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(Citation)
Plant and Cell Physiology,42(3):301-307

(Issue Date)
2001

(Resource Type)
journal article

(Version)
Accepted Manuscript

Auxin is a positive regulator for ethylene-mediated response in the growth of *Arabidopsis* roots[a]

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The requirement of auxin for the ethylene-mediated growth response in the root of *Arabidopsis thaliana* seedlings was investigated using two ethylene-resistant mutants, *aux1-7* and *eir1-1*, whose roots have been shown to have a defect in the auxin influx and efflux carriers, respectively. A 50% inhibition of growth (I_{50}) was achieved with 0.84 μl liter^{-1} ethylene in wild-type roots, but 71.3 μl liter^{-1} ethylene was required to induce I_{50} in *eir1-1* roots. In *aux1-7* roots, I_{50} was not obtained even at 1000 μl liter^{-1} ethylene. By contrast, in the presence of 10 nM 1-naphthaleneacetic acid (NAA), the concentrations of ethylene required to induce I_{50} in *eir1-1* and *aux1-7* roots were greatly reduced nearly to the level required in wild-type roots. Since the action of NAA to restore the ethylene response in *aux1-7* roots was not replaced by IAA, an increase in the intracellular level of auxin is likely to be the cause for the restoration of ethylene response. NAA at 10 nM did not inhibit root growth when

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applied solely, but it was the optimum concentration to recover the ethylene response in the mutant roots. These results suggest that auxin is a positive regulator for ethylene-induced inhibition in root elongation.

Key words: *Arabidopsis thaliana* — *AUX1* — Auxin — *EIR1* — Ethylene — Root growth.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; I_{50}, 50% inhibition of growth; NAA, 1-naphthaleneacetic acid; TIBA, 2,3,5-triiodobenzoic acid.

Introduction

Hormones control every aspect of growth and development in plants (Davies 1995) and in doing so they act in concert (Leopold and Noodén 1984). The physiological interactions between endogenous plant growth substances are important determinants in the regulation of plant development (Suttle 1988). In particular, the physiological interaction between endogenous auxin (IAA) and ethylene has been extensively studied and at least two kinds of interactions are well established. Earlier studies showed that application of exogenous auxin stimulates ethylene biosynthesis (Zimmerman and Wilcoxon 1935, Morgan and Hall 1962, 1964) and later it was shown that auxin induces the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Yang and Hoffman 1984). Secondly, it has been shown that ethylene treatment inhibits polar auxin transport and lateral transport of auxin (Morgan and Gausman 1966, Burg and Burg 1966, 1967, Lyon 1970). Suttle (1988)

clarified that ethylene reduces the number of efflux carriers for auxin in pea stems and thus inhibits the transport of auxin.

Both auxin and ethylene are endogenous regulators of root growth (Davies 1995). Although low concentrations of auxin applied to ambient solutions may stimulate root growth (Mulkey et al. 1982), the typical response of roots towards exogenous auxin is growth inhibition. Application of exogenous ethylene commonly results in inhibition of root growth (Abeles et al. 1992). Because of stimulation in ethylene production by auxin, Chadwick and Burg (1967, 1970) hypothesized that the inhibitory action of auxin on root growth is mediated by ethylene. This hypothesis was supported by Mulkey et al. (1982), but other investigators found little or no support for this idea (Andreae et al. 1968, Rauser and Horton 1975, Dubucq et al. 1978, Bucher and Pilet 1983, Eliasson et al. 1989).

In the last decade, several mutants related to auxin and ethylene have been isolated in *Arabidopsi*s. Most interestingly the mutant root having a defect in either auxin influx or efflux showed a greater resistance to ethylene in the root elongation assay. The *aux1* mutant, in which the uptake carrier protein in roots has been shown to be mutated (Bennett et al. 1996, Marchant et al. 1999), confers resistance to both ethylene and auxins (IAA and 2,4-D) (Pickett et al. 1990). At the same time the *aux1-7* roots are agravitropic (Maher and Martindale 1980). The auxin efflux mutant *eir1* was originally isolated on the basis of ethylene resistance in root growth (Roman et al. 1995). Later, it was found that the *EIR1/AGR1/AtPIN2/WAV6* gene encodes an auxin efflux carrier protein in roots (Chen et al. 1998, Luschnig et al. 1998, Müller et al. 1998, Utsuno et al. 1998). Mutation in this gene also makes the root agravitropic (Roman et al. 1995), although the *eir1-1* roots retain their ability to respond to exogenous auxin with a reduction in root growth as wild-type roots (Luschnig et al.

1998). The greater resistance towards ethylene of these auxin mutants further confirms the presence of crosstalk between these two hormones. In spite of extensive works being done in molecular level with these mutants for their genetic and molecular characterisation, the reason for the resistance of *aux1-7* and *eir1-1* roots to ethylene is still not clear. Estelle (1996) described a possible explanation for the resistance of *aux1-7* roots to ethylene; if ethylene treatment inhibits auxin efflux in *Arabidopsis* roots as in pea stem, it would increase intracellular auxin levels, while the mutation in the influx carrier AUX1 protein decreases auxin levels and provides resistance to ethylene. This idea is interesting but remains to be examined.

Since uptake of 1-naphthaleneacetic acid (NAA) is mainly due to diffusion (Delbarre et al. 1996), aux*1-7* roots show a normal sensitivity towards this chemical analogue of auxin (Yamamoto and Yamamoto 1998, Marchant et al. 1999) and the agravitropic nature of *aux1-7* roots disappears in the presence of NAA. Hence, we examined the effect of NAA on the resistance of *aux1-7* roots to ethylene and found that the mutant roots became sensitive to ethylene in the presence of NAA. Furthermore, *eir1-1* roots responded to ethylene in the presence of both IAA and NAA as wild-type roots. In the present paper we show that auxin is a positive regulator for the ethylene-mediated growth response of *Arabidopsis* roots. This finding will provide a further insight into understanding the interaction of the two hormones.

Materials and Methods

Plant Materials and Growth Condition

All mutant lines were derived from *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia. The auxin influx mutant, *aux1-7* (Pickett et al. 1990), and the auxin efflux

mutant *eir1-1* (Luschnig et al. 1998) were obtained from Arabidopsis Biological Resource Center (Columbus, Ohio, U.S.A.). These mutants were propagated as described earlier (Rahman et al. 2000).

Arabidopsis seeds were placed in a 2.6 cm Petri dish on filter paper (Advantec no.2; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) wetted with 300 μl of half-strength MS (Murashige and Skoog 1962) salt solution (pH 5.8). Two or 4 days after cold treatment at 4°C under nearly saturating humidity in the dark, seeds were irradiated to germinate for one or two days with white fluorescent lamps (FL 20SS-BRN/18, Toshiba, Tokyo, Japan) at an irradiance of about 1.6 W m^{-2}. The irradiated seeds were placed in a rectangular plastic Petri dish (6 x 4 cm) on nutrient agar containing half-strength MS salt and 1% (w/v) agar. IAA and NAA were mixed with agar medium while the temperature of agar was 45 to 50°C. Seedlings were grown on vertically oriented agar plates at 23°C under continuous irradiation.

Chemicals

IAA and NAA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Root Growth Assay

Arabidopsis seedlings were grown with IAA or NAA for 3 days under continuous irradiation at 23°C. For ethylene treatment, an agar plate containing germinated seeds was placed vertically in a sealed 140-ml plastic cylinder. Ethylene was injected with a syringe into each cylinder through a small side hole to make various concentrations of ethylene (Tsurumi and Ishizawa 1997). Ethylene and the air inside the container were

refreshed every day during the 3 day incubation period. Root length was measured under a microscope. The mean (± SE) for 10 to 15 seedlings was calculated and each assay was repeated at least three times. P values were analyzed by Student's t-test.

Results

Selective influence of NAA versus IAA on the restoration of ethylene response in aux1-7 roots

Figure 1A and B show the dose-response curves of auxin for root elongation in wild-type and *aux1-7* seedlings, respectively. In the wild-type, IAA and NAA inhibited root elongation in a similar pattern. On the other hand, *aux 1-7* roots showed a greater resistance towards IAA, while the response to NAA was similar to that of wild-type roots as reported by Yamamoto and Yamamoto (1998) and Marchant et al. (1999). Since neither 10 nM IAA nor NAA inhibited the growth of wild-type and *aux1-7* roots (Fig. 1A, B), we used this concentration of auxin to investigate its effect on the dose-response of ethylene for root elongation.

The ethylene dose-response of wild-type roots in the presence of 10 nM IAA or NAA was not so different from that of control roots grown without exogenous auxin except at 0.1 μl $liter^{-1}$ ethylene (Fig. 2A). At this concentration of ethylene the growth of roots treated with auxins was always slightly smaller compared with the control ($0.05 > P > 0.02$). In contrast, *aux1-7* roots showed a greater resistance to ethylene compared with wild-type roots as reported by Pickett et al. (1990), and application of 10 nM IAA did not change the response towards ethylene (Fig. 2B). However, in the presence of 10 nM NAA the growth inhibition of *aux1-7* roots by ethylene was accelerated almost to the level in wild-type roots (Fig. 2B). The selective influence of NAA versus IAA on the restoration of ethylene response in *aux1-7* roots suggests that

an increase in the intracellular level of auxin is required for ethylene response in this mutant root.

Ethylene response of eir1-1 roots is also restored in the presence of auxin

The NAA-induced recovery of the ethylene-mediated growth response in *aux1-7* roots prompted us to investigate the effect of NAA on another ethylene-resistant mutant, *eir1-1* (Roman et al. 1995). Figure 3 shows the dose-response curves of auxins for root growth inhibition in *eir1-1* seedlings. The growth of *eir1-1* roots was inhibited by both NAA and IAA as observed in wild-type roots (Fig. 1A). Since neither auxin showed any effect on the growth of *eir1-1* roots at 10 nM (Fig. 3), we used this concentration of auxin in the following experiments. The *eir1-1* roots exhibited a greater resistance towards ethylene in root growth inhibition as reported by Roman et al. (1995) (Fig. 4), while in the presence of NAA or IAA they displayed a normal response towards ethylene comparable to wild-type roots (Figs. 4 and 2A).

To compare the ethylene-induced inhibition in growth for wild-type, *eir1-1* and *aux1-7* roots in the absence or presence of auxins, we calculated the concentration of ethylene required to induce 50% inhibition of growth (I_{50}) (Table 1). The data are the averages from three to five experiments. In the absence of exogenous auxin, I_{50} in wild-type roots was achieved with 0.84 μl liter^{-1} ethylene, whereas 71.3 μl liter^{-1} ethylene was required to induce I_{50} in *eir1-1* roots and I_{50} of *aux1-7* was not obtained even at 1000 μl liter^{-1} ethylene. By contrast, in the presence of 10 nM NAA, the concentration of ethylene required to induce I_{50} in *eir1-1* and *aux1-7* roots was greatly reduced to 0.44 μl liter^{-1} and 0.75 μl liter^{-1}, respectively, close to the level in wild-type roots (Table 1). Although 10 nM IAA did not induce any change in the ethylene response of *aux1-7* roots, it reduced the concentration of ethylene required to induce

I_{50} to 0.6 μl liter^{-1} in *eir1-1* roots. The similar recovery of the ethylene response with NAA in the two different ethylene-resistant mutants along with the IAA-induced change in *eir1-1* roots suggest that auxin plays a pivotal role in regulating the ethylene response of *Arabidopsis* roots.

The optimum concentration of NAA to restore the ethylene response of aux1-7 roots

To evaluate the optimum concentration of NAA to restore the response of *aux1-7* roots to ethylene, we examined the effect of various concentrations of NAA on the response to 0.1 μl liter^{-1} ethylene. Figure 5A shows the dose-response curves of NAA for the growth of *aux1-7* roots in the presence and absence of ethylene. A single application of 0.1 μl liter^{-1} ethylene showed a tendency to inhibit root growth, but the inhibition was not statistically significant ($0.2 > p > 0.1$). In the presence of 1 nM and higher concentrations of NAA, however, application of 0.1 μl liter^{-1} ethylene induced a significant growth inhibition ($0.003 > p$) compared with the control. In Fig. 5B, the ethylene-induced inhibition was expressed as percentage of the elongation of control roots grown without exogenous ethylene. The percentage of ethylene-induced inhibition reached its optimum level with 10 nM NAA. A further increase in auxin concentration did not increase the percentage of ethylene-induced inhibition. These results suggest that a certain level of intracellular auxin is required for the roots to show ethylene response.

Discussion

Using two ethylene-resistant mutants, *aux1-7* and *eir1-1*, we analysed the interaction between two important hormones, auxin and ethylene. Application of 10 nM IAA or NAA restored the growth response to ethylene in *eir1-1* roots to the level

in wild-type roots, while in *aux1-7* roots only NAA was effective (Table 1). Since NAA diffuses into root cells without an aid of carrier protein (Yamamoto and Yamamoto 1998, Marchant et al. 1999), the treatment with NAA should increase the concentration of auxin in root cells. Hence, the simplest explanation for the restoration of ethylene response is that a certain level of auxin in root cells is required for sensing ethylene. This idea implies that the reduction of intracellular level of auxin may be the cause of the resistance to ethylene of these two mutant roots.

Marchant et al. (1999) reported that the uptake of [^{14}C]-2,4-D in *aux1-100* roots was about a half compared to that in wild-type roots. We also observed a similar reduction in the uptake of [^{3}H]-IAA in *aux1-7* roots (Rahman et al. 2001). Since the auxin uptake is reduced in *aux1-7* mutant, it is logical to assume that the intracellular level of auxin is lower in this mutant root compared to wild-type. This idea is consistent with the selective influence of NAA versus IAA on the restoration of ethylene response in *aux1-7* roots (Fig. 2B).

Luschnig et al. (1998) reported that in *eir1-3* roots the auxin-inducible gene *AtIAA2* expression was restricted to the root tip even after gravistimulation, while in the wild-type it extended to the elongation and differentiation zones. They also found that the *eir1* mutation blocked the inhibition of root growth caused by a high endogenous level of auxin when constructed a double mutant *eir1 alf1*. The concentration of endogenous auxin is about 10 times greater in the *alf1* mutant than in wild-type and the root length is very short, but the root length of the double mutant is normal. These results suggest that the *eir1* mutation reduces the intracellular level of auxin in the elongation zone of roots. Rashotte et al. (2000) found a reduced basipetal transport of [^{3}H]-IAA from the root tip to the elongation zone in *eir1* roots compared with wild-type. The basipetal transport of auxin in roots has been shown to occur in

the epidermis (Ohwaki and Tsurumi 1976, Tsurumi and Ohwaki 1978, Müller et al. 1998). These results suggest that the basipetal transport of IAA through the epidermis is perturbed in *eir1* roots because of a defect in the auxin efflux carrier protein. Based on these results we hypothesize that the defect of auxin efflux in *eir1* roots reduces the intracellular level of auxin in the elongation zone. Hence, the restoration of ethylene response in *eir1-1* roots by both IAA and NAA is in accordance with our idea (Fig. 4).

Yamamoto and Yamamoto (1998) and Marchant et al. (1999) showed that the agravitropic nature of *aux1-7* roots disappeared in the presence of 10 nM NAA, which enters into root cells by diffusion bypassing the auxin influx carrier (Delbarre et al. 1996). This effect of NAA was not replaced by IAA. The selective influence of NAA versus IAA was also observed in the restoration of ethylene-mediated growth response (Fig. 2B). Both the gravitropic response and ethylene response in *aux1-7* roots were recovered in the presence of NAA. These results clearly suggest that auxin is required for ethylene response as well as induction of gravitropic response. It is interesting to note that both the *eir1* and *pir1* (allelic to *aux1*) roots showed a greater resistance to a polar auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) (Fujita and Syono 1996, Luschnig et al. 1998). We suspect that the resistance to TIBA may be due to a low level of intracellular auxin in the elongation zone of the two mutant roots.

Auxin stimulates ethylene production by inducing the synthesis of ACC synthase (Yang and Hoffman 1984). So it might be argued that NAA treatment of *aux1-7* and *eir1-1* roots results in an increase in ethylene production which ultimately inhibits root growth. We rule out this possibility for the following reasons. Since the concentration of exogenous auxin required for ethylene production in roots is greater

than 0.1 or 1 μM (Chadwick and Burg 1967, Dubucq et al. 1978, Bucher and Pilet 1983, Eliasson et al. 1989), it is unlikely that application of 1 or 10 nM NAA stimulates ethylene production. Moreover, although *aux1-7* and *eir1-1* roots are resistant to ethylene (Fig. 2, 4), they respond to NAA like wild-type roots (Fig. 1, 3), suggesting that NAA-induced inhibition in growth is not mediated by ethylene. By contrast, auxin in root cells is likely to play an important role in the ethylene-mediated growth response.

Another possibility is that the ethylene-induced inhibition in growth may be mediated by ethylene-induced inhibition of auxin efflux. Estelle (1996) and Luschnig et al. (1998) discussed this possibility to explain the ethylene resistance of *aux1-7* and *eir1-1* roots. We do not completely rule out this possibility but argue against it because of the following two facts. First, Luschnig et al. (1998) found that application of ACC resulted in a strong expression of *AtIAA2* gene in the elongation zone of wild-type roots but not in *eir 1-3* roots. This suggests that the application of ethylene does not induce any accumulation of auxin in the elongation zone of *eir1-3* roots. Since in *eir1-1* roots, the efflux of auxin is already blocked, ethylene is not likely to further inhibit auxin efflux in the mutant roots. Secondly, application of 1 or 10 nM NAA induced a significant response of *aux1-7* roots to 0.1 μl liter^{-1} ethylene and these concentrations of NAA did not induce inhibition of root growth when applied solely (Fig. 5). In contrast, application of 100 nM NAA greatly inhibited the elongation of roots but the percentage of ethylene-induced inhibition was the same level as 10 nM NAA. These results suggest that a small amount of auxin is enough for the restoration of ethylene-

response and that the auxin-induced recovery of ethylene-response is mediated by a mechanism distinct from the auxin-induced growth inhibition.

Taking together, we suggest that the resistance of *eir1-1* and *aux1-7* roots towards ethylene is, at least in part, due to the lower intracellular level of auxin and that an increase in the level of auxin in root cells restores the ethylene response. The intracellular level of auxin plays a critical role in regulating the ethylene-mediated growth response and auxin is a positive regulator for this response in *Arabidopsis* roots.

We thank Dr. K. Ishizawa of Tohoku Univ. for his invaluable suggestions and the Arabidopsis Biological Resource Center of Ohio State Univ. for providing mutant seeds.

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Table 1 Ethylene concentration required for 50% inhibition (I_{50}) in the elongation of roots grown in the absence and presence of 10 nM auxin

	Ethylene concentration for I_{50} ($\mu l\ liter^{-1}$)		
	control	+ NAA	+IAA
wild-type	0.84 ± 0.12	0.40 ± 0.14	0.50 ± 0.16
eir1-1	71.3 ± 32.2	0.44 ± 0.11	0.60 ± 0.07
aux1-7	1000 <	0.75 ± 0.29	1000 <

The data were obtained from Figs. 2 and 4 and the averages from 3 to 5 experiments.

Figure legends

Fig. 1. Dose response of root elongation in wild-type (A) and *aux1-7* (B) for IAA (△) and NAA (□). *Arabidopsis* seedlings were grown for three days in the light. Data are the averages from 10 to 15 seedlings (±SE). Mean values for 100% root elongation were 7.36 ± 0.35 mm (wild-type) and 8.80 ± 0.50 mm (*aux1-7*).

Fig. 2. Dose responses of root elongation in wild-type (A) and *aux1-7* (B) for ethylene in the absence (●) and presence of 10 nM IAA (△) or 10 nM NAA (□). *Arabidopsis* seedlings were grown in 140 ml sealed plastic containers for three days in the light. Ethylene and the air inside the containers were refreshed every day. Data are the averages from 10 to 15 seedlings (±SE). Mean values for 100% root elongation were 6.31 ± 0.21 mm (wild-type) and 7.30 ± 0.46 mm (*aux1-7*).

Fig. 3. Dose response of root elongation in *eir1-1* for IAA (△) and NAA (□). *Arabidopsis* seedlings were grown for three days in the light. Data are the averages from 10 to 15 seedlings (±SE). Mean value for 100% root elongation was 5.85 ± 0.31 mm.

Fig. 4. Dose responses of root elongation in *eir1-1* for ethylene in the absence (●) and presence of 10 nM IAA (△) or NAA (□). *Arabidopsis* seedlings were grown in 140 ml sealed plastic containers for three days in the light. Ethylene and the air inside the containers were refreshed every day. Data are the averages from 10 to 15 seedlings (±SE). Mean value for 100% root elongation was 5.77 ± 0.30 mm.

Fig. 5. Dose responses of root elongation in *aux1-7* for NAA in the absence (●) and presence (○) of 0.1 μl $liter^{-1}$ ethylene (A) and the percentage of ethylene-induced inhibition compared with the elongation of control roots grown without exogenous ethylene (B). *Arabidopsis* seedlings were grown in 140 ml sealed plastic containers for three days in the light. Ethylene and the air inside the containers were refreshed every day. Data are the averages from 10 to 15 seedlings (±SE). Mean value for 100% root elongation was 5.82 ± 0.37 mm without exogenous ethylene. The values in B were calculated from A.

Fig. 1

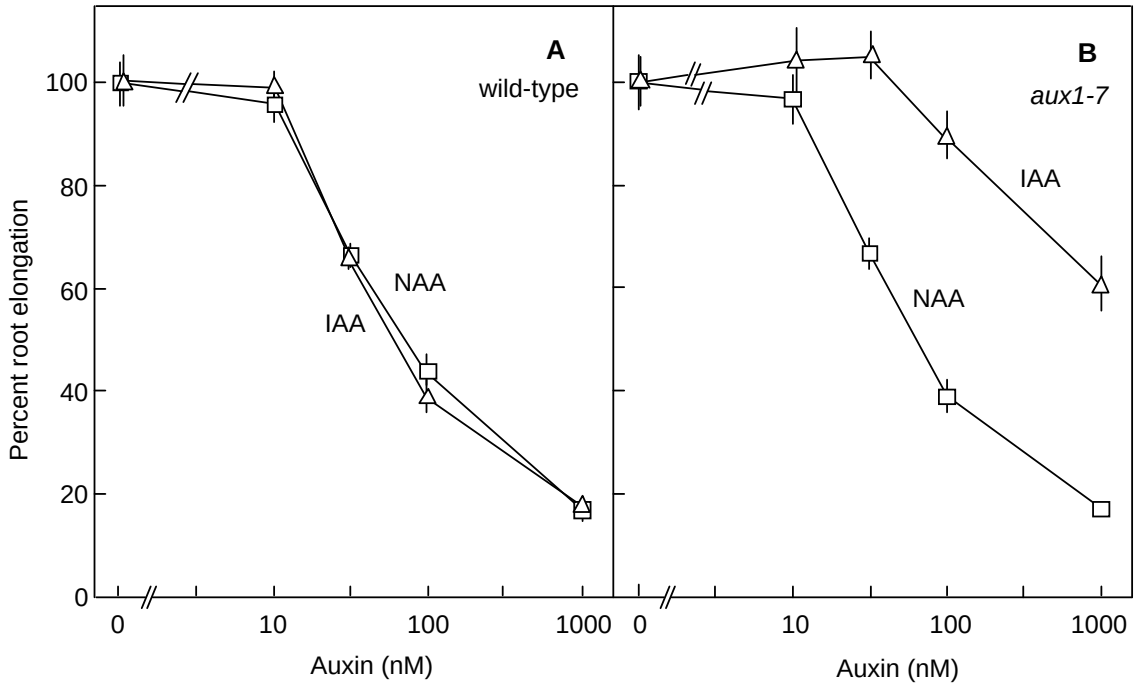

A
wild-type
B
aux1-7
Percent root elongation
100
80
60
40
20
0
NAA
IAA
IAA
NAA
0
10
100
1000
Auxin (nM)
0
10
100
1000
Auxin (nM)

Fig. 2

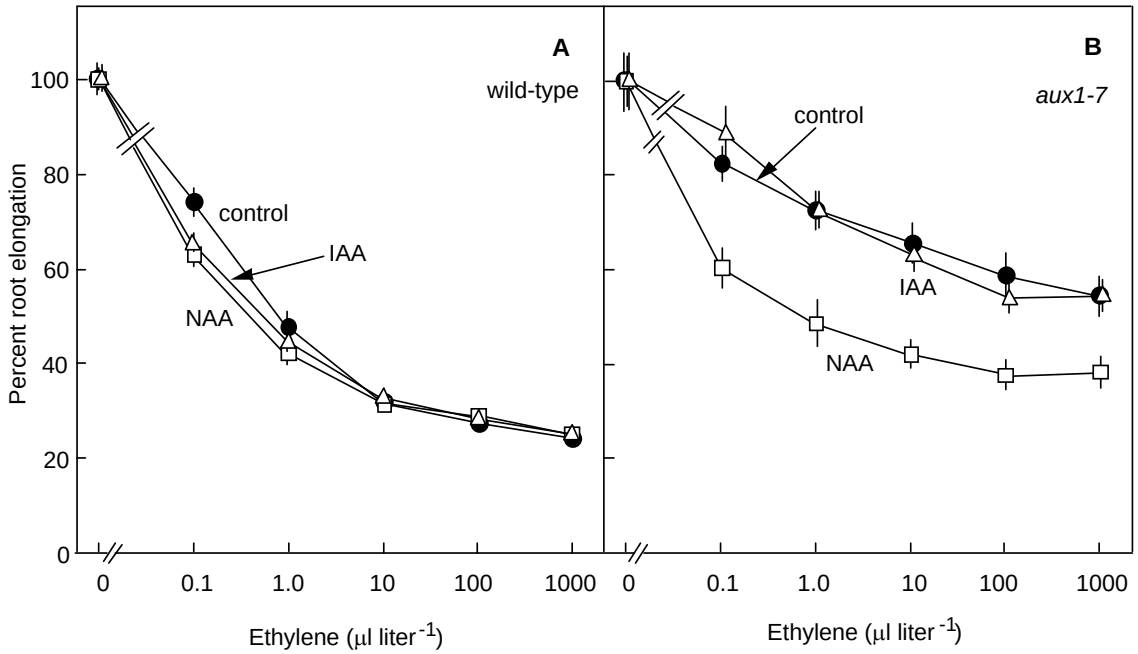

A
wild-type
B
aux1-7
control
IAA
NAA
Percent root elongation
100
80
60
40
20
0
0
0.1
1.0
10
100
1000
Ethylene (μl liter -1)

Fig. 3

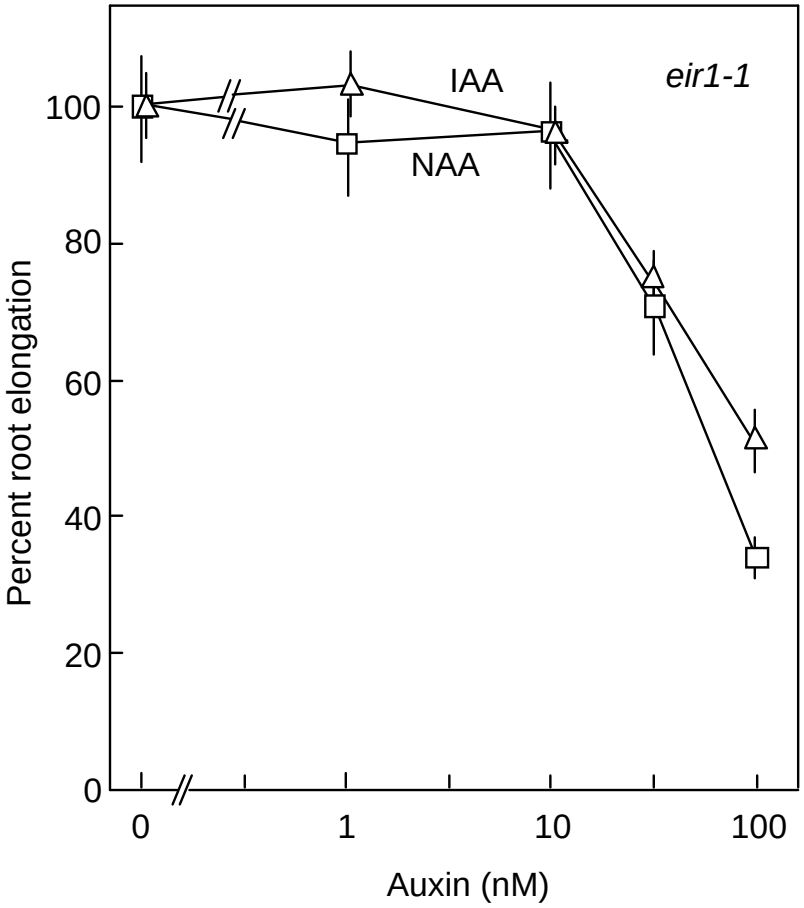

IAA
NAA
eir1-1
Percent root elongation
100
80
60
40
20
0
0
1
10
100
Auxin (nM)

Fig. 4

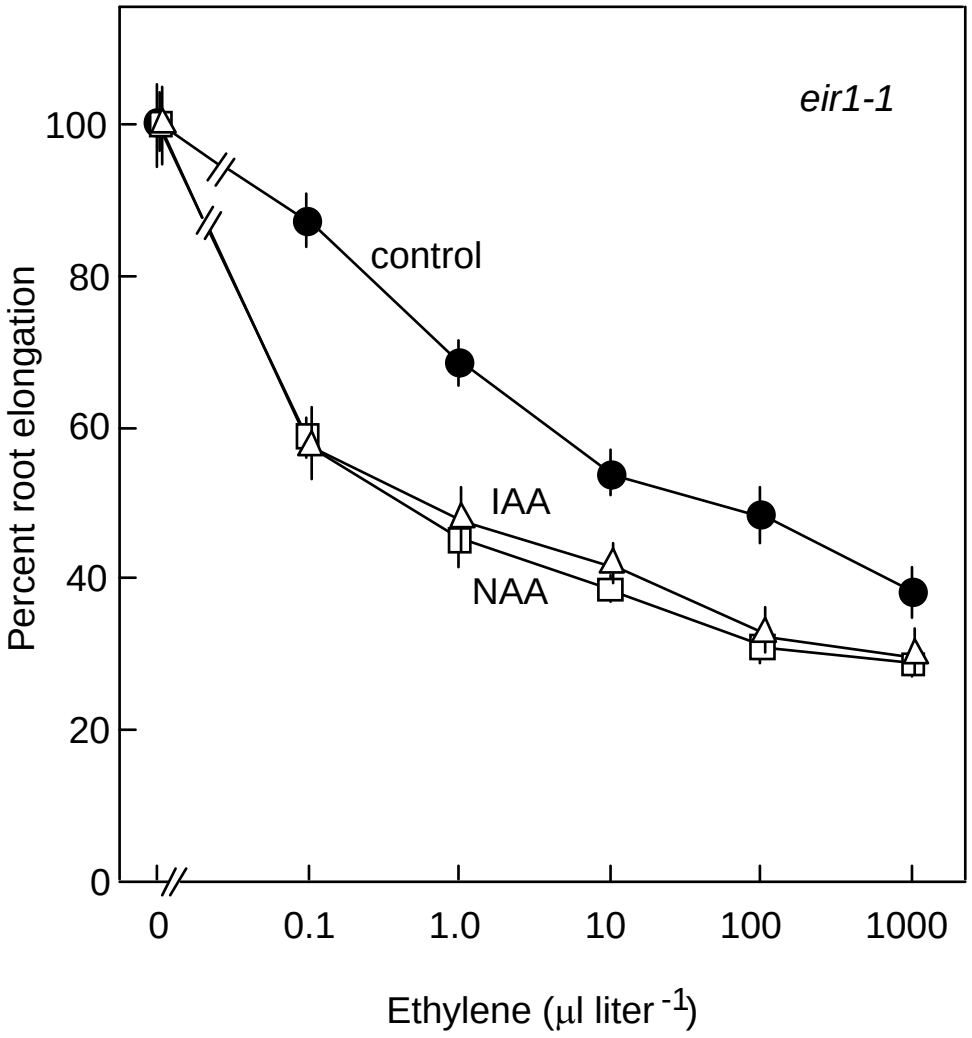
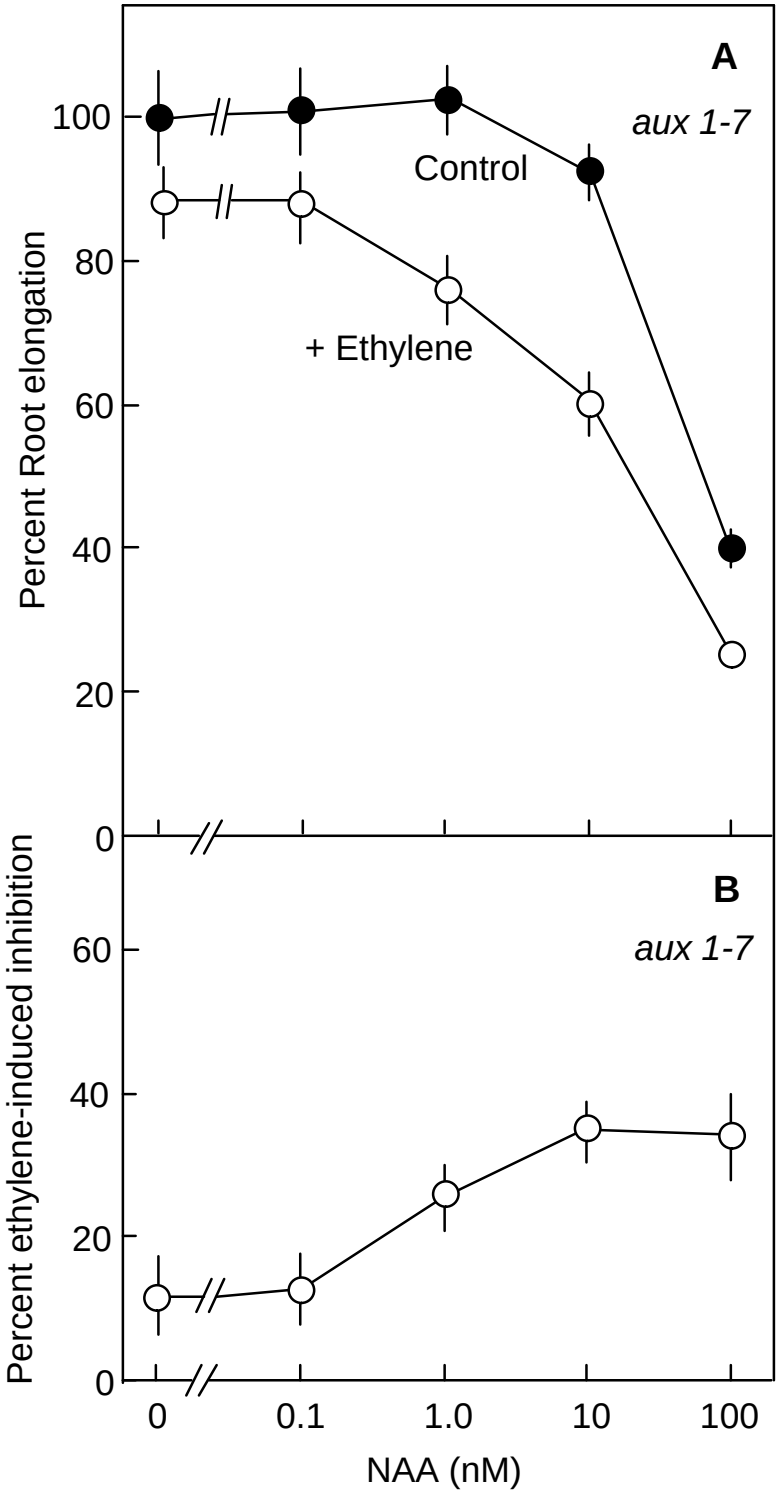

eir1-1
control
IAA
NAA
Percent root elongation
100
80
60
40
20
0
0
0.1
1.0
10
100
1000
Ethylene (μl liter -1)

Fig. 5


A
aux 1-7
Control
+ Ethylene
Percent Root elongation
100
80
60
40
20
0
B
aux 1-7
Percent ethylene-induced inhibition
60
40
20
0
0
0.1
1.0
10
100
NAA (nM)