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Genetic analysis of a seedling stress response to ethylene in *Arabidopsis*

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SUMMARY

A genetic framework has been devised for the action of genes within the ethylene-response pathway. This working model is based on the epistatic interactions among a variety of ethylene response mutations. Most of the mutations that have been described act in a linear pathway. Genes controling cell elongation in response to ethylene must, at some level, act to affect the architecture of the cytoskeleton. Genes that act late in the pathway, in mutant form, may lead to highly specific phenotypes such as the increased senitivity to taxol in the *ein6* mutant. Analysis of these downstream components may provide critical insights into the nature of ethylene's effect on the cell elongation machinery.

1. INTRODUCTION

The simple gas ethylene (C_2H_4) is an endogenous regulator of many stress responses and developmental adaptations in higher plants. These responses include the following traits: fruit ripening, flower senescence, leaf abscission, sex determination, defense responses to pathogens, and the responses to mechanical trauma (Abeles *et al.* 1992). Control of these responses to ethylene involves the complex regulation of biosynthesis and the ability to perceive ethylene and respond properly. Ethylene biosynthesis is induced before several developmentally controlled senescence processes and environmental insults also increase ethylene production (Yang & Hoffman 1984; Abeles *et al.* 1992). The biosynthetic pathway for this hormone has been extensively characterized (Yang & Hoffman 1984; Theologis 1992; Kende 1993). The rate-limiting step is the conversion of s-adenosyl-L-methionine (adoMet) to aminocyclopropane-1-carboxylic acid (ACC), which is catalysed by ACC synthase, a peridoxal phosphate-requiring enzyme. In the next step, ACC is converted to ethylene, carbon dioxide, and cyanide by ACC oxidase which is constitutive in most tissues, but is induced during fruit ripening in tomato. These two enzymes have been cloned and characterized from many plant species (reviewed in Kende 1993). ACC synthase is encoded by multigene families in all species examined, including *Arabidopsis thaliana* (reviewed in Zarembinski & Theologis 1994). Members of this gene family are transcriptionally activated by developmental signals, plant hormones, or environmental stress, thereby providing the means for a rapid induction of ethylene biosynthesis.

2. THE TRIPLE RESPONSE PHENOTYPE

The response of dark-grown seedlings to ethylene has been used extensively as a model for the effect of ethylene on plant growth (Abeles *et al.* 1992). In fact, the first illustration of a gas acting as a signalling molecule in a biological system was seen when ethylene was applied to pea seedlings (Neljubov 1901). In the presence of this gas, dicotylendous seedlings undergo dramatic morphological changes, collectively known as the 'triple response' (Knight *et al.* 1910). These changes consist of a radial swelling of the hypocotyl, an exaggeration of the apical hook, and the inhibition of both hypocotyl and root elongation (Bleecker *et al.* 1988; Gúzman & Ecker 1990). This phenotype is a stress-induced adaptation that allows seedlings to penetrate the soil without damage to the apical meristem (Darwin & Darwin 1881; Goeschl *et al.* 1966; Harpham *et al.* 1991). Physical obstruction of seedling growth leads to dramatic increases in ethylene biosynthesis which induces the development of the triple response morphology (Goeschl *et al.* 1966). Inhibitors of ethylene preception or biosynthesis prevent this morphological transformation (Kang *et al.* 1967; Beyer 1976; Gúzman & Ecker 1990).

3. COMPONENTS OF THE ETHYLENE RESPONSE PATHWAY AND THEIR ORDER OF ACTION

Wild-type *Arabidopsis thaliana* seedlings also undergo drastic morphological changes in the presence of ethylene. The total seedling length is reduced dramatically and the degree of curvature in the apical hook becomes highly exaggerated when grown in ethylene (see figure 1). The triple response phenotype has been used to dissect genetically components of the ethylene induced stress response pathway in *Arabidopsis* (Bleecker *et al.* 1988; Gúzman & Ecker 1990; Harpham *et al.* 1991; Van Der Straeten *et al.* 1992; Kieber *et al.* 1993; Roman *et al.* 1995). Eight mutant loci with an ethylene-insensitive phenotype have been identified and characterized genetically: *etr1* (Bleecker *et al.* 1988), *ein2* (Gúzman & Ecker 1990), *ein3* (Kieber *et al.* 1993), *ein4*, *ein5 ein6 ein7* (Roman *et al.* 1995) and *ain1* (Van Der Straeten *et al.* 1993) (see figure 1).

The phenotypes of double mutants have been used

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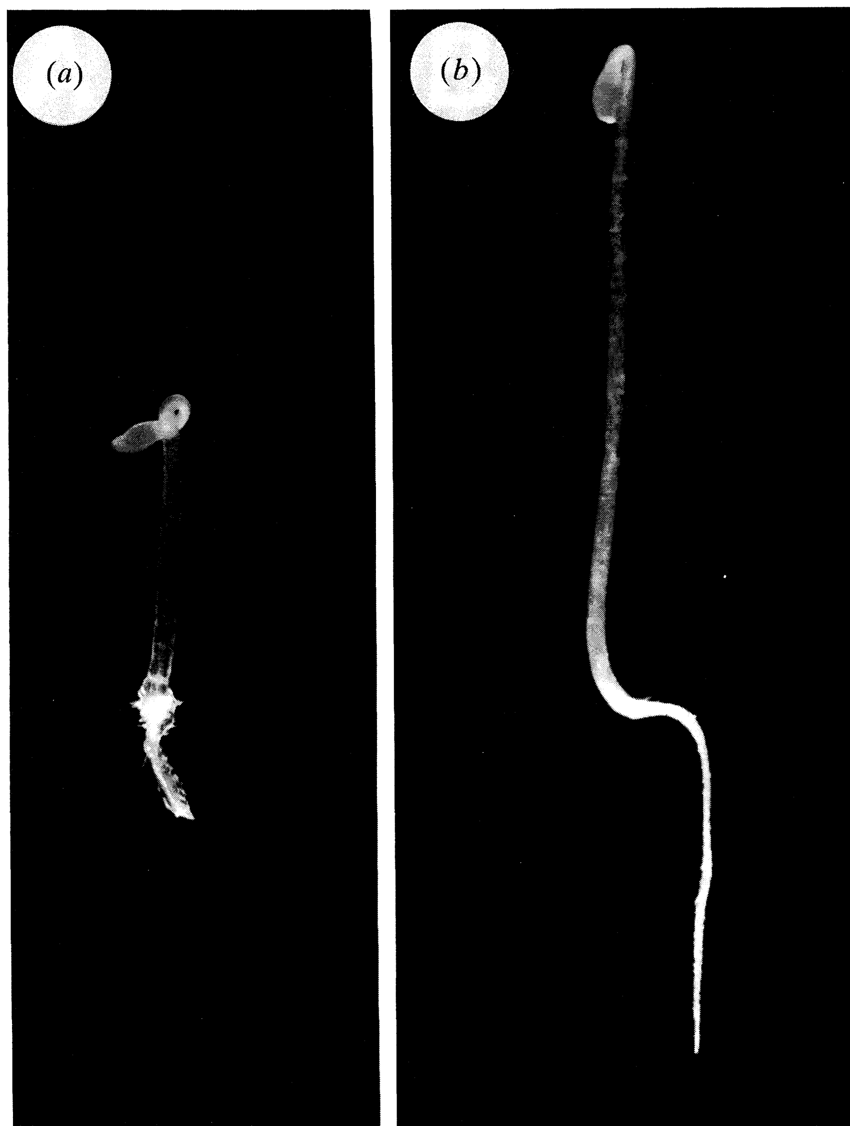

Figure 1. Ethylene insensitivity phenotype of the *ein6* mutant. Seedlings were germinated and allowed to grow in the dark for three days in the presence of 10 μl l^{-1} ethylene. (*a*) Wild-type seedlings grown in ethylene develop the triple response phenotype. (*b*) The *ein6* mutant displays an ethylene insensitive (Ein^-) seedling phenotype; a thin and elongated hypocotyl and root (seedling on the right).

to build a framework for the action of these genes within an ethylene response pathway (see figure 2). The earliest steps in the pathway are defined by the *ETR1* and *EIN4* loci. The *etr1* mutants have Ein^- phenotypes that are dominant to wild-type (Bleecker *et al.* 1988; Gúzman & Ecker 1990; Chang *et al.* 1993). This locus has recently been cloned by a map-based strategy and found to be similar to bacterial two-component histidine kinases (Chang *et al.* 1993). It is unclear whether the *etr1* mutant phenotypes result from gain-of-function or dominant-negative mutations. This question remains crucial in determining the role of the putative histidine kinase in ethylene signalling. The primary sequence of *ETR1* is similar to the *SLN1* histidine kinase in *Saccharomyces cerevisiae* (Ota & Varshavsky 1993). Both gene products are predicted to have very similar structures (Chang *et al.* 1993; Ota & Varshavsky 1993), and mutations of both genes are suppressed by second-site mutations in putative members of a MAP kinase cascade, CTR1 kinase in the ethylene pathway and PBS2/HOG1 kinases in the yeast osmosensing pathway (Kieber *et al.* 1993; Maeda *et al.* 1994). The *SLN1* gene product directly phosphorylates and inactivates the *SSK1* gene product in response to high osmolarity, which results in the inactivation of a MAP kinase phosphorylation cascade (Maeda *et al.* 1994). The *HOG1* gene product is the MAP kinase family member that is involved in this response to osmotic stress (Brewster *et al.* 1993; Maeda *et al.* 1994). Interestingly, *HOG1*-related MAP kinases are also involved in mammalian responses to osmotic and lipopolysaccharide stress signals (Weinstein *et al.* 1992; Galcheva-Gargova *et al.* 1994; Han *et al.* 1994; Rouse *et al.* 1994;Cano & Mahadevan 1995; Derijard *et al.* 1995). This signal transduction pathway may therefore represent a generalized stress-response system that is conserved in budding yeast, plants and mammals.

The order of *ETR1* and *EIN4* in the pathway is currently unknown, although the similarities be-

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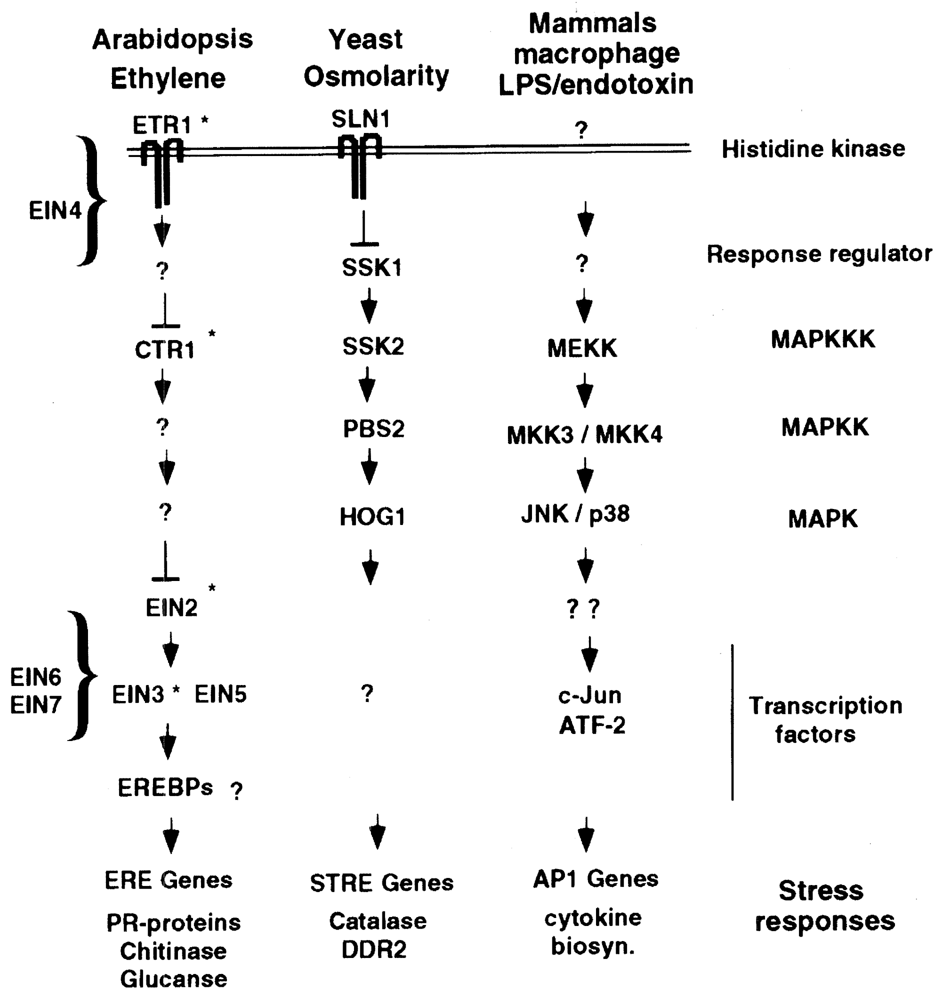

Figure 2. Similarity of the signal transduction pathway for ethylene with signal transduction pathways yeast and mammals. A model for the genetic pathway of ethylene signal transduction is shown that is consistent with epistatic relations. *ctr1-1* masks the phenotype of *etr1-3* and *ein4*; therefore, *CTR1* is shown acting after *ETR1* and *EIN4*. In this model, the *etr1* and *ein4* mutations are assumed to be dominant-negative. These genes would negatively regulate *CTR1* which acts either directly or through other proteins to regulate negatively the ethylene response pathway. These negative control points are indicated by a bar. *ein2*, *ein3*, *ein5*, *ein6*, *ein7*, are all epistatic to *ctr1*, and so their wild-type products likely act after *CTR1* in the ethylene response pathway. *EIN2* is required for the *EIN3*/*EIN5* gene activity and is shown acting before these genes. *EIN7* and *EIN6* are shown outside of the genetic pathway (acting after *ctr1*) since the double mutant analysis for these loci has not been completed. Similarity between the ethylene response pathway and the yeast (reviewed in Herskowitz 1995) and mammalian stress reponses (reviewed in Cano & Mahadevan 1995) suggests additional proteins acting before and after *CTR1*. These putative proteins are indicated by question marks.

tween *ETR1* and *SLN1* suggest putative functions for the *EIN4* gene. *EIN4* may also act after *ETR1* and have the analogous function in ethylene signal transduction in *Arabidopsis* as *SSK1* has in the osmolarity stress response in yeast. Another intriguing possibility is that *ETR1* and *EIN4* have redundant functions. Sequences similar to *ETR1* have been found within the *Arabidopsis* expressed sequence tag collection (Newman *et al.* 1994). This redundancy would account for the absence of recessive alleles at these loci. Alternatively, *EIN4* may act before *ETR1* within the ethylene signal transduction pathway. Indirect evidence suggests that the ethylene receptor contains a transition metal that could coordinate this simple gas (Burg & Burg 1967; Sisler 1990). The *ETR1* gene product does not appear to have any structure suggestive of an ethylene binding domain (Chang *et al.* 1993). Therefore, there may be other proteins which act before *ETR1* that are required for ethylene perception.

Additional screens have identified mutations that constitutively activate the triple response signalling pathway (Kieber *et al.* 1993). Mutations at the *CTR1* locus result in severe constitutive triple response phenotypes that are not reverted by inhibitors of ethylene biosynthesis or action, and the *ctr1–1* mutation is epistaitc to the *etr1* and *ein4* mutations (Kieber *et al.* 1993; Roman *et al.* 1995). These data indicate that the *CTR1* locus is a negative regulator of the ethylene response pathway. The *CTR1* gene was cloned and found to show similarity to the Raf-family (MAPKKK) of protein kinases, implicating a kinase cascade in this ethylene response (Kieber & Ecker 1993; Kieber *et al.* 1993).

The *EIN2* gene acts after *CTR1* in the ethylene signal transduction pathway (Roman *et al.* 1995). In addition to ethylene insensitivity, *ein2* mutants are also deficient in the development of disease symptoms upon infection with virulent *Pseudomonas syrringae* pv. tomato

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and *Xanthomonas campestris*. Interestingly, mutations at the *ETR1* locus do not confer this phenotype (Bent *et al.* 1992). There are several possible explanations for why *EIN2*, which acts later in the ethylene signal transduction pathway, has a phenotype not seen in the upstream gene, *ETR1*. Because *etr1–3* has a less severe phenotype than the *ein2* mutants that were tested for disease tolerance, it is possible that *etr1–3* mutants develop disease symptoms because of remaining ethylene sensitivity in these plants. Another possibility is that the pathways for tolerance to pathogens and ethylene response are distinct and that *EIN2* participates in both of these events. Cloning of the *EIN2* gene may provide insight into its dual sensitivity to ethylene and pathogens response.

The *EIN3*, *EIN5*, *EIN6* and *EIN7* genes act after *CTR1* in the ethylene signal transduction pathway. The *ein3*, *ein5*, and *ein6* mutants have a significantly less severe Ein^- phenotype than does *ein2*. In addition, Lawton *et al.* (1994) found that the ethylene induced Hevein-like gene (HEL) was induced by ethylene to higher levels in *ein3* than in either *etr1* or *ein2*. The sequence of *ein3* predicts that it gives rise to a truncated protein that should result in a severe reduction-of-function or a loss-of-function (M. Rothenberg & J. R. Ecker, unpublished data). Therefore the weak phenotype of *ein3* cannot be attributed to a simple model of reduced activity, but must be explained by the function of this gene within ethylene signal transduction. Thus the *EIN3* locus affects only a subset of the functions of *EIN2*. The molecular identities of *EIN5*, *EIN6* and *EIN7* have yet to be determined. It is possible that these mutants have a weak phenotype because they are leaky; it is also possible that these gene mutations affect only a portion of the *EIN2* functions.

(*a*) *Additional dominant ethylene insensitive loci*

To identify additional components of the ethylene signal transduction pathway in *Arabidopsis*, three day old etiolated seedlings from X-ray, diepoxybutane and fast-neutron-mutagenized M_2 pools were examined for dominant ethylene insensitive mutants. Several independent dominant mutations have been identified and some are known to be distinct from *etr1*. The D1-1, D5-3, D6-1, D13-1, and D17-1 mutations were all isolated from DEB treated lots, whereas the X3-1 and X8-3 mutations were isolated from X-ray mutagenized lots. All of these mutations show full dominance (see table 1). An additional DEB induced mutation, D11-1, may be semidominant; this designation is based on the weak Ein^- phenotype of three seedlings heterozygous for D11-1. The D13-1 and X3-1 mutations are not allelic to *etr1*. The progeny from a selfed D13-1/*etr*1-3 trans-heterozygote segregated 31 triple response seedlings out of a total of 422 progeny, indicating independent segregation ($\chi^2 = 1.02$; $p >$ 0.1). Additionally, the D13-1 mutant, when crossed onto *ein4*, failed to segregate any wild-type seedlings in 697 F_2 progenies; this mutation may therefore, be an allele of *ein4*. The progeny from a selfed X3-1/*etr1-3* trans-heterozygote segregated 39 wild-type seedlings in 731 total progeny, indicating these mutations are not

Table 1. Dominant ethylene insensitive mutations

male	female	generation	Ein	Ein	$\chi 2^b$
D1-1	wildtype[a]	F1	0	13	
		F2	14	34	0.44
D5-3	wildtype	F1	0	3	
		F2	8	22	0.04
D6-1	wildtype	F1	0	6	
		F2	14	54	0.71
D13-1	wildtype	F1	0	14	
		F2	36	93	0.62
D17-1	wildtype	F1	0	10	
		F2	25	69	0.13
X3-1	wildtype	F1	0	8	
		F2	22	63	0.07
X8-3	wildtype	F1	0	12	
			15	49	0.09

[a] wild-type denotes the wild-type Columbia strain.
[b] $p < 0.05$.

linked ($\chi^2 = 1.13$; $p > 0.1$). X3–1 mutants have not been crossed onto *ein4*. The X3-1 mutation has not been independently mapped to a chromosomal position, although X3-1 also does not appears to segregate with the *lu* visible marker (7 Lu^- progeny out of 24 F2 Ein^- plants) and therefore, the bottom of chromosome *1* and the top of chromosome *5* have been eliminated as map positions for this mutation.

The D1-1, D6-1, D17-1 and X8-3 mutations are all candidate alleles of *etr1*. Plants carrying these mutations were crossed onto the M10 chromosome *1* marker line (*ap1*, *dis1*) and F_2 Ein^- plants were examined for the independent segregation of the Ap^- phenotype. For the D1-1, D6-1, and D17-1 mutations, there were no Ap^- plants in the 24 F_2 progenies scored in each cross. These results strongly suggests linkage to the bottom of chromosome *1* ($\chi^2 = 8$; $p < 0.05$). The cross with the X8–3 mutation segregated a single Ap^- plant in 64 F_2 Ein^- progeny, also indicating linkage to the *ap1* marker ($\chi^2 = 18.7$; $p < 0.05$). Additionally, the D17–1 mutant was crossed onto both *etr1–3* and *ein4* mutants. There were no Ein^+ seedlings in 173 F_2 progeny from the cross with *etr1-3* ($\chi^2 = 11.1$; $p < 0.5$), but in 143 F_2 progeny from the cross onto *ein4*, nine wild-type seedlings were detected ($\chi^2 = 0$; $p > 0.1$). Together these data indicate that the D1-1, D6-1, D17-1, and X8-3 mutations map to the bottom of chromosome *1*, near the *etr1* locus (Bleecker *et al.* 1988; Chang *et al.* 1993). A strong indication of allelism will be found if these mutations contain changes in the predicted ETR1 protein and may provide useful biochemical information regarding the mechanism of their dominance.

4. INFLUENCE OF ETHYLENE ON CELL ELONGATION PROCESSES: THE ROLE OF *EIN6*

One mechanism by which ethylene may regulate hypocotyl and root cell elongation is through its effects on the structure of the extra-cellular matrix (Apelbaum & Burg 1971; Lang *et al.* 1982). In pea epicotyl cells, the orientation of cellulose microfibrils in the secondary cell wall realigns from a primarily transverse to a

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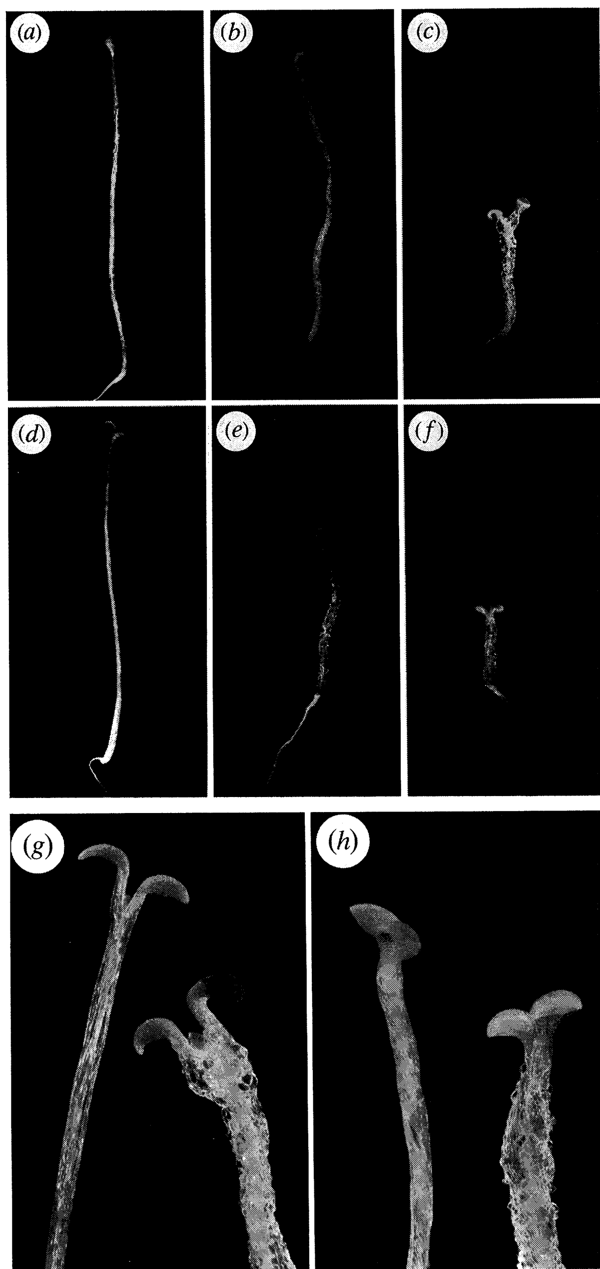

Figure 3. Effect of taxol on cell expansion in the hypocotyl of wild-type and the *ein6* mutant seedlings. Wild-type Landsberg seedlings were grown in the presence of either: (*a*) 0 μM; (*b*) 0.1 μM; or (*c*) 1.0 μM taxol for 10 days in the dark. Respresentative seedlings are shown. *ein6* seedlings were grown in the presence of either: (*d*) 0 μM; (*e*) 0.1 μM; or (*f*) 1.0 μM taxol for 10 days in the dark. (*g*) Wild type seedlings grown in the absence (left) or presence (right) of 1 μm taxol for 10 days. (*h*) Wildtype (left) or *ein6* mutant (right) seedlings grown in the presence of 0.1 μM of taxol for 10 days.

longitudinal direction in response to ethylene (Apelbaum & Burg 1972; Lang *et al.* 1982). Microfibrils laid down parallel to the long axis of the cell may constrain cell expansion to the radial axis, thereby accounting for the difference in cell shape seen in ethylene treated tissues. Underlying this shift in orientation is a parallel realignment of the cortical microtubules; it is not known how ethylene affects this realignment, or the importance of the shift in defining cell shape (Lang *et al.* 1982; Roberts *et al.* 1985; Yuan *et al.* 1994; Lloyd 1995). Cortical microtubules associate tightly with the plasma membrane, and it has been hypothesised that the microtubule array directs the cellulose synthase complex on the exterior surface of the plasma membrane (Shibaoka 1994). Recent studies of the polarity of growth and organisation of microtubule arrays in *Arabidopsis* roots suggest that re-evaluation of the role of cortical microtubules in controlling cell elongation may be necessary. Drugs such as oryzalin and taxol that perturb or promote microtubule polymerization have little effect on anisotropic growth in root cells (Baskin *et al.* 1994). Similarly, mutations that cause abnormal cell expansion in roots such as *saber* and *cobra*, have little effect on the polarity of cortical microtubules (P. Benfey, personal communication). In short, the spatial pattern of cortical microtubules may not be solely responsible for controlling anisotropic growth in root cells. Alternatively, one can speculate that the biochemical mechanisms controlling cell growth processes in shoots and roots may not be a identical.

In an attempt to sort out possible effects of the Ein$^-$ mutations on the realignment of the cortical microtubules, all of the mutants were assayed for seedling phenotypes in the presence of taxol. Taxol can stabilize microtubules from many species by inhibiting microtubule depolymerization (Schiff & Horwitz 1981), and therefore may inhibit the shift in cortical microtubules upon ethylene treatment (Heinstein & Chang 1994). The primary effect of 1 μM taxol on dark grown seedlings was an inhibition of elongation. The cells of the hypocotyl appear shorter and more round than in untreated plants (see figure 3*a*, *b*, *c*). In the presence of 0.1 μM taxol, wild-type hypocotyls are shorter relative to untreated controls and they develop a slight twist (see figure 3). The *etr1-3*, *ein2-1*, *ein3-1*, *ein4*, *ein5-1*, *ein7*, *eir1-1*, and *aux*1-21 mutants all demonstrated wild-type levels of taxol sensitivity (data not shown). However, *ein6* seedlings were significantly shorter and displayed a phenotype very similar to wild-type grown in the presence of 1 μM taxol. At 0.1 μM, this phenotype was not observed in wild-type seedlings, but a severe effect was seen in *ein6* seedlings (see figure 3). Thus, the *ein6* mutation (or a second mutation in a tightly-linked gene) causes an increase in sensitivity to taxol.

The *ein6* mutant was identified in a screen of fast neutron-mutagenized seeds of the Landsberg strain. This mutant is recessive, and has significantly reduced gametophytic transmission (Roman *et al.* 1995). Fast neutron-mutagenesis frequently induces large chromosomal aberrations (Hawkins 1979; Shirley *et al.* 1992), which may account for the reduced transmission of *ein6*. The phenotype conferred by *ein6* is substantially weaker than those conferred either *ein2-1* or *ein4* (data not shown). *ein6* was mapped to the bottom of chromosome *3* in a cross to wild-type Columbia, and lies 31.6 ± 6 cM south of the *GL1* CAPS marker and 17.6 ± 4.4 cM north of the nga112 SSLP (Roman *et al.* 1995).

The *ein6* mutant has another distinguishing phenotype when combined with the constitutive triple response mutation *ctr1*. The *ctr1* mutant has a dramatic

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adult phenotype that can be phenocopied in wild-type plants by continuous growth in the presence of ethylene (Kieber *et al.* 1993). This phenotype includes reduced leaf and petiole size and reduced elongation of the inflorescence; the result is a dramatically smaller plant. Consistent with their down stream positions in the seedling stress ethylene response pathway, the *ein2-1*, *ein3-2* and *ein5-1* double mutants with *ctr1* do not have the Ctr$^-$ adult phenotype (data not shown). However, *ein6 ctr1-1* double mutants have an adult phenotype that is very similar to that of *ctr1-1* plants. These results suggests that *EIN6* is not required for ethylene responses in the adult tissues and may act in the seedling to control cell shape in response to ethylene.

5. CONCLUSIONS AND PERSPECTIVES

The triple response phenotype of etiolated *Arabidopsis* seedlings has been used to identify genes involved in ethylene signal transduction. The triple response consists of inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl, and an exaggeration in the curvature of the apical hook. Both dominant and recessive mutations have been isolated through identification of plants that are deficient in this ethylene response. Examination of double mutant phenotypes has provided information on the relative order of these genes in the ethylene response pathway. The results demonstrate that at least ten genes are involved in the development of the triple response. Most of the mutations affect all aspects of the seedling and adult ethylene responses; these genes are most likely involved in the primary ethylene signaling pathway. Other mutations define genes that affect only a subset of ethylene responses. The phenotypes of these mutations, and their relative positions in the ethylene signal transduction pathway suggests that the development of the triple response involves hormonal interactions Roman *et al.* (1995).

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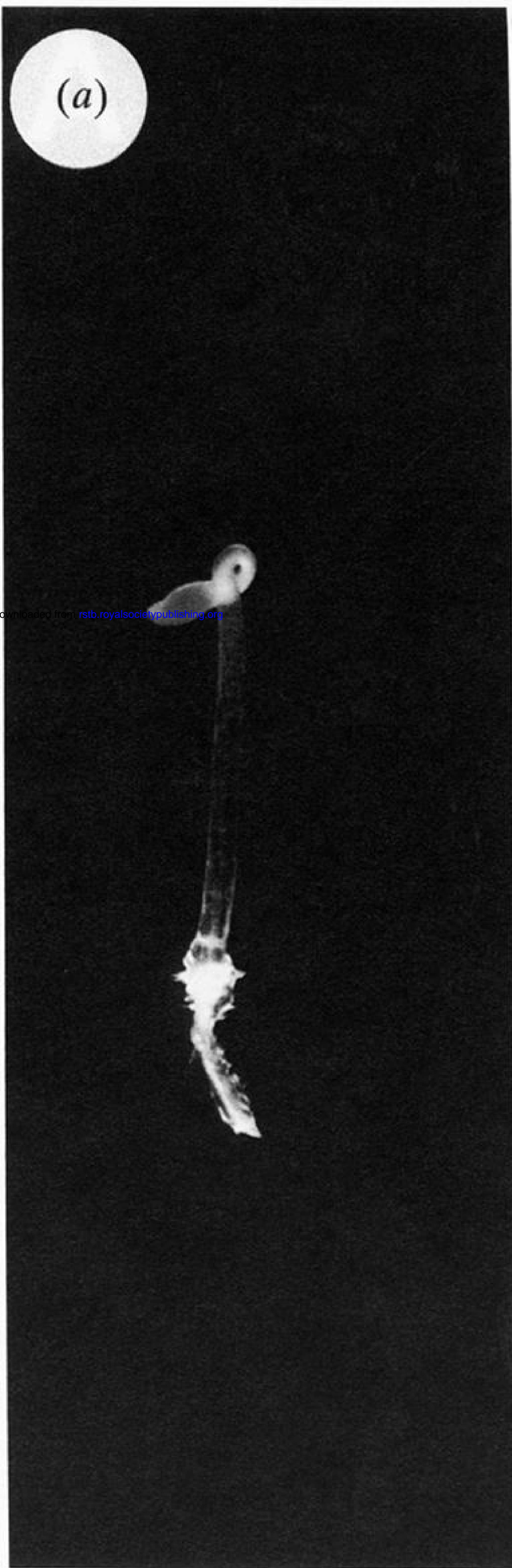

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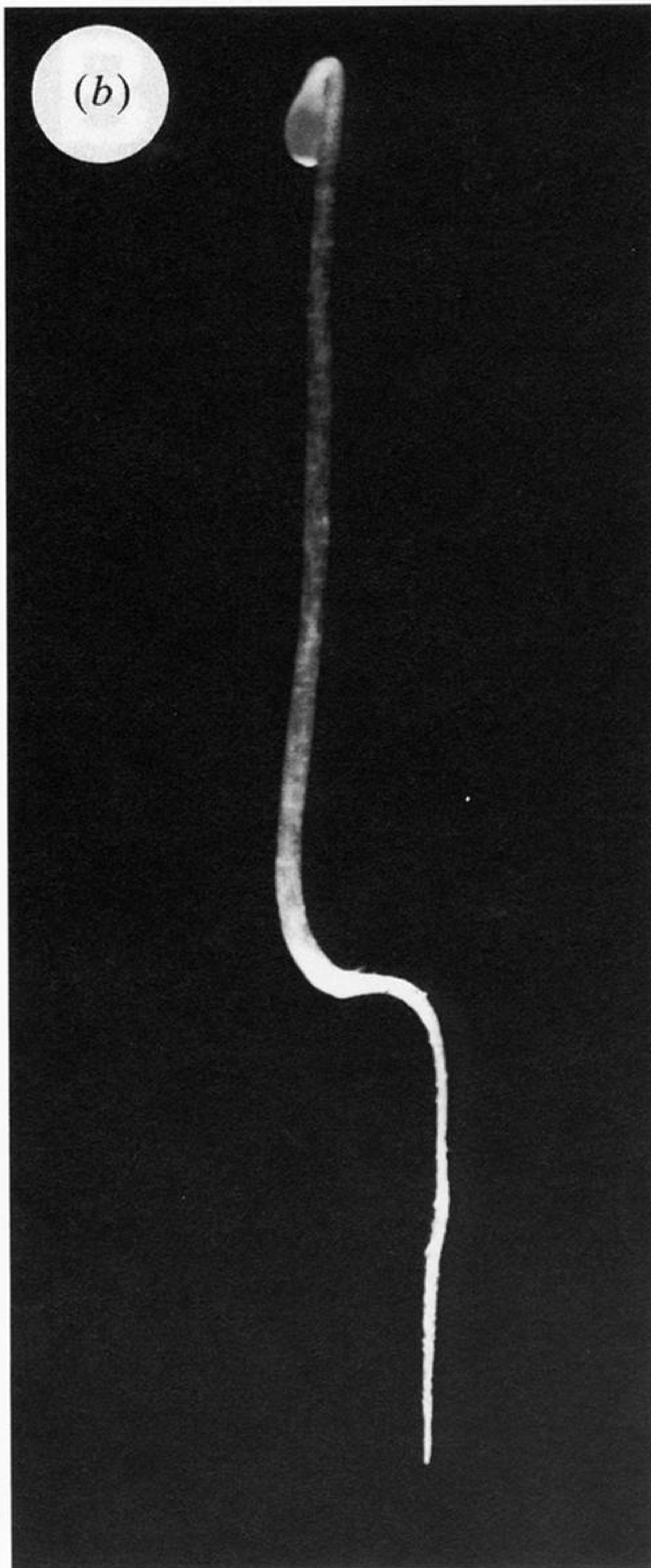

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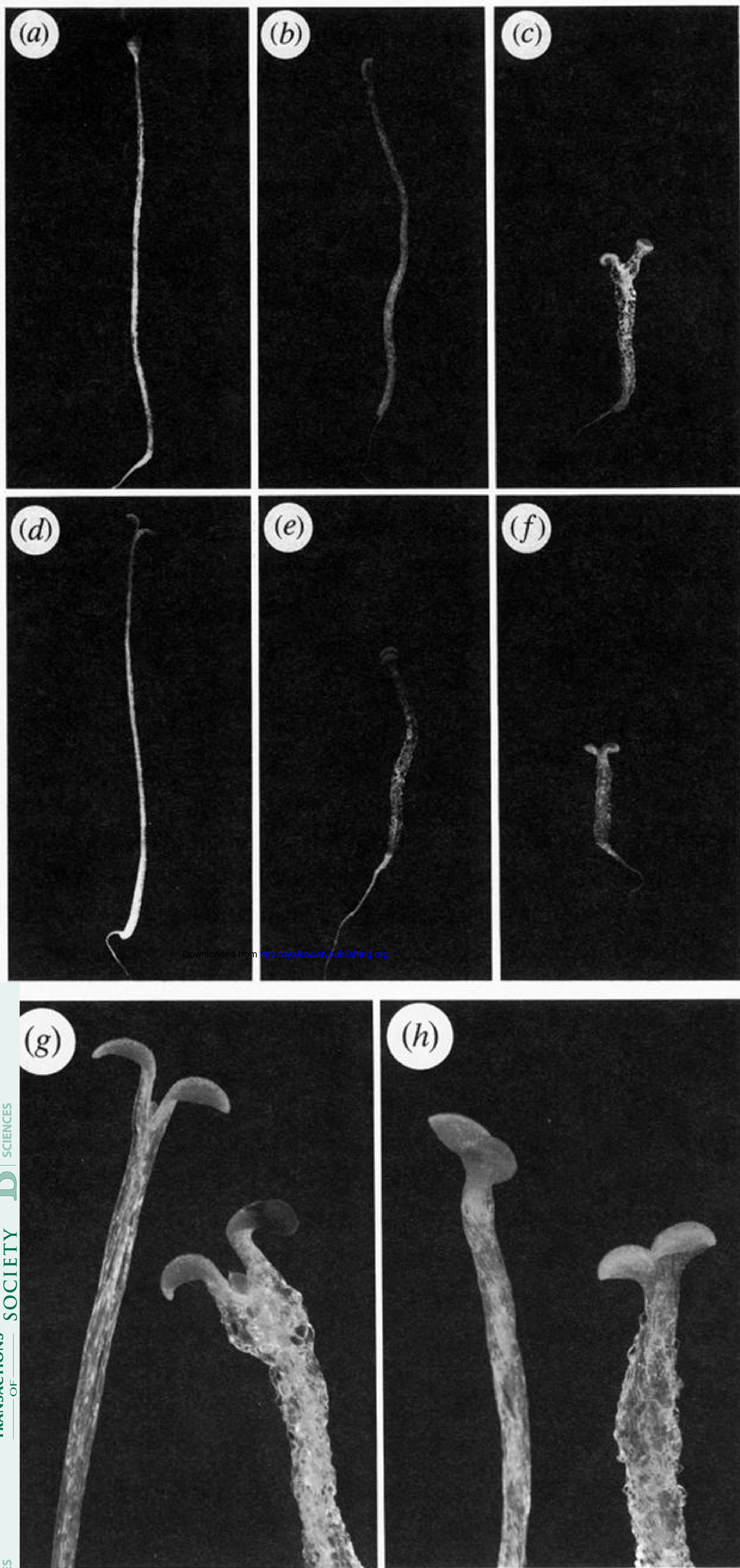
igure 1. Ethylene insensitivity phenotype of the *ein6* mutant. Seedlings were germinated and allowed to grow in the ark for three days in the presence of 10 μl l^{-1} ethylene. (*a*) Wild-type seedlings grown in ethylene develop the triple esponse phenotype. (*b*) The *ein6* mutant displays an ethylene insensitive (Ein^{-}) seedling phenotype; a thin and longated hypocotyl and root (seedling on the right).



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igure 3. Effect of taxol on cell expansion in the hypocotyl f wild-type and the *ein6* mutant seedlings. Wild-type andsberg seedlings were grown in the presence of either: (*a*) μM; (*b*) 0.1 μM; or (*c*) 1.0 μM taxol for 10 days in the dark. espresentative seedlings are shown. *ein6* seedlings were rown in the presence of either: (*d*) 0 μM; (*e*) 0.1 μM; or *f*) 1.0 μM taxol for 10 days in the dark. (*g*) Wild type eedlings grown in the absence (left) or presence (right) of μm taxol for 10 days. (*h*) Wildtype (left) or *ein6* mutant ight) seedlings grown in the presence of 0.1 μM of taxol for 0 days.