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Procuste1 mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings

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SUMMARY

Plant morphogenesis is dependent on a tight control of cell division and expansion. Cell elongation during post-embryonic hypocotyl growth is under the control of a light-regulated developmental switch. Light is generally believed to exert its effects on hypocotyl elongation through a phytochrome- and blue-light receptor- mediated inhibitory action on a so far unknown cell elongation mechanism.

We describe here a new class of allelic mutants in *Arabidopsis*, at the locus *PROCUSTE1 (prc1-1* to *-4)*, which have a hypocotyl elongation defect specifically associated with the dark-grown developmental program. Normal hypocotyl elongation is restored in plants grown in white, blue or red light. In agreement with this, the constitutive photomorphogenic mutation *cop1-6*, which induces a de-etiolated phenotype in the dark, is epistatic to *prc1-2* for the hypocotyl phenotype.

Epistasis analyses in red and blue light respectively, indicate that phytochrome B but not the blue light receptor HY4, is required for the switch from PRC1-dependent to PRC1-independent elongation.

The conditional hypocotyl growth defect is associated with a deformation of the hypocotyl surface due to an uncontrolled swelling of epidermal, cortical or endodermal cells, suggesting a defect in the structure of the expanding cell wall. A similar phenotype was observed in elongating roots, which was however, independent of the light conditions. The aerial part of mature mutant plants grown in the light was indistinguishable from the wild type.

***prc1* mutants provide a means of distinguishing, for the first time, two genetic pathways regulating hypocotyl cell elongation respectively in dark- and light-grown seedlings, whereby light not only inhibits hypocotyl growth, but also activates a *PRC1*-independent cell elongation program.**

Key words: phytochrome, cryptochrome, cell wall expansion, photomorphogenesis, skotomorphogenesis, blue light, root elongation

INTRODUCTION

Seedlings of higher plants can follow two developmental strategies depending on the surrounding light conditions. In the dark, dicotyledonous seedlings adopt a morphology and a physiology adapted to subterranean growth conditions (skotomorphogenesis): cotyledons do not expand, leaf and chloroplast development remain inhibited, whereas the hypocotyl, a stem connecting apical meristem and root in dicots, forms an apical hook and undergoes a rapid elongation. Upon exposure to light, an alternative developmental program, photomorphogenesis, is induced. Seedlings adopt a so called de-etiolated phenotype: the apical hook opens, the cotyledons expand, the chloroplasts and leaves develop, and hypocotyl growth is inhibited. The photomorphogenic pathway appears to be actively repressed in the absence of light, involving negative regulatory elements identified in *Arabidopsis* by recessive mutations such as *de-etiolated* (*det1*, Chory et al., 1989; *det2* Chory et al., 1991), and *constitutive photomorphogenic1* (*cop1*, Deng et al., 1991), *cop9* (Wei and Deng, 1992), and others (Wei et al., 1994).

Genetic and physiological data support the idea that light exerts an inhibitory action on hypocotyl growth through stimulation of phytochromes, a blue light receptor and one or more UV-B receptors (Kendrick and Kronenberg, 1994). Little is known about the molecular mechanism of cell elongation in the hypocotyl and its control by light. It is generally accepted that the cell wall is the key control point for the turgor-driven cell growth. A current model suggests that the cell wall is a highly organised and dynamic structure containing two main structural polysaccharide networks: a load-bearing cellulose/xyloglucan network, and a compression-resistant pectin network (Roberts, 1994). Cell wall expansion would be the result of the interplay between the synthesis of new components, their regulated incorporation into the existing architecture and the loosening of the structure. Hydrolases and xyloglucan endo-transglycosylases have been identified as potential wall loosening enzymes (Fry

et al., 1992; Nishitani and Tominaga, 1992; Fry, 1993). A third class of proteins, expansins, actually confer extensibility to isolated walls and seem to act on a matrix polymer tightly bound to the surface of cellulose microfibrils (McQueen-Mason et al. 1992; McQueen-Mason and Cosgrove, 1994, 1995). The role of these proteins in cell elongation in vivo still remains to be determined. Also, no information exists on the role of the biosynthesis of cell wall compounds in the regulation of cell elongation.

Genetic and physiological studies have also established a prominent role for hormones in hypocotyl elongation. Gibberellins and auxins act as stimulatory factors whereas ethylene, abcissic acid (ABA) and cytokinins have inhibitory effects (Davies, 1995). However, it is not clear at what level hormone and light signalling interact with the cell elongation mechanism to control hypocotyl growth.

As part of a research program aimed at elucidating the cell expansion process at a molecular level, we initiated a molecular-genetic study of hypocotyl elongation in *Arabidopsis thaliana*. In this species, the hypocotyl is a very simple structure, entirely generated during embryogenesis. All post-embryonic growth takes place in the absence of cell division. Hypocotyl cell elongation is extreme during dark-grown development: cells measuring approx. 10 μm in the embryo can reach a length of more than 1.0 mm (J. T. unpublished results).

In order to identify new mutants with defects specific to the 'downstream' components of the signal transduction network controlling hypocotyl cell elongation, we have screened for dark-grown seedlings showing a typical etiolated morphological phenotype (normal apical hook, unexpanded cotyledons, short root) but differing from the wild type (WT) by their failure to elongate the hypocotyl. Here we describe a new locus, *PROCUSTE1* (*PRC1*) which is essential for hypocotyl elongation in dark-grown seedlings. The growth defect of mutant hypocotyls was always associated with an uncontrolled swelling of hypocotyl cells, suggesting a role for *PRC1* in the correct assembly of the expanding cell wall. Surprisingly, the *prc1* hypocotyl growth defect was completely reversed by light. Epistasis analyses with *cop1* and the light-perception mutants *hy4* and *phyB-1*, demonstrated that *prc1* mutations uncover an alternative, phytochrome-dependent but HY4-independent pathway controlling hypocotyl cell elongation. These data indicate that light not only has an inhibitory effect on hypocotyl growth but also activates a PRC1-independent elongation program.

MATERIALS AND METHODS

Plant strains

Mutant Landsberg *erecta* lines *phyB-1* and *hy4* (2.23N) were described by Koornneef et al. (1980), *cop1-6* by McNellis et al. (1994). The γTIP-GUS transgene is a part of a T-DNA construction containing the kanamycin resistance marker (Km^R), nptII (Ludevid et al., 1992).

The *prc1* mutants were identified in a screen of individual M_2 families derived from ethyl methane sulfonate (EMS)-mutagenised seeds of the Columbia ecotype (Santoni et al., 1994). This strategy was preferred over the use of bulked M_2 seeds, in that it allows for the propagation of recessive lethal or sterile mutations through heterozygote sister plants. Among the 433 M_2 families screened, 94 albinos were found.

The *cop1-6*/*prc1-2* double mutants were picked out of an F_2 population and distinguished from *cop1-6* by their root phenotype.

Plant growth conditions

Plants were grown in vitro as described by Santoni et al. (1994) except that sucrose was omitted from the medium. Seeds were imbibed for at least 24 hours at 4°C and germination was induced by a 2-hour white light treatment (150 $\mu mol.m^{-2}.s^{-1}$). For dark conditions, plates were wrapped in four layers of aluminium foil. Day 0 of growth is defined as the time when plates were moved from 4°C to 20°C. For the observation of roots, seedlings were grown on vertically placed Petri dishes.

To prepare stock solutions, gibberellic acid GA_{4+7} was dissolved in distilled water and filter sterilised prior to storage. Naphthalene acetic acid (NAA) was dissolved in ethanol, and aminoethoxyvinylglycine (AVG) and 1-amino-cyclopropane-1-carboxylic acid (ACC) were dissolved in dimethyl sulfoxide (DMSO).

Light sources

The light sources were as follows: red: LEDs (NLS 01 n°9600, Nijssen) with a 20 nm half-band-width around 660 nm; blue: TLD 36 W/18 blue tubes (Philips) filtered through blue Plexiglas (blauw n° 627, Rohm and Haas) and with a 20 nm half-band-width around 460 nm; white light: equal number of True-Lite 65 W tubes (Duro-Lite International) and Osram L58 W/31 tubes. Dim white light was created by covering the Petri dishes with a layer of black plastic. The transmission spectrum of the plastic which selectively filters light of shorter wavelengths, can be provided upon request.

For the blue light fluence rate-response studies a threshold box unit was used as described by Peters (1992).

Hypocotyl length measurement

Seedlings (30-40) were spread out on an agar plate and magnified using a photographic enlarger. The projected image was traced with a pencil on a sheet of paper. The drawing obtained was scanned and lengths were calculated using a Visilog program (Orbovic and Kien, unpublished) which can be obtained upon request. The error bars in the figures represent the standard error of the mean (s.e.m.).

Genetic analysis

For the genetic crosses, flowers were emasculated with a fine forceps and immediately pollinated. All *prc1-1* mutants were out-crossed at least twice to the Columbia wild type. For all out-crosses we used the wild-type as the female parent.

Allelism tests were performed with a pollen donor line homozygote for the *prc1-1* mutation and the γTIP-GUS transgene. Resulting F_1 seeds were sown on a kanamycin-containing medium (50 $mg.l^{-1}$). The phenotype of seedlings was scored after 7 days of growth in darkness after which the kanamycin resistance was checked by incubating the plates in white light (150 $\mu mol.m^{-2}.s^{-1}$) for 2 weeks.

For mapping, *prc1-1* (Col0 background) was crossed with the wild-type L. *erecta*. Twenty eight seedlings homozygous for the *prc1-1* mutation were selected in the F_2 population. We determined the chromosomal location of the *prc1-1* mutation by searching linked Columbia-type alleles of cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993). The map position was further refined using 159 other *prc1-1*/*prc1-1* F_2 seedlings which were tested for the linked *CAPS LFY*. Ten seedlings carrying chromosomes recombinant between *LFY* and *prc1-1* were found. A 3-point linkage test with the microsatellite nga129 on these recombinants allowed the positioning of *prc1-1* distally from LFY (data not shown). Finally, *prc1-1* was mapped 1.7 cM distally from the m211 RFLP marker by RFLP analysis. The genetic distances were computed by the MAPMAKER program of Lander et al. (1987) applying the Kosambi mapping function.

Microscopy

For Scanning Electron Microscopy (SEM), seedlings were attached to the sample holder with a thin layer of clay. They were then rapidly frozen in undercooled liquid nitrogen and immediately transferred to

the vacuum chamber of the electron microscope. After sublimation of ice crystals on their surface, plantlets were coated with gold and examined directly.

For light microscopy, seedlings were fixed in 4% formaldehyde in 100 mM Pipes buffer, pH 6.9 and embedded in historesin (Leica, France) following the manufacturer's instructions. Sections, 5 μm thick, were cut on a Jung RM 2055 microtome, stained with 0.05% methylene blue and examined in a Nikon microphot FXA microscope.

RESULTS

Mutant isolation and genetic characterisation

prc1 mutants were identified in a screen for short hypocotyl mutants in darkness. Seedlings were grown for 7 days on sucrose-free agar medium in complete darkness. In these conditions, wild-type plants adopt a typical etiolated phenotype, i.e. an extremely long hypocotyl (up to 20 mm), a short root, and closed cotyledons which are folded over, forming an apical hook (Fig. 1). Among 433 M_2 families screened, we identified some 40 mutants with a normally etiolated phenotype but with a short hypocotyl. Four of these represented independent alleles of the same locus (Table 1), *PROCUSTE1* (*PRC1*), and mutant alleles *prc1-1* to *-4*. The wild-type phenotype of the back-crossed F_1 and the 3:1 (wild type:mutant) segregation ratio in the F_2 were consistent with a monogenic recessive mutation conferring the Prc1 phenotype (Table 2). We did not observe large allele-specific variations of the phenotype among these mutants (see below). PRC1 was mapped to the bottom of chromosome 5, 1.7 cM distal to the restriction fragment length polymorphism (RFLP) marker m211. The only known mutant that maps in this region which shows a short hypocotyl phenotype in the dark is *eto1* (Koornneef, 1994). However, physiological experiments described below, clearly distinguish the two mutant phenotypes. In addition, two known embryo lethal mutants (*emb15* and *emb16*), mapping around *yi* are known (Koornneef, 1994). The possibility that they represent strong *prc1* alleles was ruled out through complementation tests (data not shown).

Phenotype of *prc1-1* in darkness and saturating white light

The fully grown hypocotyl of dark-grown *prc1* seedlings was on average 5 times shorter than that of the wild type (Fig. 1). Hypocotyl growth of *prc1-1* seedlings reached a plateau after 7-8 days, a period comparable to that of wild-type seedlings, indicating that the shorter hypocotyl is not a result of a delayed growth or germination (Fig. 2). Using SEM we did not observe significant differences in the number of cells in epidermal cell files between *prc1-1* and wild-type dark-grown seedlings (data not shown). However, epidermal cells were significantly shorter in *prc1-1* (<0.25 mm) than in the wild type (longest cells >1 mm), grown in the same conditions (Fig. 3), demonstrating that the reduced *prc1-1* hypocotyl size resulted from a defect in cell elongation. Many epidermal cells had a very irregular shape, some being swollen and others compressed, giving the hypocotyl a highly irregular surface (Fig. 3). This deformation was most dramatic in the middle part of the hypocotyl in which the longest cells are located in the wild type. Cell shape alteration was not detected in the apical hook and cotyledons. Transverse sections made through the hypocotyl of 7-day old dark-grown *prc1-1* seedlings and observed under light microscopy indicated that its histological structure was not altered: the number of epidermal, cortical and endodermal cells was the same as in the WT (Fig. 3). Uncontrolled swelling of epidermal, cortical and endodermal cells seemed to be the cause of the irregularities observed at the surface of the *prc1* hypocotyl.

In high intensity white light (150 $\mu mol.m^{-2}.s^{-1}$) the hypocotyl of *prc1* was indistinguishable from that of wild-type seedlings: no reduction in size or differences in cell shape were observed (Fig. 1). Also, adult plants did not show any detectable aerial phenotype (Fig. 4). The deformed dark-grown

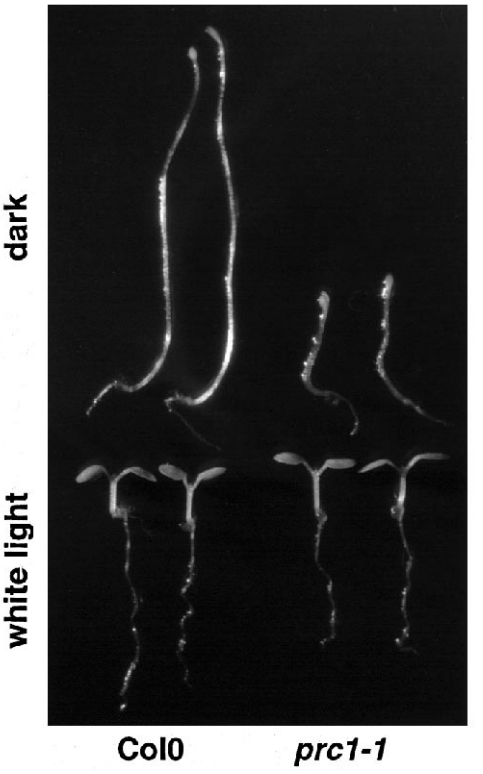

Fig. 1. Phenotype of dark or white light-grown WT (Col0) and *prc1-1* seedlings. Seedlings were grown for 7 days in complete darkness or under 16 hours of white light per day. The *prc1-1* hypocotyl had a growth defect only in darkness. Mutant roots were slightly shorter than WT both in light and in darkness. Two seedlings are shown for each condition.

Table 1. Complementation tests of *prc1* mutants

Cross	Generation	Total	WT, Kan^R	Mutant, Kan^R
prc1-2/prc1-2 × *prc1-1/prc1-1*, γTIP/γTIP	F_1	28	0	28
prc1-3/prc1-3 × *prc1-1/prc1-1*, γTIP/γTIP	F_1	18	0	18
prc1-4/prc1-4 × *prc1-1/prc1-1*, γTIP/γTIP	F_1	15	0	15

The *prc1-1* mutant line was marked with a γTIP-GUS (T-DNA-Km^R) construct to facilitate the verification of outcrossings.

Table 2. Segregation of *prc1* alleles

Cross	Generation	Total	WT	Mutant	χ^2†
WT × *prc1-1/prc1-1*	F_1	6	6	0	
	F_2	191	145	46	0.08*
WT × *prc1-2/prc1-2*	F_1	4	4	0	
	F_2	144	115	29	1.78*
WT × *prc1-3/prc1-3*	F_1	5	5	0	
	F_2	131	103	28	0.92*
WT × *prc1-4/prc1-4*	F_1	4	4	0	
	F_2	155	119	36	0.26*

*Not significant at P=0.05.
†The χ^2 value is given for the ratio of 3:1 (wild type/mutant).

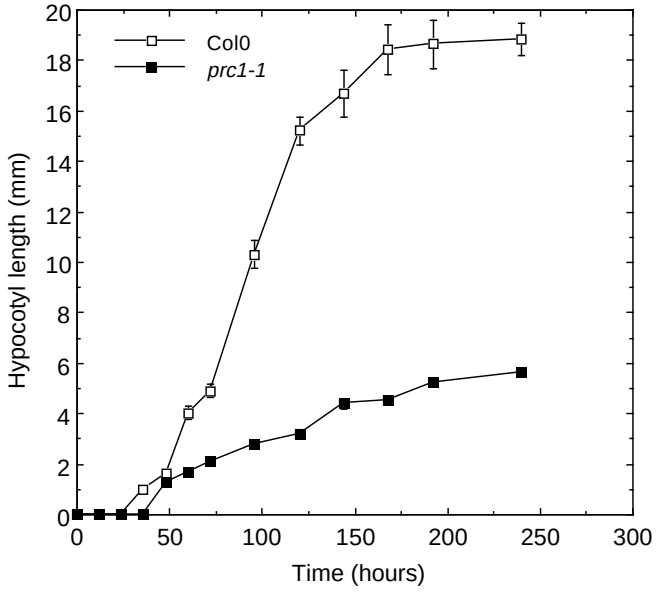

Fig. 2. Hypocotyl elongation kinetics of dark-grown WT and *prc1-1* seedlings. For each indicated time point, a different Petri dish containing WT and *prc1-1* seedlings was used.

hypocotyl remained functional: after transfer from dark to light, *prc1* seedlings underwent a normal photomorphogenesis and developed into normal looking plants.

The root morphology of the *prc1-1* mutant was altered both in dark- and light-grown seedlings (Fig. 5) as well as in adult plants (data not shown). Compared to the WT, roots of *prc1* seedlings were slightly shorter and many epidermal cells were swollen as observed in the dark-grown hypocotyl (Fig. 5). Swollen cells were not found in the tip zone of the root. Elongation of root hairs was not affected by *prc1* mutations and only their basal part was occasionally swollen (Fig. 5A), indicating that the PRC1 function is not required for tip growth.

Light microscopy of transverse sections revealed the highly irregular shape of epidermal, cortical and endodermal cells. Many cells appeared swollen, others, especially epidermal cells had collapsed, possibly as a result of the sample preparation procedure (Fig. 5B). This was never noticed in sections of WT roots.

Together, these microscopic observations strongly suggest that *prc1* mutations cause an increase in fragility of the cell wall in roots as well as in dark-grown hypocotyls.

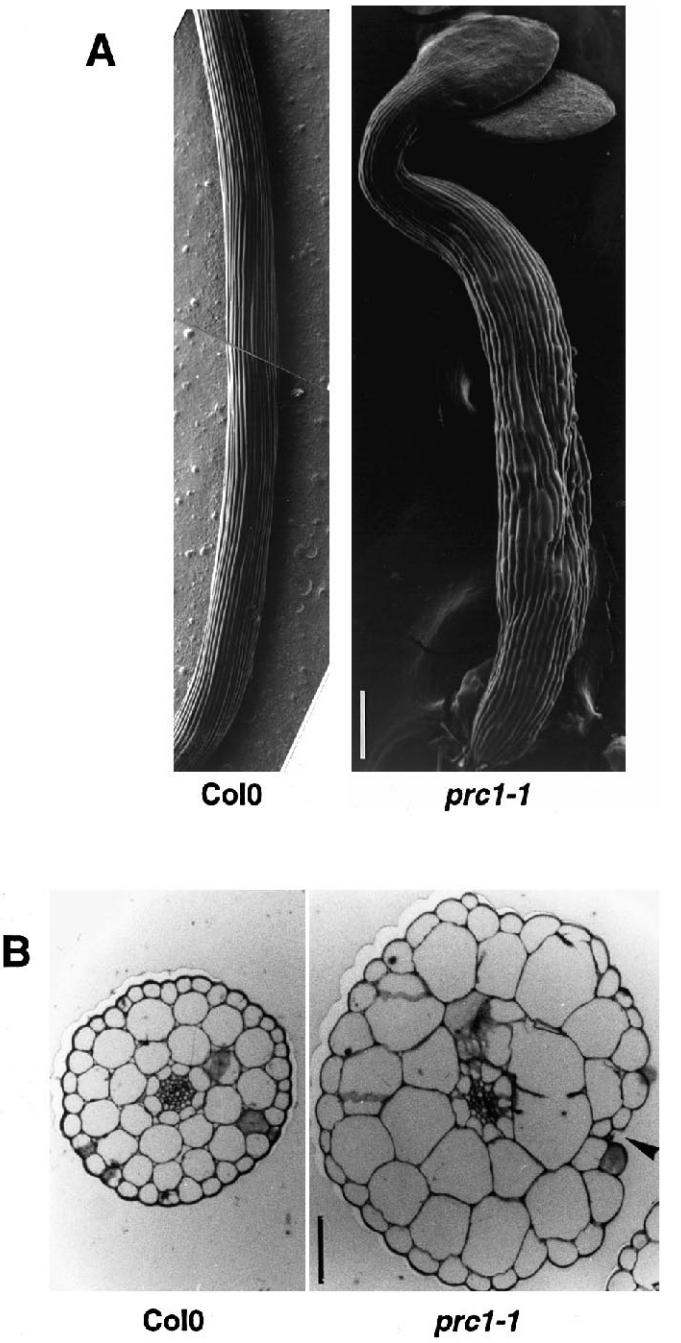

Fig. 3. Hypocotyl phenotype of dark-grown WT (Col0) and *prc1-1* seedlings. (A) Scanning electron micrograph of a 5-day old seedling. Note the irregular hypocotyl surface of *prc1-1*, especially in the middle zone of the hypocotyl. Scale bar, 250 μm. (B) Cross section of the hypocotyl of Col0 and *prc1-1* seedlings. Seedlings were grown for 7 days in darkness and cross sections were taken through the middle part of the hypocotyls. The arrowhead points to a collapsed part of the *prc1-1* hypocotyl. The diameter both of cortical and epidermal cells is irregular and larger than that of the WT. Scale bar, 70 μm.

The Prc1 phenotype is not reversed by gibberellic acid (GA) or the auxin naphtaleneacetic acid (NAA)

Gibberellins and auxins are known to promote elongation of plant aerial organs. In *Arabidopsis*, GA-deficient or -insensi-

Fig. 4. Aerial phenotype of 1-month old wild-type and *prc1-1* plants grown in the greenhouse. Scale bar, 5 cm.

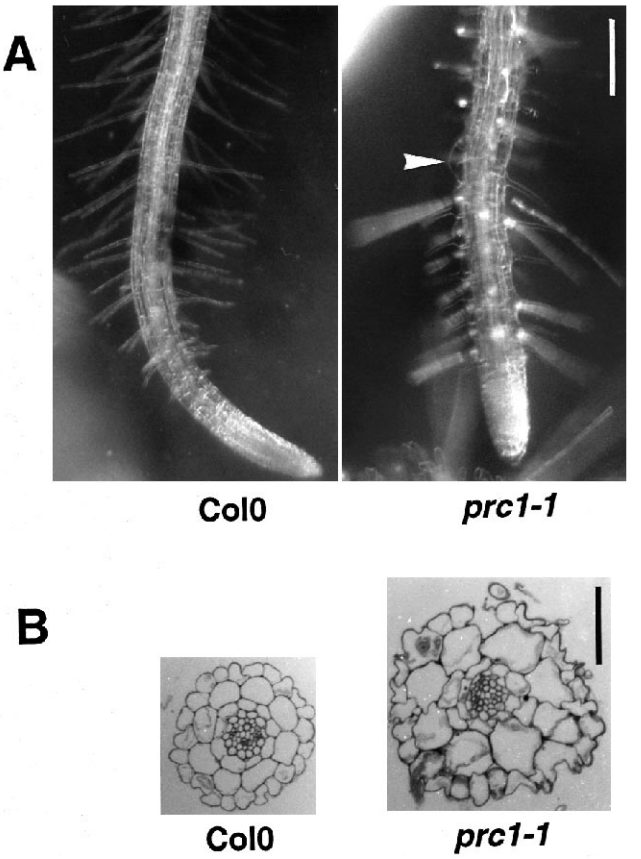

Fig. 5. Root phenotype of light-grown WT (Col0) and *prc1-1* seedlings. (A) Intact light-grown roots. Arrowhead points to a swollen epidermal cell. Scale bar, 260 μm. (B) Transverse sections through light-grown WT and mutant roots: mutant epidermal cells collapsed during the preparation of the sample; this was never observed for the WT. This phenotype is unaffected by light. Scale bar, 70 μm.

tive dwarf mutants (Finkelstein and Zeevaart, 1994) as well as auxin-resistant mutants have been described (Estelle and Klee, 1994). For both types of mutants the hypocotyl of dark-grown seedlings is shorter than that of the WT (Lincoln et al., 1990; T.D. and H.H., unpublished data).

Addition of GA_{4+7} (10^{-7} M) or the auxin analogue naphthalene acetic acid (NAA) (10^{-8} to 10^{-5} M) to the culture medium did not restore the WT phenotype to the *prc1-1* mutant. The Prc1 phenotype therefore is not a result of a simple auxin or GA deficiency. We also did not observe any resistance of *prc1-1* to inhibition of root elongation by high concentration of NAA, characteristic of auxin-resistant mutants (data not shown).

The Prc1 phenotype is not reversed by aminoethoxyvinylglycine (AVG) or by the *ein2-1* mutation

Ethylene is known for its triple response effect on dark-grown *Arabidopsis* seedlings: (1) inhibition of elongation of hypocotyls and roots, (2) increased radial expansion and (3) increase in the apical hook curvature (Ecker, 1995). Ethylene-overproducing (*eto*) and constitutive triple response (*ctr1*) mutants have been described and have indistinguishable dark-grown phenotypes (Ecker, 1995). *PRC1* does not correspond to *CTR1* based on the map position (*ctr1* maps to the top of chromosome 5; Koornneef, 1994). *ETO1*, on the other hand, is closely linked to *PRC1* (Koornneef, 1994). To investigate the possibility that the Prc1 phenotype is caused by ethylene overproduction, or a new mutation conferring a constitutive ethylene response specific for hypocotyl elongation, a double mutant *ein2-1/prc1-1* was constructed. *ein2-1* is an ethylene insensitive mutant and EIN2 acts downstream of CTR1 in the ethylene signal transduction pathway (Ecker, 1995). As expected, when grown in the dark on 10^{-4} M 1-amino-cyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, wild-type seedlings showed the triple response phenotype (Fig. 6), whereas *ein2-1* seedlings were completely insensitive. *prc1-1* also showed a triple response on ACC, including a reduction in hypocotyl length (Fig. 6) and an exaggerated apical hook (data not shown), demonstrating its ethylene sensitivity. The *ein2-1* mutation did not alleviate the *prc1-1* growth defect in the double mutant combination, indicating that the Prc1 phenotype is not caused by a simple ethylene overproduction, nor a constitutive ethylene response mutation upstream of *ein2-1*. Ethylene overproduction as a cause for the *prc1* growth defect was further ruled out by the failure to alleviate the *prc1-1* dark-grown phenotype with 10 μM AVG (aminoethoxyvinylglycine), an artificial inhibitor of the 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (data not shown). Note that *ein2-1/prc1-1* hypocotyls showed a slight increase in size compared to *prc1-1*, both in the absence and presence of ACC, again confirming the sensitivity of *prc1-1* for ethylene inhibition of hypocotyl growth. This might indicate that the *PRC1* and ethylene act via separate pathways on hypocotyl growth, assuming that the *prc1-1* mutation is not leaky.

The *prc1-1* hypocotyl is longer under dim white light than in darkness

As shown above, *prc1* seedlings appear normal in white light. In these conditions, hypocotyl growth is inhibited through the action of phytochrome as well as blue light and UV-B

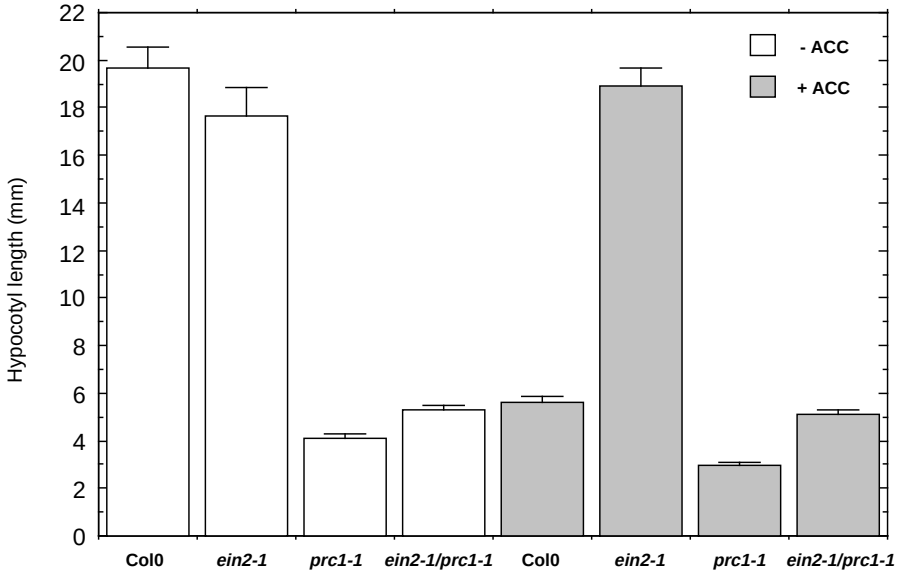

Fig. 6. Effect of ACC and the *ein2-1* mutation on the *prc1-1* hypocotyl elongation. Seeds were sown on a medium with or without 10^{-4} M ACC and seedlings were grown in the dark for 7 days.

receptors (Koornneef et al., 1980; Kendrick and Kronenberg, 1994). Reducing the light intensity relieves the growth inhibition in WT plants (Kendrick and Kronenberg, 1994). To investigate the effect of the *prc1* mutation on the increased hypocotyl growth observed in these conditions, we compared the hypocotyl length for WT and *prc1-1* seedlings after 7 days of growth in dim white light enriched for longer wave lengths, in the dark or in saturating white light. As expected, the hypocotyl length of WT seedlings increased in dim light and reached a maximum in the dark. Surprisingly, for *prc1-1*, hypocotyl growth was also promoted in dim light, however to a lesser extent than WT seedlings (Fig. 7).

These results show that the hypocotyl elongation defect in *prc1-1* is conditional: in the presence of light, the hypocotyl at least partially retains the ability to elongate.

Growth promotion of the *prc1-1* hypocotyl also occurs in dim blue or red light

To test whether the increased elongation of the *prc1-1* hypocotyl observed in dim white light involves light of specific wavelengths, we first established fluence rate-response curves of the hypocotyl length for blue light (Fig. 8). As shown previously (Liscum et al., 1992), blue light inhibited elongation of the WT hypocotyl, following a simple sigmoid dose-response curve. For *prc1-1*, however, the hypocotyl length increased with increasing fluence rates, reached a peak at 1 $\mu mol.m^{-2}.s^{-1}$ but dropped and followed exactly the WT curve for higher fluence rates. A similar growth promoting effect was observed under dim red light (20 $\mu mol.m^{-2}.s^{-1}$; see below in Fig. 11).

Therefore, elongation of the *prc1-1* hypocotyl can be stimulated both in dim blue and red light. Since blue light also can stimulate phytochrome (Kendrick and Kronenberg, 1994), we cannot conclude from these data whether phytochrome, a blue light receptor or both are responsible for the growth promoting effect.

Abnormal cell shape in the *prc1-1* hypocotyl is correlated with the extent of the growth defect

The surface of the hypocotyl of WT and *prc1-1* plants grown in different blue light intensities was studied using SEM (Fig. 8). At higher fluence rates no irregularities were observed at the hypocotyl surface as long as the hypocotyl length of *prc1-1* remained indistinguishable from the WT (Fig. 8C,H and 8D,I). At very low fluence rates, as in darkness, the *prc1-1* hypocotyl was very short and highly deformed (Fig. 8A,E). At 1.28 $\mu mol.m^{-2}.s^{-1}$, the *prc1-1* hypocotyl was only slightly shorter than WT. Under these conditions, we occasionally observed a bulging cell among other normal looking epidermal cells (Fig. 8B, 8G). This abrupt cell swelling of individual cells rather than a coordinated gradual isodiametric growth of all hypocotyl cells suggests the involvement of a rupture of the cell wall and not an alteration in the control of the direction of cell expansion.

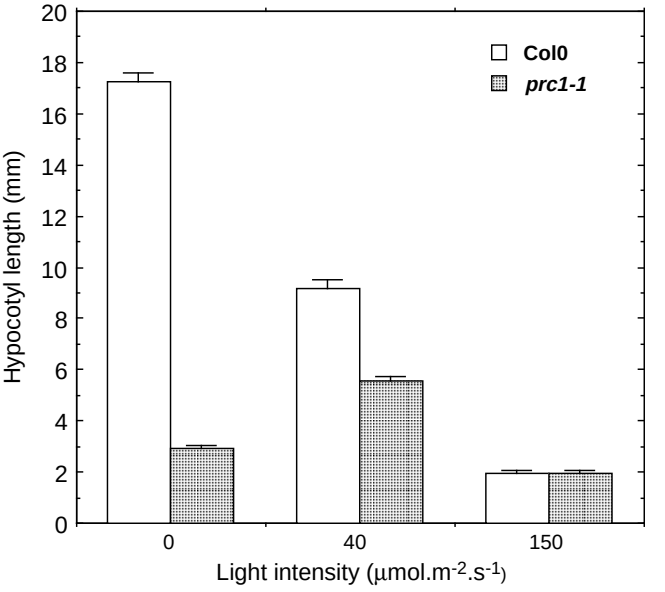

Fig. 7. Dim white light stimulates hypocotyl elongation of *prc1* seedlings. Dim white light (40 $\mu mol.m^{-2}.s^{-1}$) was obtained by covering the Petri dish with one layer of semi-transparent black plastic. Light conditions were the same as for Fig. 1.

The Prc1 hypocotyl phenotype is not strictly dependent on the growth rate

A number of conditional root cell expansion mutants have been described (Baskin et al., 1992; Hauser et al., 1995). In these plants, the mutant root phenotype (increased lateral cell expansion) is only observed in conditions stimulating root growth (high concentration of sucrose or high temperature). With this in mind, the elongation defect of *prc1* seedlings in darkness and low fluence rate light also might simply reflect a growth rate dependency on PRC1 in WT plants. To test this hypothesis, we grew 4 different *prc1* allelic mutants in the dark at 5 different temperatures to study the effect of the reduction of the hypocotyl growth rate on the Prc1 phenotype (Fig. 9). The length of both WT and mutant seedlings decreased with decreasing temperature. We did not observe any increase in hypocotyl length of the mutant at lower temperatures, something that could be expected if the Prc1 phenotype was strictly dependent on the growth rate. It is also noteworthy that we did not observe large allele-specific variations in length at the different temperatures.

Similar results were obtained when the hypocotyl growth rate in the dark was diminished by reducing the endogenous GA concentration with a *ga1* mutation introgressed in the *prc1-1* mutant (data not shown).

These results demonstrate the absolute requirement of PRC1 for hypocotyl growth in the dark, independently from the actual growth rate.

The *constitutive photomorphogenic* mutant *cop1-6* is epistatic to *prc1-2* for the hypocotyl phenotype

The results reported above show that the hypocotyl growth defect of *prc1-1* can be suppressed in dim red or blue light. The de-etiolation response induced under these light conditions is under the control of a negative regulatory element identified by the loss-of-function mutation *constitutive photomorphogenic1* (*cop1*) (Deng et al., 1991). Seedlings homozygous for a leaky mutant allele at this locus, e.g. *cop1-6* (McNellis et al., 1994) display a short hypocotyl and fully expanded cotyledons even in complete darkness.

To investigate whether the suppression of the *prc1* growth defect in light is strictly dependent on the presence of light or is part of the photomorphogenic developmental program, we constructed a double mutant *cop1-6/prc1-2* and studied its phenotype after 7 days of growth in the dark. *cop1-6/prc1-2*

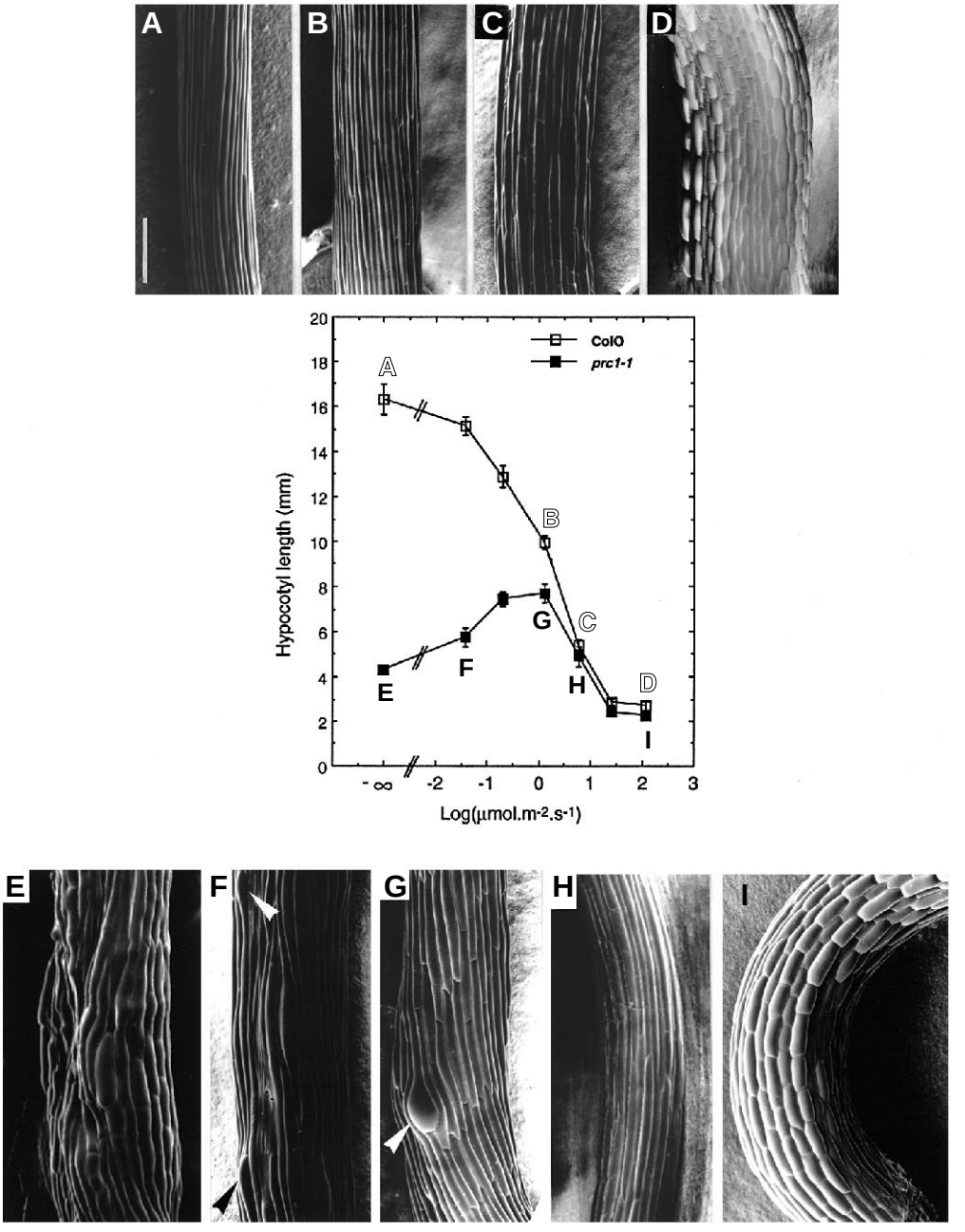

Fig. 8. Hypocotyl length and SEM of WT and *prc1-1* seedlings grown for 5 days under different fluence rates of continuous blue light. Letters on the curves refer to conditions for which scanning electron micrographs were performed. (A-D) Micrographs of hypocotyls of wild-type (Col0) seedlings grown in light intensities of 0, 1.28, 5.79 and 117.39 µmol.m^{-2}.s^{-1}, respectively. (E-I) Micrographs of hypocotyls of *prc1-1* seedlings grown in light intensities of 0, 0.04, 1.28, 5.79 and 117.39µmol.m^{-2}.s^{-1}, respectively. Scanning electron micrographs show the middle region of a hypocotyl. Arrowheads point to some of the abnormally shaped epidermal cells. Scale bar (A-I), 165 µm.

seedlings were fully de-etiolated with a hypocotyl indistinguishable from *cop1-6*; no additive effects were observed between the two mutations (Fig. 10A,B). Moreover, no deformation of the surface could be detected. In contrast, the light-independent root phenotype of *prc1-2* was still visible in the double mutant. The same result was observed with the *prc1-1* allele (data not shown). The aerial part of light-grown *cop1-6/prc1-2* double mutants was also indistinguishable from the *cop1-6* single mutant both for seedlings and adult plants.

In conclusion, the derepression of the photomorphogenic program in the dark by the *cop1-6* mutation entirely suppressed the prc1-2 hypocotyl phenotype.

This result also rules out the requirement for photosynthesis in the observed restoration of normal elongation in light, which we were able to confirm using the photobleaching herbicide norfluorazon, or a *prc1-1/albino* double mutant (data not shown).

Interactions of *prc1* with photoreceptor mutants

The experiments described above strongly suggest that the PRC1 gene product is required for hypocotyl elongation specifically in darkness. Derepression of the photomorphogenic program either by light or by the mutation *cop1-6*, suppresses the requirement for PRC1. Using double mutants, we next investigated the involvement of phytochrome B and the putative blue light receptor HY4 in the transition from PRC1-dependent to PRC1-independent hypocotyl elongation.

phyB-1

The results of Fig. 11A,B show that 20 μmol.m^{-2}.s^{-1} of red light suffice to de-etiolate wild-type seedlings: cotyledons are expanded and green, and hypocotyl elongation is inhibited. As previously described (Quail et al., 1995), PHYB is required for this de-etiolation, as indicated by the reduced cotyledon expansion and the long hypocotyl phenotype of *phyB-1* seedlings under these conditions.

De-etiolation with red light also reverted the *prc1-1* hypocotyl growth defect. Double mutant *phyB-1/prc1-1* seedlings were indistinguishable from *prc1-1* seedlings in the dark. The growth of *phyB-1/prc1-1* hypocotyls was stimulated in red light, however they never reached the length of *phyB-1* hypocotyls and remained slightly deformed (data not shown). This result shows that the incomplete de-etiolated phenotype caused by the absence of PHYB, was also reflected in an incomplete reversion of the *prc1*-associated growth defect in the double mutant. In conclusion, PHYB is not only required for the red light-induced inhibition of hypocotyl growth, but paradoxically, also activates the transition from PRC1-dependent to PRC1-independent elongation. *phyB-1* is a null allele (Reed et al., 1993) suggesting that other members of the phytochrome family also may play a role in this transition.

hy4

Fig. 11C,D shows that 28 μmol.m^{-2}.s^{-1} of blue light completely de-etiolated wild-type seedlings, with a stronger hypocotyl inhibition than that observed in 20 μmol.m^{-2}.s^{-1} of red light (Fig. 11A), confirming previous results (Young et al., 1992). This de-etiolation is partially mediated by the blue light receptor HY4 (or CRY, Ahmad and Cashmore, 1993; Lin et al., 1995) as judged from the long hypocotyl (about 80% of the length reached in darkness) and the partially expanded cotyledons of the *hy4* mutant. *prc1-1* mutants were indistinguishable from wild type under these blue light conditions, showing that blue light can revert the *prc1-1* hypocotyl growth defect, which also confirms the results of Fig. 8.

In the dark, the phenotype of the double mutant *hy4*/*prc1-1* was identical to *prc1-1*. Surprisingly, in blue light, the aerial part of *hy4*/*prc1-1* seedlings was indistinguishable from *hy4*, including the absence of any deformation of the hypocotyl surface.

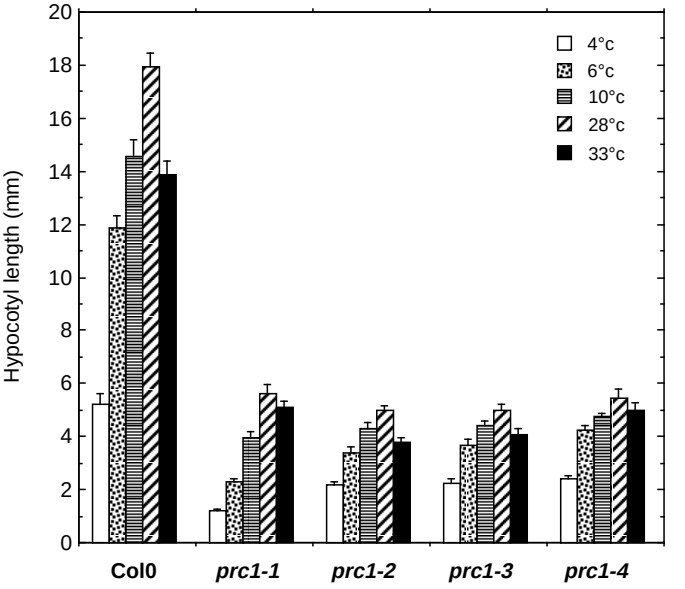

Fig. 9. Influence of temperature on the hypocotyl length of dark-grown WT and *prc1* mutants. Seedlings were grown continuously for 7 days at the indicated temperature.

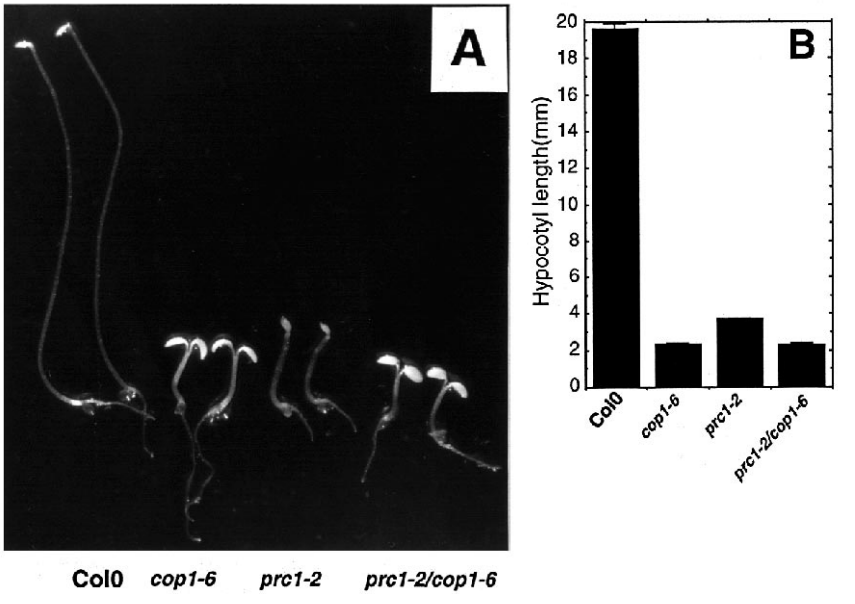

Fig. 10. Influence of the *cop1-6* mutation on the hypocotyl phenotype of *prc1-2* in darkness. (A) Phenotype and (B) hypocotyl length of seedlings.

In conclusion, HY4 is required for complete cotyledon expansion and hypocotyl growth inhibition in high fluence blue light, however the blue light receptor does not mediate the blue light-induction of the PRC1-independent elongation program. These results also demonstrate that the complete restoration of the *prc1*-associated growth defect can occur even in conditions under which the elongation rate is comparable to that of dark-grown wild-type seedlings.

DISCUSSION

PRC1 is required for hypocotyl elongation in dark-grown seedlings

Our results demonstrate that PRC1 is essential for cell elongation in the hypocotyl of seedlings grown in the dark, and in the root regardless of the light conditions. The Prc1 phenotype is not the result of a deficiency in either GA or auxin synthesis, nor of increased ethylene production. The growth defect is not diminished in conditions that slow down the growth rate as observed for a class of root elongation mutants (Hauser et al., 1995).

Evidence for a cell wall defect in dark-grown *prc1* seedlings

The elongation defect is always associated with a deformation of the hypocotyl surface resulting from an uncontrolled swelling of epidermal, cortical or endodermal cells. The deformation is most pronounced in physiological conditions that provoke the largest hypocotyl growth defects in the mutants and was not observed in cells above the growing zone of the hypocotyl. In conditions in which the *prc1* hypocotyl has a very limited growth defect, individual bulging cells could be observed among normal looking epidermal cells, suggesting the involvement of an all-or-nothing response, reminiscent of a rupture of the cell wall rather than a coordinated reorientation of the axis of expansion. These observations suggest that the *prc1* mutations cause a structural deregulation in the primary cell wall of growing cells. Interestingly, sublethal concentrations of the cellulose biosynthesis inhibitor, DCB (2,6-dichlorobenzonitrile; Delmer, 1987) cause a short and deformed hypocotyl phenotype in the WT, reminiscent of *prc1* mutants (data not shown). Also, a mutant (*mur1*) in which L-fucose reaches less than 2% of the WT level, and showing as a result, altered cell wall mechanical properties (Reiter et al., 1993), showed a deformed, short hypocotyl phenotype in dark-grown conditions (H.H., unpublished observations).

Other mutants with specific cell elongation defects in roots and/or aerial parts have been described previously (Baskin et al., 1992; Hauser et al., 1995; Feldmann et al., 1989, Bowman, 1994). The cloning of two loci, *SABRE* and *DIMINUTO-DWARF1*, has thus far failed to provide any clues as to the nature of the gene products involved (Aeschbacher et al., 1995; Takahashi et al., 1995). Interestingly, at least one conditional abnormal root-expansion mutant class, *rsw*, is partially defective for the incorporation of radiolabelled glucose into the cellulosic wall fraction (Baskin et al., 1995).

The biochemical and cytological analysis of the cell wall of

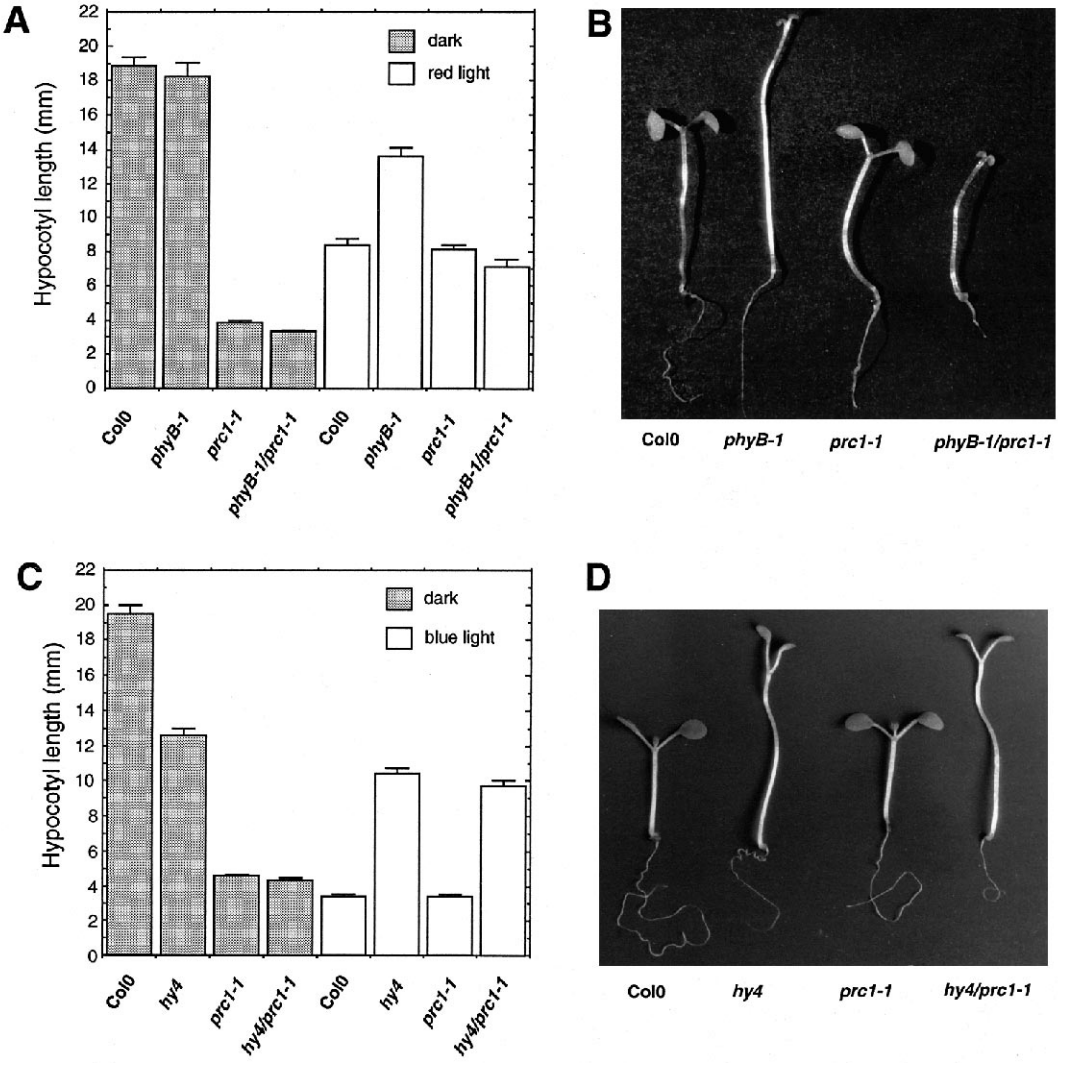

Fig. 11. Influence of the *phyB-1* or *hy4* mutation on the hypocotyl growth of *prc1-1* under red light or blue light, respectively. (A) Hypocotyl length of dark- or red-light-grown seedlings. (B) Phenotype of the red-light-grown seedlings. In A and B red light was 20 μmol.m^{-2}.s^{-1}. (C) Hypocotyl length of dark or blue light grown seedlings. (D) Phenotype of the blue-light-grown seedlings. In C and D blue light was 28 μmol.m^{-2}.s^{-1}.

etiolated *prc1* hypocotyls will demonstrate whether the growth defect of these mutants is also associated with an alteration in the structure of the expanding cell wall.

prc1 mutants uncover a second phytochrome-dependent genetic pathway controlling hypocotyl elongation in light-grown seedlings

The *prc1*-associated hypocotyl growth defect is reversed in a fluence rate-dependent fashion by white, blue or red light. In addition, we did not observe any growth defects in the light-grown adult plant. The restoration of normal hypocotyl growth in *prc1* mutants is not strictly dependent on light, but on the derepression of the photomorphogenic developmental program as judged by the absence of the *prc1*-associated hypocotyl growth defect in a *cop1-6 /prc1* double mutant, even in complete darkness.

A simple model explaining the conditional phenotype of *prc1* is shown in Fig. 12. We postulate that light not only has an inhibitory effect on cell elongation in the hypocotyl, but paradoxically also activates a function or a complex of functions, referred to as 'X', participating in the cell elongation process and allowing elongation without the involvement of PRC1. The PRC1 function itself is either constitutively expressed or may disappear in light. Based on this interpretation, we can distinguish two pathways controlling cell elongation in the hypocotyl: a PRC1-dependent pathway, during skotomorphogenesis, and a PRC1-independent pathway, during photomorphogenesis. These 2 elongation programs are not mutually exclusive and both can be activated under dim light.

What could be the function of X? PRC1 and X might represent the same class of proteins, encoded by two members of a gene family, but with a different light-dependent regulation. Alternatively X might represent a more complex physiological change associated with the light-induced developmental transition. For instance, if PRC1 has a function in the expansion of the cell wall, it is conceivable that light induces changes in the structure of the cell wall in such a way that expansion can occur without the involvement of PRC1. In accordance with this idea, Morvan et al. (1991) showed for flax seedlings that light can induce important changes in the polysaccharide composition of hypocotyl cell walls.

These two different elongation strategies might reflect an adaptation to two different environments: a subterranean environment for dark-grown seedlings and an aerial environment in the light, presumably imposing different constraints on the growth of the seedling. For instance, etiolated hypocotyls need to grow fast, consuming a minimum of food reserves, elongate even under high mechanical stress, but may require less resistance to bending stress and desiccation. In these conditions cell elongation in the hypocotyl may have characteristics in common with that of the root, which is consistent with the light-independent root phenotype of *prc1* mutants. In contrast, hypocotyls growing above the soil presumably require more resistance to lateral stress and desiccation. A detailed comparison of the physico-chemical and biochemical characteristics of the hypocotyls of etiolated and de-etiolated seedlings may provide more insight into potential adaptations to these different environments and their relationship to the PRC1 function.

The results with the *phyB-1/prc1-1* and *hy4/prc1-1* double mutants showed that the induction of the *PRC1*-independent hypocotyl elongation program involves at least PHYB but not the blue light receptor HY4. However, the current data do not exclude the potential involvement of other phytochromes (PHYA, PHY?) and/or blue light receptors other than HY4.

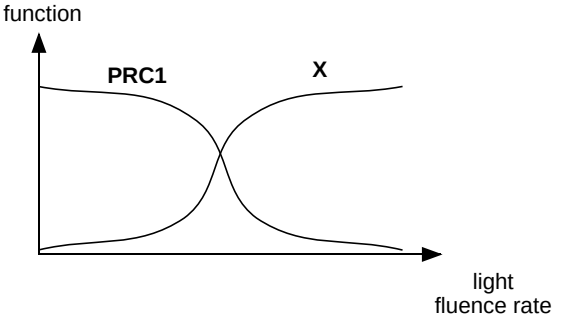

Fig. 12. Diagram to explain the conditional hypocotyl growth defect of the *prc1* mutant. The ordinate axis represents the requirement for PRC1 or hypothetical function 'X' for hypocotyl elongation. In darkness, PRC1 is required for hypocotyl growth. In increasing light intensities, a new function, 'X', is expressed which renders PRC1 dispensable for elongation. Our results do not rule out the possibility that in light-grown seedlings PRC1 remains active and carries out a function overlapping with that of X. The function X may be a PRC1-like activity with a contrasting light regulation or it may represent a developmentally regulated change in the hypocotyl cell elongation mechanism.

In conclusion, de-etiolation involves, besides the inhibition of hypocotyl growth, the activation of an alternative, PRC1-independent elongation program. Both processes are at least partially mediated by phytochrome and COP1. In contrast, HY4 mediates growth inhibition by blue light, but is not involved in the activation of the PRC1-independent elongation program.

The *PRC1* locus has been fine mapped and a contig of YACs (yeast artificial chromosomes) covering the locus has been constructed (T. D., unpublished data). The cloning of this gene will pave the way for the molecular-genetic analysis of the mechanism of cell expansion and its control by light.

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