old seedlings were treated with different concentrations of $CuSO_4$ for 9 h and the lengths of newly grown roots were measured after the Cu-treated plants were transferred to 1/2 MS medium for another 2 d. Data are presented as relative root growth compared with control values. (B) Root meristem zone size of 5-day-old Arabidopsis seedlings treated with different $CuSO_4$ concentrations for 9 h. (C) Root elongation zone size of 5-day-old Arabidopsis seedlings treated with different $CuSO_4$ concentrations for 9 h. (D) Excess Cu does not affect stem cell niche potential, as monitored by the *QC25::GUS*, *QC46::GUS* and *SCR::GFP* reporters. Bars = 50 μm. (F) Excess Cu affects cell cycle activity of the root meristem, as monitored by the *CYCB1;1::GUS* reporter. Bars = 50 μm. For B and C, error bars represent the SD, and asterisks indicate significant differences at $^{**}P < 0.01$ and $^{***}P < 0.005$ (Student's *t*-test).

that the percentage of GUS-stained cells in the root meristem was significantly reduced in Cu-treated roots compared with that in control roots (Fig. 1E), suggesting that excess Cu reduced the competence of meristematic cells to divide. Taken together, our data indicated that Cu inhibited primary root growth by affecting both the elongation and meristem zones. Excess Cu reduced meristem size by affecting meristematic cell division potential.

Ethylene-mediated signaling is not required for the inhibitory action of Cu on primary root elongation

Ethylene is often associated with stress (Potters et al. 2009). It also stimulates root hair formation (Pitts et al. 1998) and inhibits root growth primarily by affecting cell elongation (Swarup et al. 2007). Excess Cu also substantially inhibits cell elongation and stimulates root hair formation (Pasternak et al. 2005). Thus, whether ethylene is involved in Cu-inhibited primary root elongation or not was investigated. For this purose, we employed an ethylene-insensitive mutant *ein2-1* that affects a membrane-associated signal transduction component of the ethylene response (McGrath and Ecker 1998). The mutant seedlings were exposed to varying concentrations of $CuSO_4$ (40, 50 and 60 μM), and the lengths of newly grown roots were statistically analyzed after the treated seedlings were transferred to 1/2 MS medium for 2 d. Primary root elongation was inhibited by 23, 48 and 69% in *ein2-1* exposed to 40, 50 and 60 μM $CuSO_4$ respectively, similar to Cu inhibition in wild-type plants (21, 44 and 68% with 40, 50 and 60 μM 50 $CuSO_4$ respectively) (Fig. 2A). If both *ein2-1* and wild-type plants were grown on 1/2 MS medium containing varying concentrations of $CuSO_4$ (0, 25, 40, 50 and 60 μM) continuously for 5 d and primary root lengths were statistically analyzed, similar inhibitory effects of $CuSO_4$ on the root elongation in *ein2-1* and wild-type plants were observed (Fig. 2B). These data showing that $CuSO_4$ had a similar impact on inhibition of root elongation in both *ein2-1* and wild-type plants suggested that the inhibition of primary root elongation by Cu was not due to the ethylene signaling pathway, in contrast to the observation in Al-induced inhibition of root elongation (Sun et al. 2010).

Auxin is redistributed in roots exposed to excess copper

Auxin's role as another key regulator of root development is also reported in stress responses by changes in auxin homeostasis and distribution (Wang et al. 2009, Li et al. 2011). To analyze whether or not Cu-induced inhibition of primary root elongation is mediated by auxin redistribution, differential auxin responses were assayed with an auxin-responsive *DR5::GUS* marker line, whose pattern of expression provides reliable information on auxin distribution (Ulmasov et al. 1997a, Sabatini et al. 1999). If the seedlings were not exposed to excess Cu, *DR5* expression was mainly enriched in the QC, columella initial cells, and columella cells of the root cap, as

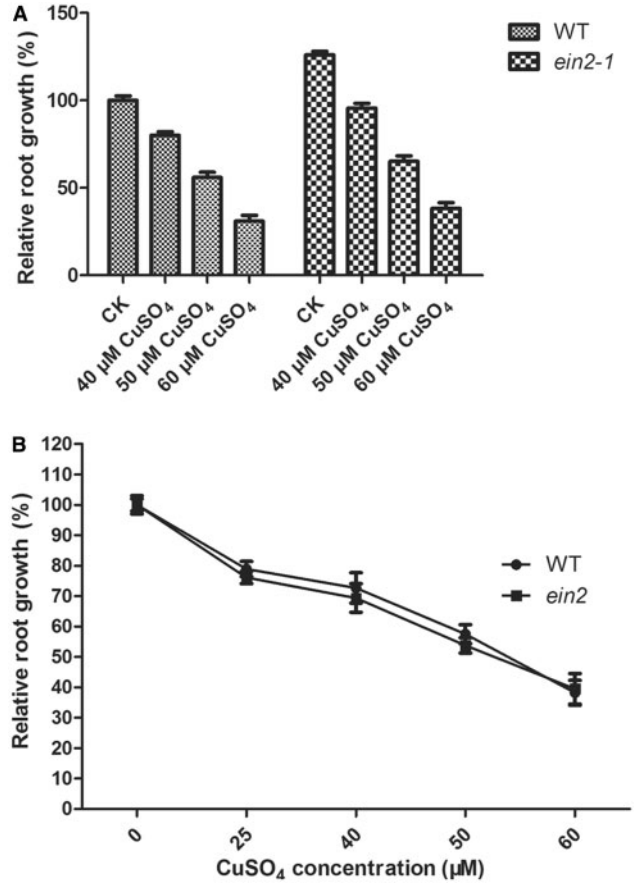

Fig. 2 Effect of $CuSO_4$ on primary root elongation of both wild-type and *ein2-1* plants. (A) Five-day-old seedlings of both the wild type and the *ein2-1* mutant were exposed to 40, 50 and 60 μM $CuSO_4$ for 9 h. Then the treated seedlings were transferred to 1/2 MS medium for continued growth for 2 d and the lengths of newly grown roots were measured. (B) Both wild-type and *ein2-1* plants were exposed to 40, 50 and 60 μM $CuSO_4$ for 5 d, and then root lengths were measured. Data are presented as relative root growth compared with control values.

reported previously (Sabatini et al. 1999). However, excess Cu (40, 50 and 60 μM) changed the *DR5::GUS* expression pattern by increasing its activity in both the root meristem and elongation zones and decreasing its expression in columella cells (Fig. 3A), indicating Cu regulation of auxin distribution in the roots.

Furthermore, a time course of the effect of Cu on auxin redistribution was carried out. These experiments indicated that Cu-induced auxin redistribution was positively dependent on the age of Cu-treated seedlings. If 5-day-old seedlings were treated with 50 μM $CuSO_4$ for 3 h, the initial changes in the *DR5::GUS* expression pattern can be observed in Cu-treated roots compared with control roots (Fig. 3B). The auxin activities were dramatically elevated in both root meristem and elongation zones, with a decrease in columella cells when the plants were exposed to 50 μM $CuSO_4$ for 6 or 9 h (Fig. 3B). Then, the seedlings treated with 50 μM $CuSO_4$ for 9 h were transferred to 1/2 MS medium for continued growth, and both the lengths of newly grown roots and the auxin activity

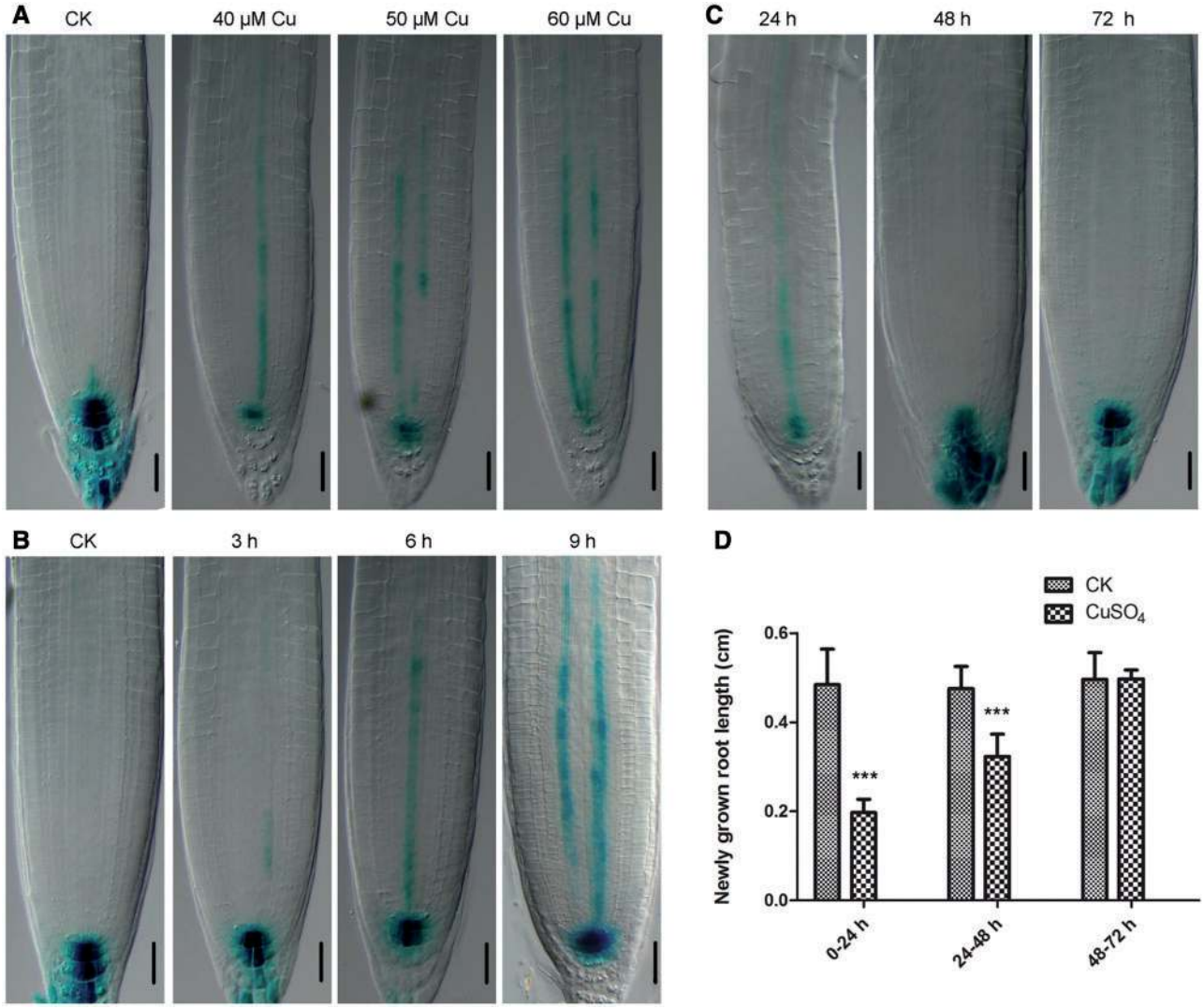

Fig. 3 Effect of $CuSO_4$ on auxin redistribution in the roots. (A) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of 5-day-old seedlings treated with 0, 40, 50 and 60 μM $CuSO_4$ for 9 h. Bars = 40 μm. (B) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of 5-day-old seedlings treated with 50 μM $CuSO_4$ for 3, 6 or 9 h. Bars = 40 μm. (C) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of 5-day-old seedlings treated with 50 μM $CuSO_4$ for 9 h and then transferred to 1/2 MS medium for 24, 48 or 72 h. Bars = 40 μm. (D) Five-day-old seedlings were treated with 50 μM $CuSO_4$ for 9 h and transferred to 1/2 MS medium for continued growth. Then, the lengths of newly grown roots during the periods of 0–24, 24–48 or 48–72 h on 1/2 MS medium were measured and statistically analyzed. Means and error bars were calculated from three repeats with >25 plants each. ***indicates significant differences at $P < 0.005$ (Student's *t*-test).

distribution were examined. Our assays showed that the changed pattern of auxin activity in both the root meristem and elongation zones was still maintained 24 h after the transfer. Accordingly, primary root elongation of the treated seedlings during 0–24 h after the transfer was also repressed (**Fig. 3C, D**). However, similar *DR5::GUS* expression patterns were observed in the roots of both untreated plants and Cu-treated seedlings 48 or 72 h after the transfer (**Fig. 3A, C**). In agreement with these changes in auxin redistribution, no inhibitory effect on primary root elongation was assayed in the Cu-treated seedlings compared with untreated control by measuring the lengths of the newly grown roots during 48–72 h after the transfer (**Fig. 3C, D**). These data suggested that Cu inhibition of primary root elongation was due to its ability to modulate auxin distribution.

PIN1 but not PIN2 or AUX1 is required for copper-induced auxin redistribution

It has been showed that auxin transport regulates root development by controlling local auxin distribution in roots, and this PAT is mediated by influx carriers of the AUX/LAX family and efflux carriers of the PIN family (Vieten et al. 2007, Křeček et al. 2009, Péret et al. 2012). Thus, the Cu-regulated auxin distribution for the inhibition of primary root elongation could be modulated by auxin carriers. To test this, the *aux1-7*

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DR5::GUS mutant was exposed to Cu, and auxin activities were assayed based on GUS staining. Our results indicated that the Cu-treated *aux1-7 DR5::GUS* mutant had increased auxin activities in both the root meristem and elongation zones, as did Cu-treated *DR5::GUS* plants (**Fig. 4A**). In addition, the *AUX1-YFP* (yellow fluorescent protein) expression pattern in the roots of *AUX1::AUX1-YFP* plants were not affected by Cu treatment compared with that in untreated *AUX1::AUX1-YFP* roots (**Fig. 4B, C**). These data indicated that *AUX1* was not involved in Cu-regulated auxin redistribution. They also implied that Cu inhibition of primary root elongation may not be affected by *AUX1*. Indeed, this was evidenced by the observations that a similar inhibition of primary root elongation was found in both Cu-treated *aux1-7* and wild-type plants (**Fig. 4F, G**).

A recent report indicates that both *AUX1* and *PIN2* function in Al-induced inhibition of root elongation (Sun et al. 2010). To test the possible involvement of *PIN2* in Cu-modulated auxin distribution, the *pin2 DR5::GUS* mutant was treated with 50 μM $CuSO_4$. Cu treatment resulted in higher auxin activities in both the root meristem and elongation zones of the *pin2 DR5::GUS* mutant, as observed in Cu-treated *DR5::GUS* plants (**Fig. 4A**). However, this Cu treatment did not change the *PIN2-GFP* expression profile in *PIN2::PIN2-GFP* plants (**Fig. 4D, E**). These results combined with genetic data showing that both Cu-treated *pin2* and wild-type plants exhibited similar inhibition of primary root elongation indicated that *PIN2* did not participate in Cu-mediated auxin redistribution for its inhibitory role in primary root elongation (**Fig. 4F, G**).

PIN1, another auxin efflux carrier, was then assayed for its possible involvement and it was shown to play a role in Cu-mediated auxin distribution and inhibition of primary root elongation in our further experiments. To perform these

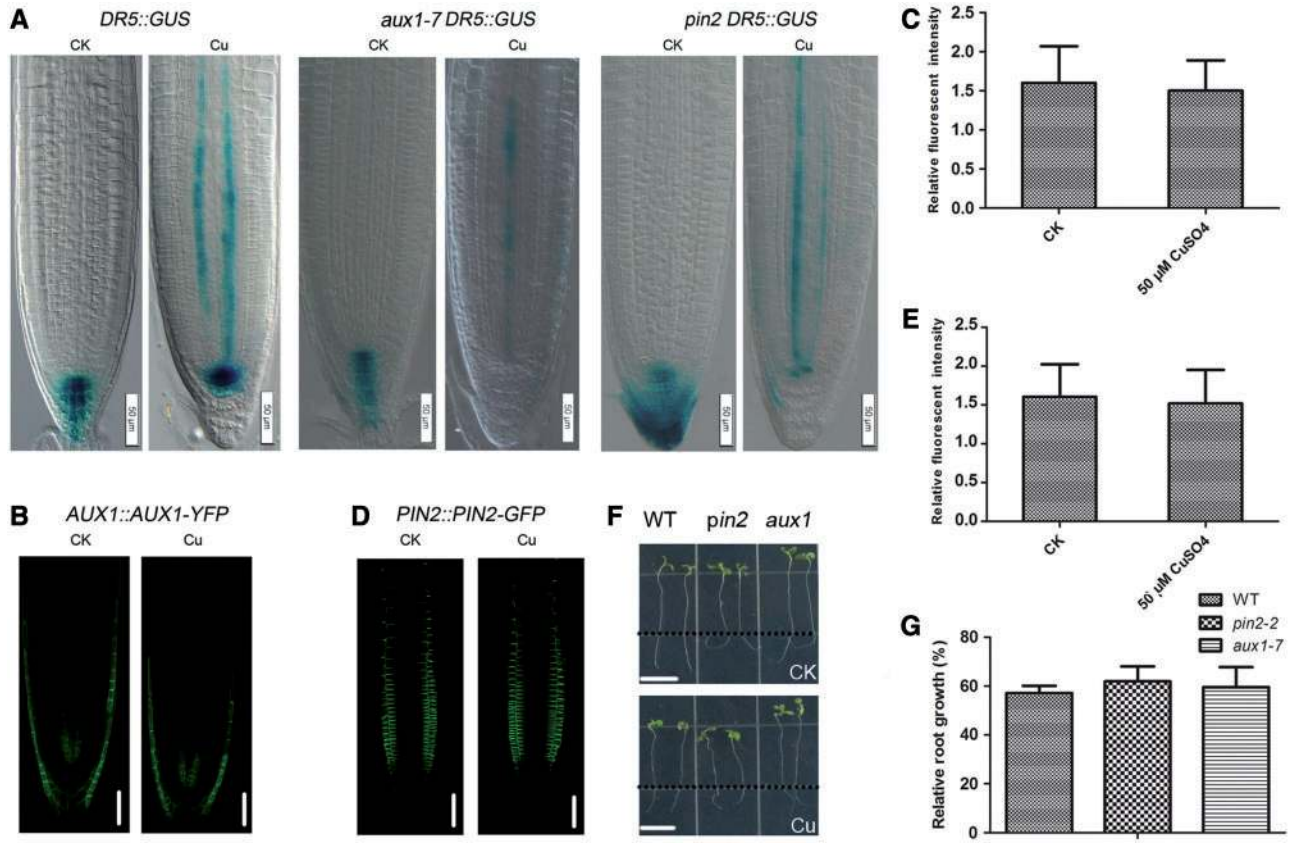

Fig. 4 Effect of $CuSO_4$ on auxin redistribution and primary root elongation of wild-type and auxin polar transport mutants, *aux1-7* and *pin2* plants. (A) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of wild-type and auxin polar transport mutants, *aux1-7* and *pin2* treated without (CK) or with 50 μM $CuSO_4$ (Cu). Bars = 50 μm. (B) Effect of $CuSO_4$ on *AUX-YFP* expression in *AUX1::AUX1-YFP* plants. Bars = 50 μm. (C) Quantification of fluorescence by image analysis of confocal sections. *AUX1-YFP* expression was not affected by Cu treatment. (D) Effect of $CuSO_4$ on *PIN2-GFP* expression of *PIN2::PIN2-GFP* plants. Bars = 50 μm. (E) Quantification of fluorescence by image analysis of confocal sections. *PIN2-GFP* expression was not affected by Cu treatment. (F and G) Five-day-old seedlings of the wild type, *pin2* and *aux1-7* were exposed to 50 μM $CuSO_4$ for 9 h and then were transferred (dotted line) to 1/2 MS medium for another 2 d (F). The lengths of newly grown roots were measured and the data are presented as relative root growth compared with control values, and are given as means ± SD of >20 roots (G). The lengths of roots newly grown for an additional 2 d of untreated wild-type, *pin2* and *aux1-7* were 0.95 ± 0.11, 0.87 ± 0.1 and 1.15 ± 0.19 cm, respectively. When the seedlings were subjected to 50 μM $CuSO_4$, the newly grown root lengths of treated wild-type, *pin2* and *aux1-7* were 0.55 ± 0.12, 0.53 ± 0.1 and 0.69 ± 0.17 cm, respectively. Bars = 1 cm.

experiments, the *pin1* mutant was crossed with the *DR5rev::GFP* marker line for monitoring auxin distribution in roots (Benková et al. 2003), and the resulting progeny were used. Our results showed that higher auxin activities assayed in both the meristem and elongation zones in Cu-treated *DR5rev::GFP* plants were not detected in *pin1 DR5rev::GFP* exposed to varying concentrations of $CuSO_4$ (40, 50 and 60 μM) (**Fig. 5A**), indicating *PIN1*'s involvement in Cu-regulated auxin distribution. This was probably due to Cu-modulated *PIN1* expression because PIN1–GFP accumulation was dramatically decreased in the roots exposed to excess copper (**Fig. 5B, C**). A time course of the effect of Cu on *PIN1* expression was carried out. These experiments indicated that Cu-repressed *PIN1* expression was positively dependent on the duration of Cu treatment (**Fig. 5B, D**). Furthermore, *pin1-1* seedlings were exposed to excess Cu and the primary root lengths were statistically analyzed as above. We found that when the seedlings were subjected to different concentrations of $CuSO_4$ (40, 50 and 60 μM), the root elongation in wild-type plants was reduced by 21, 44 and 68%, respectively, whereas the root elongation of *pin1-1* seedlings was reduced by only 14, 26 and 52%, indicating that root elongation in *pin1-1* was relatively insensitive to Cu compared with that of wild-type plants (**Fig. 5E, F**). Taken together, our data revealed that Cu inhibited primary root elongation at least partly by changes in auxin distribution via its modulation of the expression of *PIN1*, but not that of *AUX1* or *PIN2*.

Increased H_2O_2 in Cu-treated seedlings does not contribute to Cu-regulated auxin redistribution

H_2O_2, as an important second messenger, is often associated with many types of stress. It has been documented that Cu stress can result in higher H_2O_2 level in leaves (Drążkiewicz et al. 2004). Arabidopsis treated with either paraquat or a H_2O_2 derivative had short primary roots (Pasternak et al. 2005). In addition, the inhibition of root elongation is also evidenced in the transgenic line *35S::UPB1-3YFP* with a higher H_2O_2 level (Tsukagoshi et al. 2010). These data suggest that Cu may inhibit root elongation by changes in the H_2O_2 level. Thus, the possible role of H_2O_2 in Cu inhibition of primary root elongation was investigated in our study. First, H_2O_2 was assayed with 3′-(*p*-hydroxyphenyl) fluorescein (HPF) which is known to stain H_2O_2 (Dunand et al. 2007). HPF fluorescence indicating the H_2O_2 level was substantially increased up to 1.46-fold in the roots after Cu treatment compared with untreated control. Potassium iodide (KI) is also reported to scavenge H_2O_2, and its application to the transgenic line *35S::UPB1-3YFP* can partially rescue H_2O_2 inhibition of the root meristem zone (Tsukagoshi et al. 2010). In our experiments, the seedlings were treated in the presence of excess Cu with or without KI and the lengths of newly grown roots of treated seedlings were measured. Our results showed that exogenous application of both Cu and KI together scavenged the increased H_2O_2 in Cu-treated seedlings (**Fig. 6A, B**) and resulted in a less inhibitory effect on root elongation compared with Cu treatment alone (**Fig. 6C**). These results demonstrated that Cu-mediated inhibition of root elongation can be partially rescued by KI, suggesting the involvement of H_2O_2 in Cu-mediated inhibition of the root elongation. To investigate whether increased H_2O_2 could influence the Cu-induced auxin redistribution, auxin distribution was examined in the roots of *DR5::GUS* seedlings in the presence of excess Cu with or without KI. We found a similar auxin distribution in the roots either treated with Cu and KI together or exposed to Cu alone (**Fig. 6D**). To test further whether the increased H_2O_2 could influence the Cu-modulated expression of *PIN1*, *PIN1* expression was analyzed in the roots of *PIN1::PIN1-GFP* seedlings treated with Cu plus KI or Cu alone. A similar inhibition of *PIN1* expression was assayed in the roots of Cu-treated seedlings with or without KI (**Fig. 6E, F**). These data indicated that Cu could induce H_2O_2 accumulation to inhibit primary root elongation, but the increased H_2O_2 did not contribute to Cu-induced auxin redistribution. This conclusion was further evidenced by the finding that higher auxin activities assayed in both the meristem and elongation zones in Cu-treated *DR5::GUS* plants were not detected in H_2O_2-treated *DR5::GUS* plants (**Fig. 6G**).

Discussion

Exposure of plants to mild chronic stress can induce stress-induced morphogenic responses, such as a blockage of cell division, an inhibition of root elongation and a stimulation of lateral organs. The combination of ROS, auxin and ethylene is integrated at the cellular level, leading to the stress-induced morphogenic response phenotype (Potters et al. 2007, Potters et al. 2009). Cu as an essential microelement is highly toxic when in excess. Exposure of plants to excess Cu led to morphological alterations including reduced number and size of leaves, decreased cotyledon area, short primary root and higher root hair density (Pasternak et al. 2005, Pető et al. 2011). It is also known that plants exposed to excess Cu can decrease the accumulation of free Cu ions in cells by regulating Cu uptake, chelation and efflux (Puig and Thiele 2002, Prohaska 2008, Burkhead et al. 2009, Puig and Peñarrubia 2009) and modifying the hormone and NO level (Pető et al. 2011). However, the mechanism of Cu modulation in these processes remains to be further elucidated. In our study, excess Cu was shown to inhibit primary root elongation, and the inhibition of root elongation was positively dependent on $CuSO_4$ concentrations, similar to the finding of previous reports (Chen et al. 2011). Further experiments showed that higher $CuSO_4$ treatments reduced not only the elongation zone, but also the root meristem zone. The detailed examinations indicated that excess Cu reduced meristem size by affecting meristematic cell division potential.

These observed phenotypic changes in Cu-treated roots are probably related to auxin because maintenance of auxin homeostasis and the particular pattern of auxin distribution in roots are necessary for normal root development (Blilou

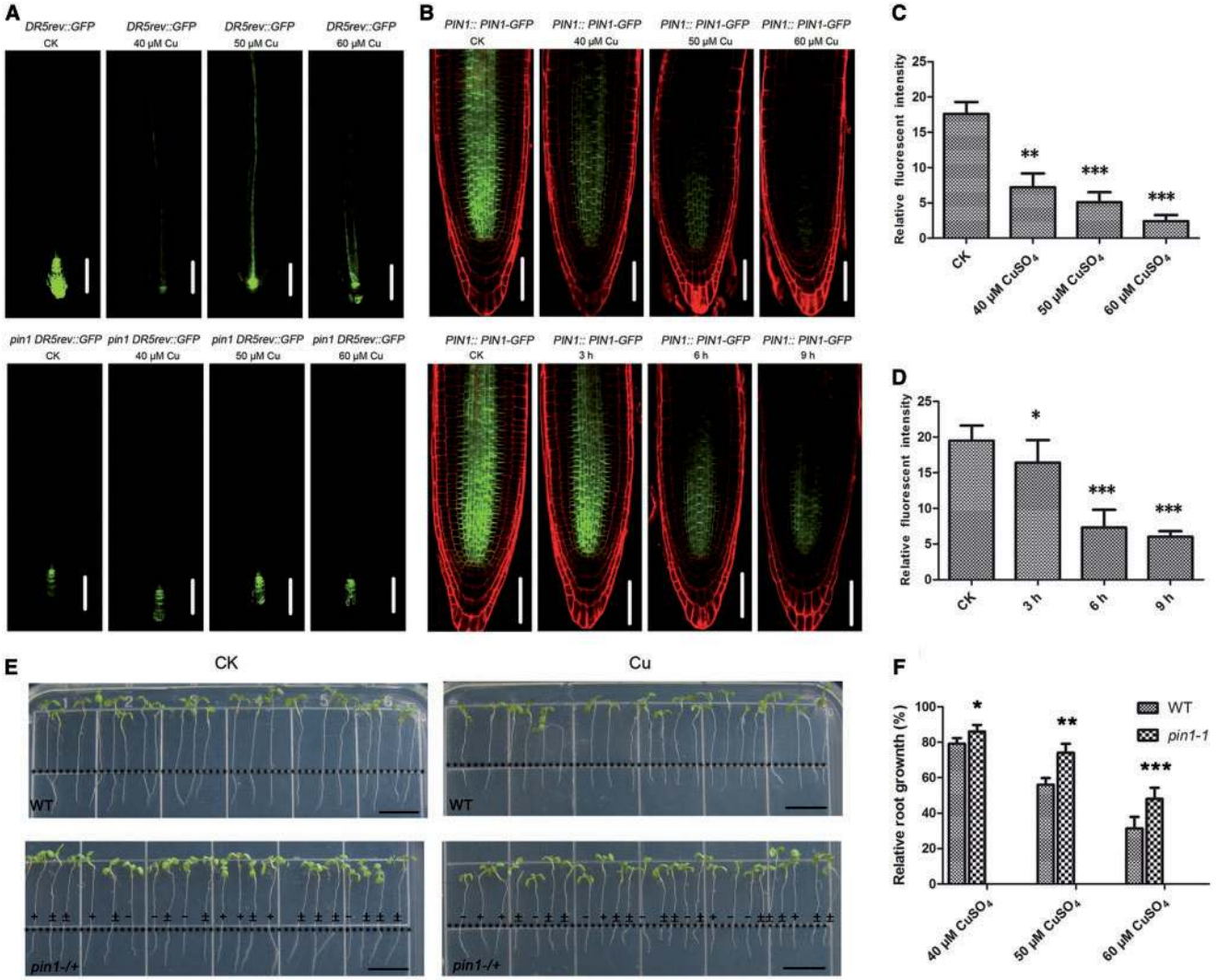

Fig. 5 PIN1 is required for Cu-induced auxin redistribution. (A) *DR5* activities for auxin distribution were assayed with *DR5-GFP* signals in the roots of $DR5_{rev}$::*GFP* and *pin1* $DR5_{rev}$::*GFP* plants. Bars = 50 μm. (B) Effect of $CuSO_4$ on *PIN1-GFP* expression in *PIN1*::*PIN1-GFP* plants. Bars = 50 μm. (C and D) Quantification of fluorescence by image analysis of confocal sections. *PIN1-GFP* expression was dramatically repressed in the roots of *PIN1*::*PIN1-GFP* plants exposed to excess Cu compared with untreated control (C) and the inhibition of *PIN1-GFP* expression was positively dependent on the duration of Cu treatment (D). (E and F) Five-day-old seedlings of wild-type and *pin1-1* were exposed to different concentrations of $CuSO_4$ (40, 50 and 60 μM) for 9 h, and then were transferred (dotted line) to 1/2 MS medium for another 2 d. The pictures of the roots for the wild type and *pin1-1* mutant treated with (Cu) or without (CK) 50 μM $CuSO_4$ are shown as a representative (E). The lengths of newly grown roots were measured and statistically analyzed. Data are presented as relative root growth compared with control values, and are given as the means ± SD of >20 roots (F). The lengths of roots newly grown for an additional 2 d for untreated wild-type and *pin1-1* were 0.943 ± 0.09 and 0.8 ± 0.08 cm, respectively. The lengths of newly grown roots of wild-type and *pin1-1* plants were reduced to 0.745 ± 0.11 and 0.69 ± 0.1 cm by 40 μM $CuSO_4$, 0.53 ± 0.07 and 0.592 ± 0.06 cm by 50 μM $CuSO_4$, and 0.29 ± 0.07 and 0.38 ± 0.11 cm by 60 μM $CuSO_4$, respectively. For the experiments with the *pin1-1* mutant, the seeds from *pin1-1* heterozygote plants were used because the *pin1-1* homozygote is infertile. After primary root lengths were recorded, the seedlings were identified to be wild-type (+), *pin1-1* homozygote (−) or heterozygote (±) by PCR. Only data for primary root lengths from the seedlings of the *pin1-1* homozygote were used for statistical analysis. For C, D and F, error bars represent the SD, and asterisks indicate significant differences at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ (Student's *t*-test).

et al. 2005, Overvoorde et al. 2010). Our results further showed higher auxin activities in both the meristem and elongation zones in Cu-treated roots, revealing that this overaccumulation of auxin in both the meristem and elongation zone may result in reduced cell division and cell elongation for the growth arrest of the primary roots.

It has been documented that auxin homeostasis and distribution patterns are markedly disturbed in primary roots under Cd, Al and salt stresses (Potters et al. 2009, Wang et al. 2009, Sun et al. 2010), and a good correlation between lateral root formation and Cd-induced changes in auxin distribution is reported (Potters et al. 2009). PAT plays an important role in root growth

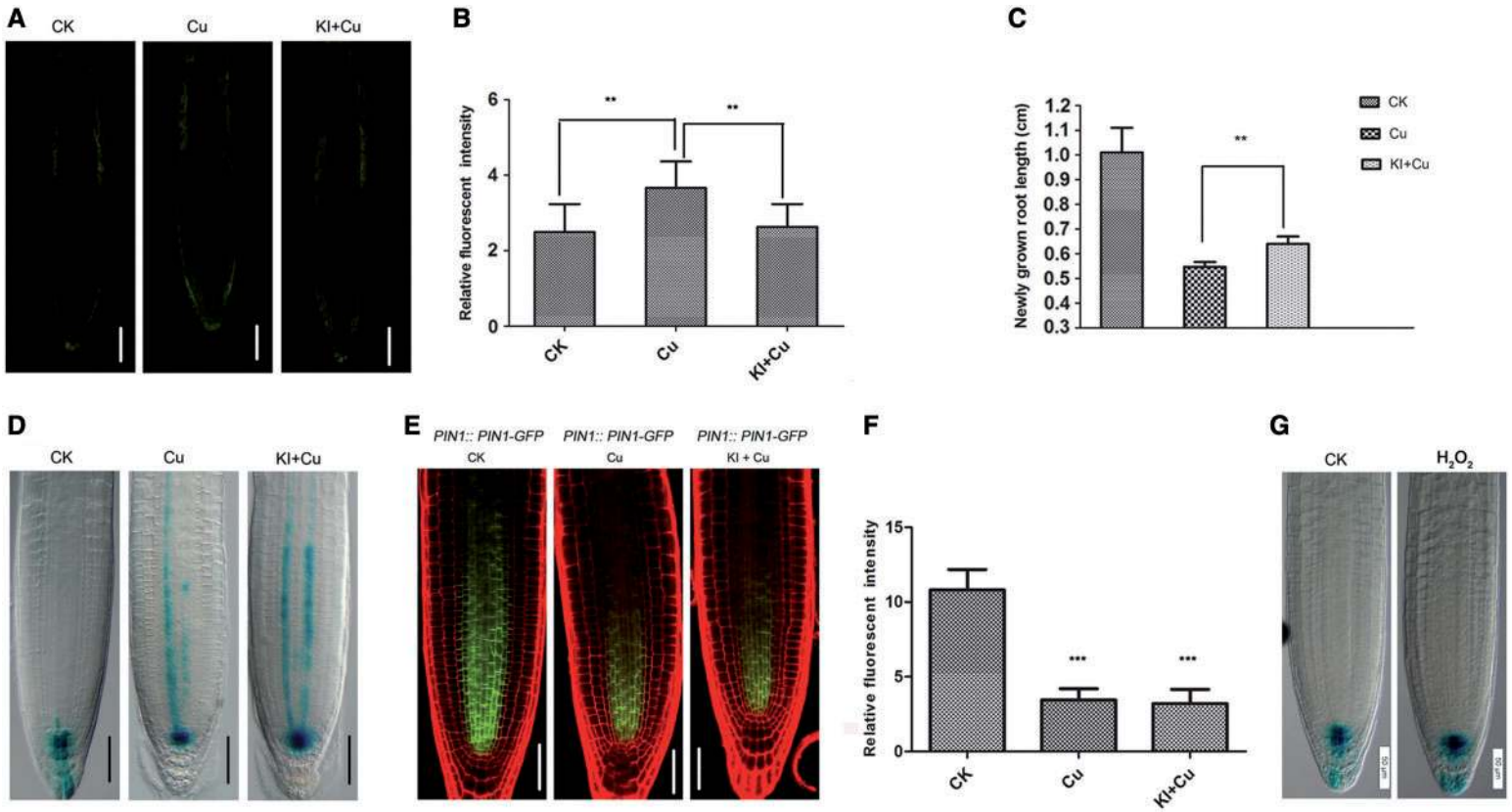

Fig. 6 Effect of KI on Cu-induced changes of auxin distribution and the inhibition of primary root elongation. (A) Effect of $CuSO_4$ on the H_2O_2 content in the roots. The roots of 5-day-old seedlings were stained with HPF for H_2O_2 levels. Bars = 50 μm. (B) The quantification of HPF fluorescence signals in the roots. The H_2O_2 level was significantly increased in Cu-treated roots compared with that in the untreated control, and Cu-induced H_2O_2 can be scavenged by KI treatment. (C) Effect of KI on Cu-mediated inhibition of primary root elongation. Five-day-old seedlings were exposed to either 50 μM $CuSO_4$ and 1 mM KI together or 50 μM $CuSO_4$ alone for 9 h, and the lengths of newly grown roots were measured after the treated plants were transferred to 1/2 MS medium for another 2 d. (D) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of *DR5::GUS* plants either treated with both 50 μM $CuSO_4$ and 1 mM KI together or exposed to 50 μM $CuSO_4$ alone. Bars = 50 μm. (E) Effect of KI on copper-mediated *PIN1-GFP* expression in *PIN1::PIN1-GFP* plants. Bars = 50 μm. (F) Quantification of fluorescence by image analysis of confocal sections. KI treatment did not affect Cu-repressed accumulation of PIN1–GFP protein. (G) Histochemical staining with X-Gluc of *DR5::GUS* activity in the roots of *DR5::GUS* plants treated with or without 100 μM H_2O_2 for 9 h. Bars = 50 μm. For B, C and F, error bars represent the SD, and asterisks indicate significant differences at $^{**}P < 0.01$ and $^{***}P < 0.005$ (Student's *t*-test).

by mediating auxin homeostasis and distribution (Friml et al. 2002, Blilou et al. 2005). When our experiments were carried out to explore the possible role of auxin carriers of PAT in Cu-inhibited root elongation, the data revealed that it was *PIN1* rather than *AUX1* or *PIN2* that acted as a key element in Cu-regulated auxin distribution. However, in a previous study (Sun et al. 2010), Al was also reported to alter the patterns of auxin accumulation and distribution in root via *AUX1*- and *PIN2*-mediated PAT when root elongation is inhibited in Al-treated seedlings. The differences in the modulation of auxin distribution between Cu- and Al-mediated inhibition of root elongation suggest that the changes in auxin distribution could be a common mechanism underlying metal-mediated inhibition of root elongation, but different auxin carriers are employed in auxin redistribution induced by distinct metal stresses. However, why different carriers are needed and how these carriers are modulated in different metal-mediated processes remains unknown.

Ethylene, another important phytohormone, is often associated with stress. It has been shown that exposure of Arabidopsis seedlings to $AlCl_3$ leads to a rapid ethylene evolution and marked inhibition of root elongation (Sun et al. 2010). Application of the ethylene precursor ACC could mimic the effect of phosphorus deficiency in inducing root hairs (Zhang et al. 2003). However, ethylene did not promote lateral root formation under phosphorus deprivation (López-Bucio et al. 2002). Therefore, ethylene is unlikely to be associated with all aspects of the stress-induced morphogenic response phenotype. In our study, both wild-type plants and *ein2-1*, an ethylene-insensitive mutant, showed essentially the same inhibition of primary root elongation when exposed to higher concentrations of $CuSO_4$, implying that ethylene is unlikely to be associated with the primary root inhibition caused by excess Cu.

ROS act as an important second messenger in regulating root growth, stomatal movement and seed germination (Kwak et al. 2003). Many data have revealed that environmental stresses could affect ROS levels, the concentration of antioxidants and their oxidation states (Apel and Hirt 2004). In our study, a substantially increased H_2O_2 level was shown in roots exposed to toxic Cu concentrations. It was also reported that auxin and ROS are two important molecular signals in abiotic stresses, and these two signaling pathways impact extensively on each other (Kovtun et al. 2000, Joo et al. 2001, Kwak et al. 2006). In our experiments to explore the possible factors involved in Cu inhibition of primary root elongation, both ROS and auxin were found to be modulated in the roots exposed to excess Cu. However, the increased H_2O_2 did not contribute to Cu-induced auxin redistribution for the Cu-mediated inhibitory role in primary root elongation. These findings indicate that ROS and auxin are two independent pathways in the process of Cu-induced root inhibition. In agreement, with this, Tsukagoshi et al. (2010) showed that ROS control the transition from cell proliferation to differentiation in roots via a separate pathway different from auxin signaling.

In summary, Cu as an essential microelement can inhibit primary root elongation by reducing both the elongation zone and meristem zone when in excess. This inhibitory role was mediated partly by modulating auxin redistribution via *PIN1*, but not *PIN2* or *AUX1*.

Materials and Methods

Plant materials and growth conditions

The published transgenic and mutant lines are *PIN1::PIN1-GFP* (Benková et al. 2003); *DR5rev::GFP* (Blilou et al. 2005); *PIN2::PIN2-GFP* (Blilou et al. 2005); *AUX1::AUX1-YFP* (Swarup et al. 2005); *cycB1;1::GUS* (Colon-Carmona et al. 1999); DR5::*GUS* (Ulmasov et al. 1997b); and *SCR::GFP* (Wysocka-Diller et al. 2000). The lines *QC25::GUS*, *QC46::GUS*, *pin1* (SALK_047613), *pin2* (CS859601), *aux1-7* (CS3047) and *ein2-1* (CS3071) were obtained from the Arabidopsis Biological Resource Centre. The transgenic lines were obtained by crossing the respective lines above, and confirmed by PCR.

Arabidopsis thaliana seeds were surface sterilized with 5% bleach for 5 min, washed three times with sterile water, placed at 4°C for 3 d and then planted on agar medium containing 1/2 MS medium (Murashige and Skoog 1962) (supplemented with 1% agar and 1% sucrose, pH 5.8) at 23°C and 100 μmol m^{-2} s^{-1} illumination under 16 h light/8 h dark conditions.

Root elongation assays

To study the inhibitory effect of $CuSO_4$ on root elongation, 5-day-old Arabidopsis seedlings were exposed to 1/2 MS medium supplemented with $CuSO_4$ at concentrations of 0, 40, 50 and 60 μM for 9 h, and then transferred to 1/2 MS medium for another 2 d. The lengths of newly grown roots were measured and statistically analyzed. Results are expressed as the mean ± SD ($n > 20$ roots). Each measurement was performed in at least three biological replications. When the experiments were carried out with the *pin1-1* mutant, the seeds from *pin1-1* heterozygote plants were used because the *pin1-1* homozygote is infertile. After primary root lengths were recorded, the seedlings were identified to be wild type, *pin1-1* homozygote or heterozygote by PCR. Only data for primary root lengths from the seedlings of the *pin1* homozygote were used for statistical analysis.

GUS staining

GUS staining was carried out according to the methods described in the literature (Hu et al. 2010). Briefly, seedlings were incubated at 37°C in staining solution {100 mM sodium phosphate buffer, pH 7.5, containing 10.0 mM EDTA, pH 8.0, 0.5 mM $K_3[Fe(CN)_6]$ and 0.5 mM $K_4[Fe(CN)_6]$, 0.1% Triton X-100 and 1.0 mM 5-bromo-chloro-3-indolyl-β-D- glucuronide}. The GUS staining time was dependent on the transgenic marker lines: 2 h for *DR5::GUS*, 6 h for *cycB1;1::GUS* and 12 h for both *QC46::GUS* and *QC25::GUS*.

Microscopic analyses

Confocal microscopy was performed using an Olympus FluoView 1000-Confocal laser scanning microscope according to the manufacturer's instructions. GFP lines were mounted with 20 μg ml^{-1} propidium iodide (PI) while YFP lines were mounted with ddH_2O.

For the observation of the root meristem zone, elongation zone and GUS staining, the seedlings were mounted with clearing solution (8 g of chloral hydrate, 2 ml of water and 1 ml of glycerol) on glass slides, examined under an Olympus BX60 differential interference contrast (DIC) microscope and photographed by a charge-coupled device (CCD) Olympus dp72.

ROS assays

HPF (Alexis Biochemical) staining procedures were performed as described in the literature (Dunand et al. 2007). Briefly, 5-day-old Arabidopsis seedlings were exposed to 1/2 MS medium supplemented with 50 μM $CuSO_4$ for 9 h. The seedlings were pre-incubated in 0.1 M phosphate buffer (pH 6.1), and incubated for 2 min in the same buffer containing 5 μM HPF. The reaction was stopped by transferring the seedlings into 0.2 M phosphate buffer (pH 6.1).

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