

# RELATIONSHIP BETWEEN CADMIUM-INDUCED ROOT SUBAPICAL HAIR DEVELOPMENT AND ETHYLENE BIOSYNTHESIS IN OILSEED RAPE SEEDLINGS

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It has long been observed that toxic heavy metals at different concentrations can induce root hair development in plants. In oilseed rape we studied ethylene levels and root hair initiation under Cd<sup>2+</sup> stress. Growth of the primary root was inhibited but close to root tips the development of subapical root hairs was significantly stimulated by Cd<sup>2+</sup> at 30 μM. Versus the control, the distance between the root tip and the root hair zone and the length of the epidermal cell in the elongation zone were significantly reduced by Cd<sup>2+</sup> at the same concentration. Exogenous application of Cd<sup>2+</sup> and 1-aminocyclopropane-1-carboxylate (ACC) to roots had similar effects on subapical root hair development. Hair density increase and hair elongation in the presence of Cd<sup>2+</sup> were reduced by the ethylene inhibitors CoCl<sub>2</sub> at 15 μM and aminooxyacetic acid (AOA) at 10 μM. Exposing roots to Cd<sup>2+</sup> caused a rapid increase in superoxide radical (O<sub>2</sub><sup>•-</sup>) production in the root hair differentiation zone, and at the tips of emerging and newly formed root hairs. Cd<sup>2+</sup>-induced O<sub>2</sub><sup>•-</sup> production at the growing hair tips was blocked in the presence of AOA. Our findings suggest that Cd<sup>2+</sup>-induced ethylene signaling may act upstream of O<sub>2</sub><sup>•-</sup>. Cd<sup>2+</sup> promotion of O<sub>2</sub><sup>•-</sup> production may operate through an ethylene signaling pathway, and O<sub>2</sub><sup>•-</sup> itself may stimulate root hair elongation.

**Key words:** Cadmium, root hairs, ethylene, superoxide radical, *Brassica napus*.

## INTRODUCTION

Seed germination is a critical phase in the life cycle of plants which sometimes occurs under unfavorable conditions such as cadmium contamination of soil. Cd<sup>2+</sup> is one of the most important heavy metal pollutants, highly damaging to plants (Sanita di Toppi and Gabbrielli, 1999). Most research has focused on the phytotoxic effects of Cd<sup>2+</sup> on seed germination and subsequent growth (Zhang et al., 2010; Smiri, 2011). There appears to be little information on the effect of Cd<sup>2+</sup> on root hairs. Reduced root growth is a general symptom indicating toxic effects on plants, but increased root hair elongation and root hair density has been observed under treatment with cadmium (Kuriakose and Prasad, 2008; Kopittke et al., 2010). The mechanisms of changes in the initiation and growth of root hairs under Cd<sup>2+</sup> stress are not well understood.

In most vascular plants, root hairs are subcellular extensions that form on root epidermal cells. The root epidermis is organized into discrete files consisting of a linear array of hair-bearing cells (trichoblasts) and hairless cells (atrachoblasts). Trichoblast length has been shown to be closely related to root hair density and distribution (Dolan, 1996). Genetic (Schiefelbein and Somenrille, 1990) and environmental factors (López-Bucio et al., 2003) contribute to the pattern of root hair development. Ethylene production in a wide variety of plant species is low under normal physiological conditions but is stimulated under stress. Ethylene signaling plays an important role in low phosphorus (Zhang et al., 2003; Niu et al., 2012), low iron (Müller and Schmidt, 2004) and low potassium (Jung et al., 2009) induced plant responses by increasing the density of subapical root hairs or mediating cluster root formation (Zaid et al., 2003). Similarly to nutrient deficiency, several metals have

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been shown to increase ethylene production by induction of changes in ethylene biosynthesis, including  $\text{Cd}^{2+}$  (Chen and Kao, 1995; Fuhrer, 1982),  $\text{Cu}^{2+}$  (Sandmann and Böger, 1980) and  $\text{Al}^{3+}$  (Sun et al., 2007). Thus it is reasonable to suggest that ethylene might mediate root hair growth in response to  $\text{Cd}^{2+}$  stress.

One of the major consequences of heavy metals stress is increased ROS production, which usually causes oxidative damage. However, there is growing evidence for a positive role for ROS in the growing zone since they are required for elongation growth (Schützendübel et al., 2001; Rodríguez et al., 2002). In maize roots  $\text{O}_2^{\cdot-}$  (superoxide production) can be converted to  $\text{H}_2\text{O}_2$  and  $\cdot\text{OH}$ , which control root elongation and root hair cell polar growth (Foreman et al., 2003; Liskay et al., 2004).

Both reactive oxygen species and ethylene may be involved in the control of root hair formation and growth, and there may be a relationship between ethylene synthesis and  $\text{O}_2^{\cdot-}$  production under  $\text{Cd}^{2+}$  stress. In this study we examined root and root hair development under  $\text{Cd}^{2+}$  stress, using chemical approaches. We discuss the role of ethylene synthesis and  $\text{O}_2^{\cdot-}$  production in  $\text{Cd}^{2+}$ -induced subapical root hair development.

## MATERIAL AND METHODS

### PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of oilseed rape (*Brassica napus*) cv. Shanghaiqing were sterilized with 10% NaOCl solution (v/v) for 5 min, followed by four rinses in sterile water. The disinfected seeds were placed on filter paper saturated with sterile water in Petri dishes for 48 h in the dark at 25°C. Fifteen seedlings were transferred to new Petri dishes (12 cm) filled with treatment solutions. All treatment solutions contained 500  $\mu\text{M}$   $\text{CaCl}_2$ .

### TREATMENT OF ROOTS

To study the short-term effects of  $\text{Cd}^{2+}$  and ACC (1-aminocyclopropane-1-carboxylate) on root hair growth, 15 2-day-old seedlings of uniform root length (~15 mm) were transferred to dishes with solutions containing either 30  $\mu\text{M}$   $\text{Cd}^{2+}$  or 10  $\mu\text{M}$  ACC for 12 h at 25°C, with 500  $\mu\text{M}$   $\text{CaCl}_2$  solution as control, 3 dishes per condition. Solution pH was adjusted to 6.0. Primary root length, root hair density, hair length and the length of trichoblast cells in the root elongation zone were measured after 12 h treatment.

To examine the possible requirement of ethylene in Cd-induced changes in root morphology, we

tested the effect of Cd on root elongation and subapical root hair development in the presence of ethylene biosynthesis inhibitors. Seedlings of uniform root length were treated with 500  $\mu\text{M}$   $\text{CaCl}_2$  (control), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  (Cd), 10  $\mu\text{M}$  ACC (ACC), 15  $\mu\text{M}$   $\text{CoCl}_2$  (Co), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  plus 15  $\mu\text{M}$   $\text{CoCl}_2$  (Cd+Co), 10  $\mu\text{M}$  ACC plus 15  $\mu\text{M}$   $\text{CoCl}_2$  (ACC+Co), 10  $\mu\text{M}$  AOA (aminooxyacetic acid) (AOA), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  plus 10  $\mu\text{M}$  AOA (Cd+AOA) or 10  $\mu\text{M}$  ACC plus 10  $\mu\text{M}$  AOA (ACC+AOA) for 12 h.

### MICROSCOPY

Roots hairs were stained with 1% (w/v) methylene blue solution for 1 min at room temperature and placed on glass microscope slides, then photographed with a digital camera attached to the microscope. Root hair density was estimated by counting root hairs and bulges visible within a 1.5 mm segment in the root hair zone behind root tips (Lombardo et al., 2006). Root hairs were counted three-dimensionally by adjusting the microscope's plane of focus (Ma et al., 2001). In all experiments, 12 plants randomly sampled from 45 plants were measured for each treatment.

For each root we measured the length of root hairs in a 1 mm region starting 0.5 mm above the root hair differentiation zone (n=5 root hairs) of 12 plants per treatment. Root hairs were photographed with a Canon 550D digital camera and their length was then measured from the images with Photoshop 6.0 (Ma et al., 2001).

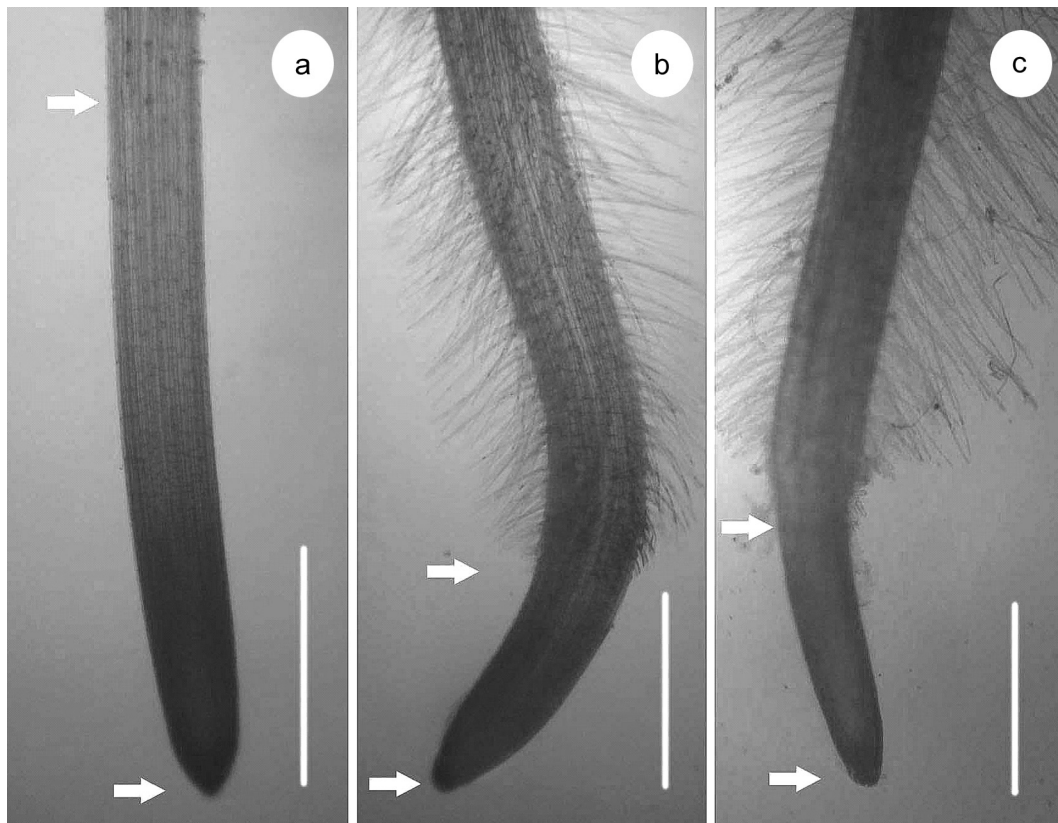
For each root the length of trichoblast cells in elongation zone was measured (5 trichoblast cells per root, 12 plants per treatment) from digital images with Photoshop 6.0.

The distance from the root tip to the first visible initiating root hair (bulge) of oilseed rape seedlings was measured under a microscope (n=12).

### HISTOCHEMICAL STAINING OF ROOTS FOR $\text{O}_2^{\cdot-}$

Histochemical staining of roots for  $\text{O}_2^{\cdot-}$  was performed according to Liskay et al. (2004) with some modifications. The sites of  $\text{O}_2^{\cdot-}$  production in roots were localized by incubating intact roots in 0.5 mM nitroblue tetrazolium chloride (NBT) dissolved in 100 mM phosphate buffer, pH 7.5, for 5 min at 25°C. After that time, incubation was interrupted by replacing the NBT solution with phosphate buffer. NBT forms an insoluble formazan product upon reduction by  $\text{O}_2^{\cdot-}$ .

To assess the effect of  $\text{Cd}^{2+}$ , ACC or ethylene inhibitor on  $\text{O}_2^{\cdot-}$  accumulation in the root differentiation zone, two-day-old seedlings were treated with 500  $\mu\text{M}$   $\text{CaCl}_2$  (control), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  (Cd), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  plus 10  $\mu\text{M}$  AOA (Cd+AOA), 10  $\mu\text{M}$  ACC (ACC)



**Fig. 1.** Effect of  $\text{Cd}^{2+}$  and ACC on root hair morphology in oilseed rape seedlings. (a) Control, (b)  $\text{Cd}^{2+}$ , (c) ACC. Bar = 1.0 mm. Photographs show plants representative of the 12 plants analyzed in each experiment.

or 10  $\mu\text{M}$  ACC plus 10  $\mu\text{M}$  AOA (ACC+AOA) for 12 h, after which the roots were stained with NBT.

To determine whether  $\text{Cd}^{2+}$  or ACC-induced  $\text{O}_2^-$  production in root hair tips can be further reduced by blocking ethylene signaling in roots, seedlings were subjected to 30  $\mu\text{M}$   $\text{Cd}^{2+}$  or 10  $\mu\text{M}$  ACC for 12 h, followed by another 3 h incubation with 10  $\mu\text{M}$  AOA. The roots were stained with NBT.

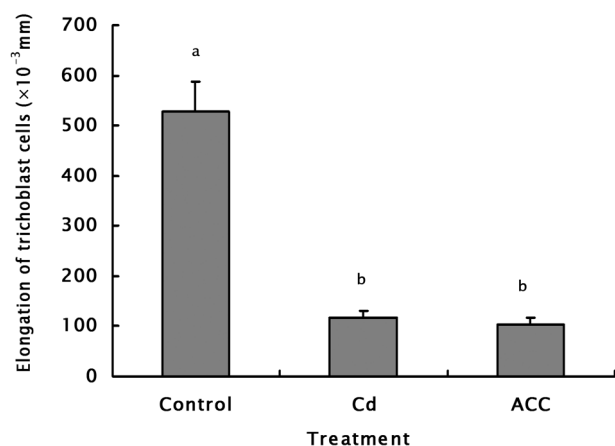
#### STATISTICAL ANALYSIS

The significance of differences in mean values between treatments was tested by one-way ANOVA followed by Duncan's multiple range tests at  $P < 0.05$  using SPSS11.5.

## RESULTS

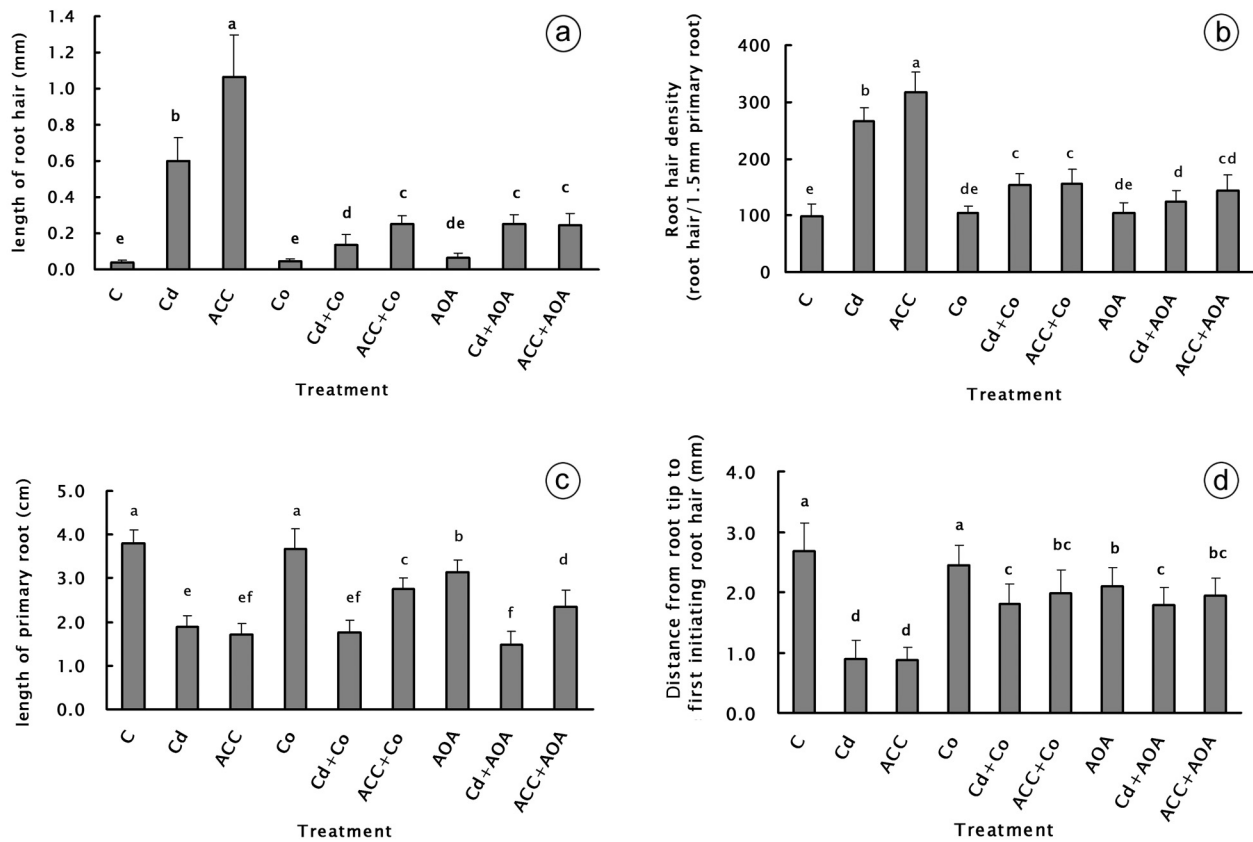
### ROOT MORPHOLOGY IN RESPONSE TO $\text{Cd}$ STRESS AND ETHYLENE

Plants grown in control conditions showed almost no root hairs in the zone close to the primary root tip (Fig. 1a). The pattern of root hair development was greatly affected by treatment with  $\text{Cd}^{2+}$ . Though



**Fig. 2.** Effect of  $\text{Cd}^{2+}$  and ACC on elongation of trichoblast cells in the root elongation zone. Two-day-old seedlings were treated with 500  $\mu\text{M}$   $\text{CaCl}_2$  (control), 30  $\mu\text{M}$   $\text{Cd}^{2+}$  (Cd) or 10  $\mu\text{M}$  ACC (ACC) for 12 h. Data are means  $\pm$  SE. Values bearing different letters differ significantly at  $P < 0.05$ .

the primary root was significantly shorter than in the control after 12 h exposure to 30  $\mu\text{M}$   $\text{Cd}^{2+}$  (Fig. 3c), root hair growth did not appear to be inhibited by  $\text{Cd}^{2+}$  at the same concentration.



**Fig. 3.** Effect of  $\text{Co}^{2+}$  and AOA on root hair growth (a), root hair density (b), primary root length (c), and distance from root tip to first initiating root hair (d) of oilseed rape seedlings in the presence of  $\text{Cd}^{2+}$  or ACC. Data are means  $\pm$  SE. Values bearing different letters differ significantly at  $P < 0.05$ .

Cadmium treatment significantly increased root hair density and root hair length near the root tips (Figs. 1b, 3a,b). The distance between the root tip and the root hair zone decreased sharply (Fig. 3d). These observations indicate that  $\text{Cd}^{2+}$  at  $30 \mu\text{M}$  is a positive regulator of root hair development.

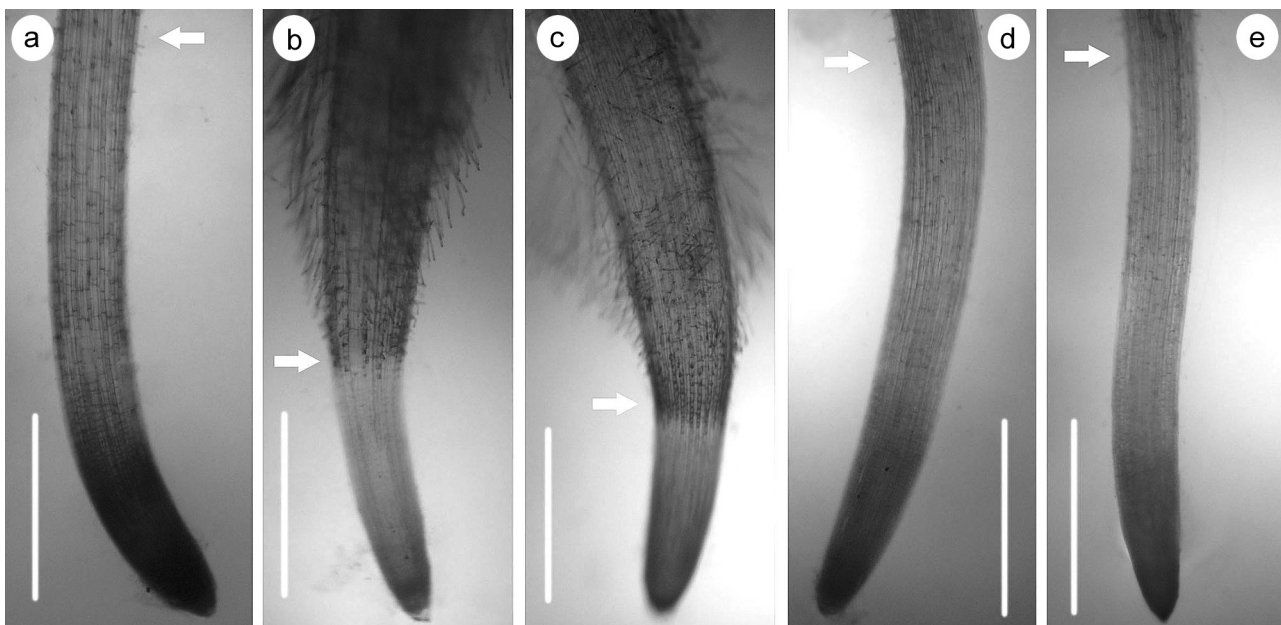
Previous studies showed that  $\text{Cd}^{2+}$  can stimulate ethylene biosynthesis, suggesting to us that  $\text{Cd}^{2+}$ -induced elevated ethylene production probably is involved in  $\text{Cd}^{2+}$ -induced root hair formation and elongation. To test this hypothesis we grew seedlings in the presence of the ethylene precursor ACC. After 12 h of culture on plates we assessed the effects of ACC on root morphology. Root growth and root hair growth were strongly affected by ethylene. As seen in Figure 1c, primary root elongation was markedly inhibited by treating roots with  $10 \mu\text{M}$  ACC for 12 h and the root hairs were denser, longer, and closer to the root tip.

Trichoblast elongation at the primary root surface is closely related to root hair density and distribution, so we compared epidermal cell length in the elongation zone. Elongation of these cells was

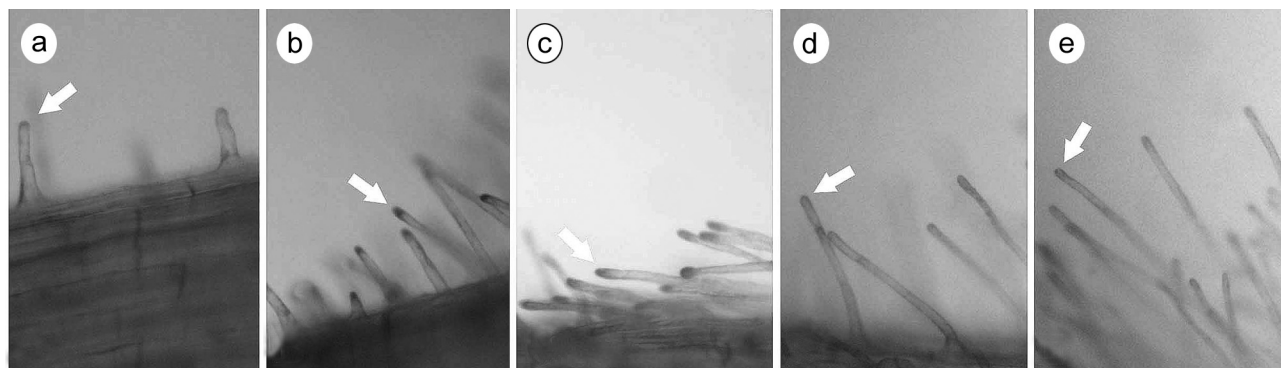
strongly inhibited by exposure to  $\text{Cd}^{2+}$  or ACC, as shown by their short length (Fig. 2). Taken together, these data suggest that  $\text{Cd}^{2+}$  and ethylene have similar effects on root development in oilseed rape.

#### EFFECT OF ETHYLENE INHIBITORS ON ROOT DEVELOPMENT UNDER TREATMENT WITH Cd AND ACC

The similarities in root hair response to  $\text{Cd}^{2+}$  and ACC suggest a role for ethylene in mediating this stress response. To see whether there is a relationship between ethylene and  $\text{Cd}^{2+}$ -induced root hair development near the root tips, we made an experiment under  $\text{Cd}^{2+}$  treatment to test the effect of ethylene biosynthesis inhibitors AOA and  $\text{Co}^{2+}$  on root hairs growth. Root hair length and root hair density were measured after 12 h treatments in 2-day-old treated seedlings as shown in Figure 3a,b. Though  $10 \mu\text{M}$   $\text{Co}^{2+}$  had no effect on root hair length and root hair density in the absence of  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$  significantly reduced  $\text{Cd}^{2+}$ -promoted root hair length and root hair density at the same site.



**Fig. 4.** Histochemical localization of  $O_2^-$  accumulation in roots with emerging root hairs. (a) Control, (b)  $Cd^{2+}$ , (c) ACC, (d)  $Cd^{2+}$  plus AOA, (e) ACC plus AOA. Bars = 1.0 mm. Photographs show representative plants chosen from 12 plants analyzed in each experiment. White arrows indicate hair differentiation zone with forming root hairs.



**Fig. 5.** Histochemical localization of  $O_2^-$  accumulation in root hair tips. White arrows indicate NBT precipitates in root hair tips. (a) Control, (b)  $Cd^{2+}$ , (c) ACC, (d)  $Cd^{2+}$  plus AOA, (e) ACC plus AOA. Photographs show representative plants chosen from 12 plants analyzed in each experiment.

Another inhibitor, AOA, had a similar effect. These results indicate that ethylene production is involved in  $Cd^{2+}$ -induced subapical root hair growth.

Root elongation was inhibited by ACC, but that effect was partially ameliorated when ACC treatment was combined with AOA or  $Co^{2+}$  (Fig. 3c). To see whether  $Cd^{2+}$  inhibits primary root growth via ethylene production, we tested the effect of  $Cd^{2+}$  on root elongation in the presence of the ethylene biosynthesis antagonists AOA or  $Co^{2+}$ . Primary root length did not change significantly under  $Cd^{2+}$  treatment when AOA or  $Co^{2+}$  were added, but in those conditions the distance between the root tip and the root hair zone was much longer (Fig. 3d).

#### ETHYLENE AND $Cd$ ENHANCE $O_2^-$ GENERATION IN THE HAIR DIFFERENTIATION ZONE AND IN ROOT HAIR TIPS

Previous studies have shown that ROS production by plasma membrane-bound NADPH oxidase is a basic requirement for the tip growth of root hairs (Jones et al., 2007). We used NBT to localize  $O_2^-$  production in roots. Under control conditions only small amounts of  $O_2^-$  were observed, spread diffusely over the surface of the hair differentiation zone (Fig. 4a). In contrast, under  $Cd^{2+}$  and ACC exposure the same site showed intense blue formazan staining, clearly indicating accumulation of  $O_2^-$  where root hair growth was initiating (Fig. 4b,c). When roots were exposed to  $Cd^{2+}$  and AOA together,

the Cd<sup>2+</sup>-induced formazan staining was less intense (Fig. 4d). The effect was the same for roots exposed to ACC and AOA together (Fig. 4e).

There was very little staining at the tip of root hairs in the absence of Cd<sup>2+</sup> (Fig. 5a), but root hairs were strongly stained at the growing tip in the presence of Cd<sup>2+</sup> (Fig. 5b) and ACC (Fig. 5c). Accumulation of insoluble blue formazan precipitates in hair tips was reduced by subsequent addition of AOA (Fig. 5d,e), indicating that Cd<sup>2+</sup>-induced and ACC-induced O<sub>2</sub><sup>•-</sup> production in root hair tips was blocked by an ethylene inhibitor.

## DISCUSSION

The most common effect of Cd toxicity on plants is stunted growth. This study demonstrated that root hair density and length were significantly stimulated and root hairs developed closer to the root tips when the plants were treated with Cd<sup>2+</sup>. Similar effects have been observed in different plants treated with various cadmium concentrations (Kuriakose and Prasad, 2008; Kopittke et al., 2010). More trichoblasts per unit of root length resulted in increased numbers of root hairs per unit of root length (Tanimoto, 1995). Our measurements of the lengths of trichoblasts from which root hairs arise indicate that trichoblast length decreased under Cd<sup>2+</sup> treatment (Fig. 2), so the Cd<sup>2+</sup>-induced shortening of trichoblast cells can be considered a cause of the increased root hair density.

In *Arabidopsis* the ethylene burst induced by Al<sup>3+</sup> treatment is closely associated with rapid inhibition of primary root elongation (Sun et al., 2010). If ethylene biosynthesis is also involved in Cd<sup>2+</sup>-induced inhibition of primary root elongation, then applying the ethylene inhibitors Co<sup>2+</sup> or AOA should have ameliorated the inhibiting effect of Cd<sup>2+</sup> on root elongation. However, we found that primary root length was not affected by Co<sup>2+</sup> or AOA under Cd<sup>2+</sup> stress. Unlike under Cd<sup>2+</sup> stress, ACC-induced inhibition of primary root elongation was indeed ameliorated by treatment with Co<sup>2+</sup> or AOA. These results suggest that ethylene is not the sole or the important regulator of primary root development under Cd<sup>2+</sup> stress. In fact, Cd<sup>2+</sup> can exert a direct impact on the plant cell. The inhibition of root elongation may be the result of binding of Cd<sup>2+</sup> to sulphhydryl groups in enzymes (Das et al., 1997), or excessive production of reactive oxygen species, which may damage membranes, nucleic acids and proteins (Berlett and Stadtman, 1997; Maksymiec, 2007) and thereby inhibit root elongation.

At their growing tips roots have apical meristems which are responsible for elongating the root tip. Cd<sup>2+</sup> may directly affect apical meristems via the

mechanisms mentioned above, inhibiting apex cell division (Fusconi et al., 2007; Zou et al., 2012). It seems reasonable to speculate that the Cd<sup>2+</sup>-induced decrease in primary root length does not act via ethylene; it may act through direct inhibition of epidermal cell elongation and apical meristem division.

The root hairs and primary root responded differently to the same cadmium concentration. Co<sup>2+</sup> at 15 µM or AOA at 10 µM significantly reduced Cd<sup>2+</sup>-promoted root hair length and root hair density. In the absence of Cd<sup>2+</sup>, however, no obvious inhibition of root hair growth by Co<sup>2+</sup> or AOA was observed. These results indicate that ethylene production is more important for root hair development in Cd<sup>2+</sup>-stressed plants than under normal conditions. These results are consistent with previous findings that exogenous ethylene-induced root hair formation required the ethylene signaling pathway, but that the normal developmental pathway for root hair formation is independent of the ethylene pathway (Pitts et al., 1998; Cho and Cosgrove, 2002)

Under normal growth conditions, reactive oxygen is necessary for cell elongation (Schützendübel et al., 2001; Causin et al., 2012), but excessive production of ROS during environmental stresses could cause oxidative damage and then inhibit growth (Schützendübel and Polle, 2002). Whether ROS becomes a damaging molecule depends on the balance between ROS production and scavenging (Gill and Tuteja, 2010). In this experiment, O<sub>2</sub><sup>•-</sup> was found to accumulate in growing root hair tips in the presence of Cd<sup>2+</sup> and ACC, suggesting that O<sub>2</sub><sup>•-</sup> was under the control of enzymatic and non-enzymatic antioxidant defense systems, rather than causing oxidative damage to root hair cells.

Previous results showed that root hair growth is coupled to a highly localized increase of NBT formazan precipitates in hair tips (Dolan et al., 1994). By the same token, treatments that decreased the O<sub>2</sub><sup>•-</sup> concentration in root hairs reduced root hair elongation (Foreman et al., 2003). This shows that O<sub>2</sub><sup>•-</sup> is a basic requirement for the tip growth of root hairs. Since subapical root hair elongation is promoted by Cd<sup>2+</sup> or ACC and reduced by the ethylene synthesis inhibitors Co<sup>2+</sup> or AOA, as we have shown, it strongly suggests a relationship between ethylene synthesis and O<sub>2</sub><sup>•-</sup> production under Cd<sup>2+</sup> stress. Unlike the controls, roots grown in the presence of Cd<sup>2+</sup> or ACC accumulated large amounts of O<sub>2</sub><sup>•-</sup> in the cell walls of their differentiation zone and in the tips of subapical root hairs. This localization is consistent with the role assigned to ROS in the formation of root hairs. Ethylene inhibitors greatly reduced Cd<sup>2+</sup>-induced ROS production and decreased subapical root hair elongation and density, which indicates that ethylene signaling acts

upstream of the processes that produce  $O_2^{\cdot-}$ . Taken together, these results suggest that  $Cd^{2+}$ -induced subapical root hair development is mediated by ethylene signaling, which might play a role in mediating ROS accumulation in the differentiation zone which in turn results in subapical root hair initiation and elongation.

Most uptake of macro- and micronutrients and water occurs through the root hairs (Gilroy and Jones, 2000).  $Cd^{2+}$  toxicity is based in part on decreased uptake of mineral nutrients and water (Barcelo et al., 1986; Gussarson et al., 1996; Sandalio et al., 2001) resulting from cadmium-induced inhibition of root growth. Our results suggest that subapical root hair growth is less sensitive than root growth to the action of  $Cd^{2+}$ . Oilseed rape seems able to promote root hair growth to prevent loss of nutrients and water.

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