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An Arabidopsis Brassinosteroid-Dependent Mutant Is Blocked in Cell Elongation

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Cell elongation is a developmental process that is regulated by light and phytohormones and is of critical importance for plant growth. Mutants defective in their response to light and various hormones are often dwarfs. The dwarfed phenotype results because of a failure in normal cell elongation. Little is known, however, about the basis of dwarfism as a common element in these diverse signaling pathways and the nature of the cellular functions responsible for cell elongation. Here, we describe an Arabidopsis mutant, *dwarf4* (*dwf4*), whose phenotype can be rescued with exogenously supplied brassinolide. *dwf4* mutants display features of light-regulatory mutants, but the dwarfed phenotype is entirely and specifically brassinosteroid dependent; no other hormone can rescue *dwf4* to a wild-type phenotype. Therefore, an intact brassinosteroid system is an absolute requirement for cell elongation.

INTRODUCTION

Plant growth is accomplished by orderly cell division and tightly regulated cell expansion. In plants, the contribution of cell expansion to growth is of much greater significance than in most other organisms; all plant organs owe their final size to a period of significant cell elongation, which usually follows active cell division. In Arabidopsis, cell elongation is largely responsible for hypocotyl growth in germinating seedlings and extension of inflorescences (bolting) at the end of vegetative growth. Coordinate control of plant growth is regulated by both external stimuli and internal mechanisms. Of the external signals, the most obvious is light (Deng, 1994). Light inhibits hypocotyl elongation and promotes cotyledon expansion and leaf development in seedlings, and photoperiod is crucial for flower initiation in a large number of species.

The internal components of plant signaling are generally mediated by chemical growth regulators (phytohormones; reviewed in Klee and Estelle, 1991). Gibberellic acid (GA) and cytokinins promote flowering; in addition, GA stimulates stem elongation, whereas cytokinins have the opposite effect, reducing apical dominance by stimulating increased axillary shoot formation. Conversely, auxins promote apical dominance and stimulate elongation by a process postulated to require acidification of the cell wall by a K^+-dependent H^+-pumping ATPase (Rayle and Cleland, 1977). A less well understood class of plant growth substances is the brassinosteroids (BRs; Mitchell et al., 1970; Grove et al., 1979; Mandava, 1988). Although several such compounds have been identified and are known to effect elongation of cells in various plant tissues, their biosynthesis, regulation, and mechanism of action have only recently begun to be elucidated (reviewed in Clouse, 1996; Fujioka and Sakurai, 1997).

In Arabidopsis, a number of mutations have been identified that affect either light-dependent or hormone signaling pathways and whose pleiotropic phenotypes include defects in cell elongation. The majority of these mutants also have other alterations in their phenotypes. The range of phenotypes in the *deetiolated* (*det*) and *constitutive photomorphogenic* (*cop*) light-regulatory mutants is broad. Mutations in *DET1*, *COP1*, *COP8*, *COP9*, *COP10*, and *COP11* result in constitutive derepression of substantial portions of the photomorphogenic program (Chory et al., 1989b; Deng and Quail, 1992; Wei and Deng, 1992; Wei et al., 1994), whereas mutations in *COP4* seem to affect only morphology and gene expression (Hou et al., 1993). The only invariant phenotype in this class of light-regulatory mutants is a substantial reduction in height in both light and darkness.

A number of hormone mutations also lead to reduced plant height. At least five GA mutants have been described as being reduced in stature (Koornneef and Van der Veen, 1980). GA biosynthetic mutants may also have no or defective flower development and are marked by an absence of viable pollen. Reduced levels of endogenous gibberellins are also a characteristic (Barendse et al., 1986; Talon et al., 1990), and their phenotype can be nearly restored to that of

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the wild type by the addition of exogenous GA. Another hormone mutation, *auxin resistant2* (*axr2*), results in plants with a dwarf phenotype both in the light and in darkness as well as increased resistance to high levels of auxin, ethylene, and abscisic acid (Timpte et al., 1992). An interesting relationship exists between light regulation and cytokinin levels. Arabidopsis seedlings grown in the dark in the presence of cytokinins have open cotyledons, initiate chloroplast differentiation and leaf development, and activate transcription from the chlorophyll *a*/*b* binding protein gene (*CAB*) promoter. Importantly, they also display a cytokinin dose-dependent dwarf phenotype. Moreover, *det1* and *det2* have a decreased requirement for cytokinins in tissue culture and appear to be saturated for a cytokinin-dependent delay in senescence (Chory et al., 1994).

The most recently discovered class of plant growth substances, the BRs, has been to date the least studied; however, rapid progress toward understanding BR biosynthesis and regulation is now being made (Yokota, 1997). Three Arabidopsis mutants with a dwarf phenotype, *dwarf1* (*dwf1*; Kauschmann et al., 1996; B.P. Dilkes and K.A. Feldmann, unpublished data), *constitutive photomorphogenesis and dwarfism* (*cpd*; Szekeres et al., 1996), and *det2* (Li et al., 1996), have been shown to be defective in steroid biosynthesis. In addition, BRs have been shown to cause elongation in a variety of mutants with a dwarfed phenotype (Szekeres et al., 1996). Interestingly, many of these mutants were previously identified as being defective in light-mediated (*cop*, *det*, and *fusca* [*fus*; another group of mutants with some members perturbed in light-regulated growth]) or hormone-mediated (*axr2*) regulation. Evidently, many of the signaling pathways regulated by light and phytohormones result in defective cell elongation.

Currently, little is known about the downstream events that occur in response to these signals and thereby directly control cell size. This is because the biochemical and cell biological processes involved have thus far been difficult to address. In addition, there is little information about the integration of regulatory signals converging at the cell from different signaling pathways and the ways they are coordinately controlled. In particular, the interaction of light and hormones in the control of cell elongation is not clear. Additional mutants in each of these pathways may help to further unravel these interactions.

In this work, we describe *dwf4*, an Arabidopsis mutant in which the major defect of the mutation is a block in cell elongation. This mutant also shares some characteristics of light-grown plants when grown in the dark; we present evidence suggesting that this latter phenotype is a consequence of defective cell elongation. Like *det2* and *cpd*, *dwf4* is rescued to a wild-type phenotype by exogenous brassinolide (BL). Because the defect in cell elongation blocks both the response to darkness and the effects of exogenously supplied GA and auxin, our results suggest that steroid signaling is necessary for normal plant growth and development.

RESULTS

Isolation of *dwf4*

As formally defined, a plant with a dwarf phenotype is one that has a short, robust stem and short, dark green leaves. *dwf1* is the first dwarf isolated via T-DNA mutagenesis (Feldmann and Marks, 1987) and has been described in detail (Feldmann et al., 1989; referred to as *diminuto* in Takahashi et al. [1995] and Szekeres et al. [1996]). In a screen of 14,000 transformed families (T_3), a number of additional dwarfs were identified. Two independent lines were found that segregated for a similar phenotype: both were shorter than *dwf1*, but their rosette diameter was comparable to that mutant. These dwarfs were also essentially infertile. The most striking aspect of the morphology of these mutants is their similarity to *det2* (Chory et al., 1991). For this reason, further analysis was conducted with these lines. After being found to be allelic to each other, both were designated as *dwf4.* The *dwf4* mutation was subsequently shown to be inherited as a monogenic, recessive Mendelian trait that, in *dwf4-1*, cosegregates with the dominant kanamycin resistance marker contained in the T-DNA (data not shown), suggesting that the mutation in this line may be a disrupted, tagged allele. *dwf4*-2 also contains a single kanamycin resistance marker, but it failed to cosegregate with the dwarf phenotype (data not shown). Two additional alleles (*dwf4-3* and *dwf4-4*) were identified among dwarf mutants obtained from the Nottingham Arabidopsis Resource Centre (Nottingham, UK; N365 and N374, respectively; Figure 1). Unless otherwise indicated, all experiments presented below were performed with *dwf4-1.*

The *dwf4* Phenotype

As shown in Figure 1, *dwf4* mutants are significantly smaller than the wild type and are dark green in color. They have short, rounded leaves. Again, the *dwf4* phenotype is reminiscent of the light-regulatory mutant *det2* (Chory et al., 1991); however, complementation analysis has shown that the two mutations are not allelic, with the *dwf4* mutation mapping to the lower arm of chromosome 3 and *det2* mapping to chromosome 2 (Chory et al., 1991). The results presented in Table 1 show that soil-grown *dwf4* plants attained a height of $<$3 cm at 5 weeks, whereas wild-type plants grew to $>$25 cm. Moreover, individual organs, such as leaves, were invariably shorter in dwarf plants. *dwf4* siliques were also markedly shorter than those of the wild type and were infertile. The loss of fertility of *dwf4* was due to the reduced length of the stamen filaments relative to the gynoecium, which resulted in mature pollen deposition on the ovary wall rather than on the stigmatic surface. Hand pollination of *dwf4* flowers with either mutant or wild-type pollen resulted in good seed set without significantly changing the size of the siliques.

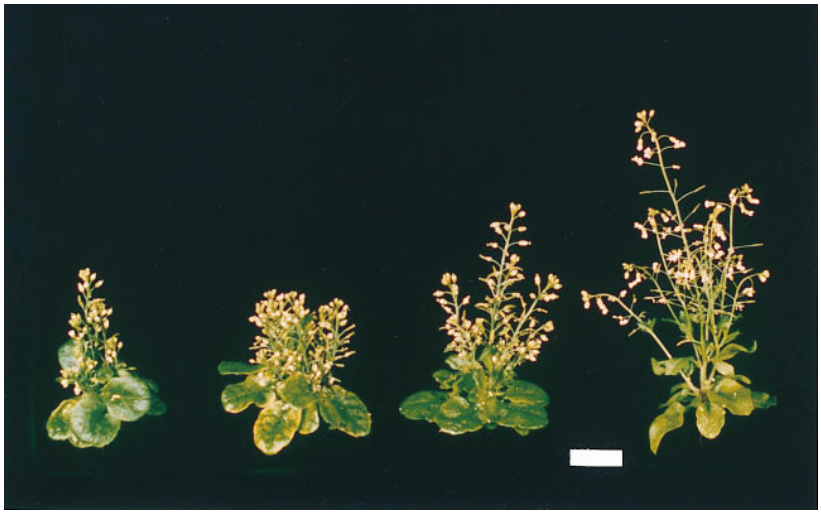

Figure 1. Morphological Comparison of *dwf4* Alleles at 5 Weeks after Germination.

The wild type is >25 cm tall at this age (data not shown). *dwf4*-1 (left) and *dwf4-2* (second from left) are in the Wassilewskija background; *dwf4-3* (second from right) and *dwf4-4* (right) are in the Enkheim background. Bar = 1 cm.

Another feature of *dwf4* plants is a reduction in apical dominance, as is evident by the threefold increase in the number of inflorescences at 5 weeks of age (Table 1). Mutants also have twice the number of rosette leaves, which may be explained by a prolonged vegetative phase in the *dwf4* plants. Development of flowers on the primary inflorescence is delayed by ~4 days in *dwf4*, but the flowering phase is significantly longer in the mutant, with senescence of the last flower occurring at ~98 days compared with ~57 days for the wild type. One result of this delay in senescence is that *dwf4* plants contain almost three times the number of siliques as does the wild type (Table 1).

Table 1. The Development of Wild-Type and *dwf4-1* Plants

Measurements	Wild Type[a]	*dwf4-1*[a]
Five weeks		
Height	25.8 ± 2.6 cm	2.8 ± 0.3 cm
Leaf blade length[b]	1.72 ± 0.36 cm	0.96 ± 0.15 cm
Leaf blade width[b]	0.77 ± 0.10 cm	0.99 ± 0.18 cm
No. inflorescences	3.6 ± 0.5	10.5 ± 1.4
No. rosette leaves	7.1 ± 0.9	13.5 ± 1.3
Other		
Start of flowering	21.5 days	25.9 days
Mature silique length	1.16 ± 0.07 cm	0.29 ± 0 cm
No. seeds per silique	37.7 ± 3.3	0.0
Final no. of siliques	336.5 ± 90.6	988.4 ± 214.2
Height at maturity	27.0 ± 2.7 cm	11.6 ± 1.0 cm

[a] Results shown are the average ±SD of measurements taken from 10 plants.

[b] Measurements taken from the second pair of leaves.

The reduced stature observed in soil-grown *dwf4* was also observed in hypocotyls of agar-grown seedlings. Measurements of hypocotyl length over time, shown in Figure 2, indicated that not only are *dwf4* seedlings shorter than wild-type seedlings immediately after germination but also that the rate of growth was retarded in the mutants. In addition, *dwf4* hypocotyls reached their terminal length in <5 days, whereas wild-type seedlings continued to grow.

The Growth Defect of *dwf4* Is Due to a Reduction in Cell Length

Both the short stature and the reduced growth rate of *dwf4* could be due to a defect in cell division or cell elongation or both. To distinguish between these possibilities, we analyzed sections from 7-day-old hypocotyls and 5-week-old inflorescence stems, by light microscopy, as described in Methods. To minimize variations due to the developmental stage of the sample, we always took the stem sections from the fourth internode. Figures 3C and 3D show that the average cell size in *dwf4* is significantly smaller than in wild-type plants (Figures 3A and 3B), whereas no differences were detected in the number of cells along the length of either organ between the wild type and *dwf4* (data not shown). Therefore, the short stature and reduced organ length of *dwf4* are largely or exclusively due to a failure of individual cells to elongate. No differences were observed in the number of cell layers contained in the wild type and *dwf4.*

The small size of *dwf4* cells offers a possible explanation for the dark green color of the mutant plants. Chlorophyll measurements were taken, and leaf mesophyll protoplasts

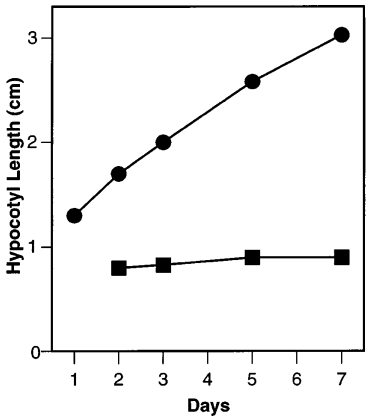

Figure 2. Comparison of Wild-Type and *dwf4* Hypocotyl Growth Rates.

Circles indicate wild type; squares indicate *dwf4.* Each data point represents the average of 10 seedlings.

were prepared, stained, and measured to visualize and count chloroplasts, as described in Methods. Although there were no significant differences in total chlorophyll content, the chlorophyll *a*/*b* ratio, or the absorption spectra between wild-type plants and mutants (data not shown), the mean plane area (the apparent two-dimensional surface area of mounted cells) of *dwf4* leaf mesophyll protoplasts was 376 mm^2, whereas that of wild-type protoplasts was 599 mm^2. The two-dimensional comparison of plane area represents a dramatic reduction in volume for *dwf4* cells. However, the number of chloroplasts per cell was only slightly lower: the mean number of chloroplasts per cell was 40 for *dwf4* and 44 for the wild type. Therefore, *dwf4* cells contain a greatly increased number of chloroplasts per unit cell volume. As a consequence, the chloroplasts are brought closer to each other, making the color of the leaves appear darker. Chloroplast size was the same in both lines (data not shown).

dwf4 Is Specifically Rescued by BRs

The reduced length of cells in *dwf4* hypocotyls and inflorescence stems is indicative of a failure of these cells to elongate during development. A variety of endogenous and environmental signals is responsible for stimulating elongation in plants; therefore, a series of experiments was performed to determine whether *dwf4* is affected in a specific signaling pathway or is blocked in elongation as a response to various signals.

Of the endogenous (hormonal) signals that might be deficient in dwarf plants, an obvious candidate is GA, because gibberellin-deficient mutants are shorter in stature than are the wild-type plants (Koornneef and Van der Veen, 1980). Our results, however, indicate that *dwf4* is not defective in the synthesis of gibberellins. When germinated on 10^{-5} M GA, wild-type seedlings demonstrated an elongation response (Figure 4), whereas *dwf4* seedlings responded minimally, if at all. At 10^{-4} M GA, wild-type seedlings elongated slightly more than at 10^{-5} M (data not shown), but the *dwf4* seedlings were essentially saturated for elongation at 10^{-5} M GA. Similar results were obtained when soil-grown plants were sprayed with 1 mM GA once inflorescences first became visible: *dwf4* inflorescence stems elongated by only 28% above the untreated controls, whereas those of the wild type elongated by 45% above the untreated controls (data not shown). Mutants that owe their reduced stature to decreased levels of endogenous gibberellins can be fully rescued by added hormone (Koornneef and Van der Veen, 1980; Talon et al., 1990). In addition, *dwf4* seeds germinate in the absence of exogenously supplied GA. Our results therefore suggest that *dwf4* is not deficient in endogenous GA. A corollary conclusion from this experiment is the demonstration that *dwf4* is capable of detecting GA; that is, it is not likely to be affected in signal perception but rather is defective in the extent to which it can respond to this signal.

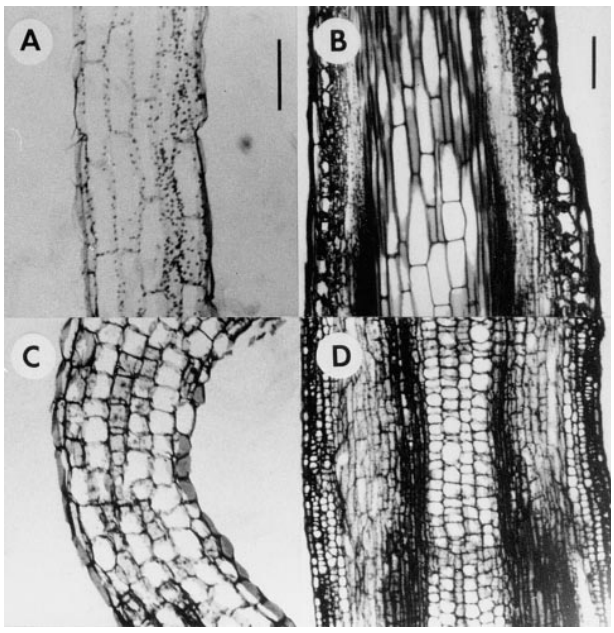

Figure 3. Longitudinal Sections of Wild-Type and *dwf4* Tissues.

(A) Hypocotyl from a 7-day-old wild-type plant. Average cell length is 92.7 μm.
(B) Stem from a 5-week-old wild-type plant. Average cell length is 79.2 μm.
(C) Hypocotyl from a 7-day-old *dwf4* plant. Average cell length is 32.2 μm.
(D) Stem from a 5-week-old *dwf4* plant. Average cell length is 15.8 μm.
(A) and **(C)** are at the same magnification; **(B)** and **(D)** are at the same magnification. Bars in **(A)** and **(B)** = 100 μm.

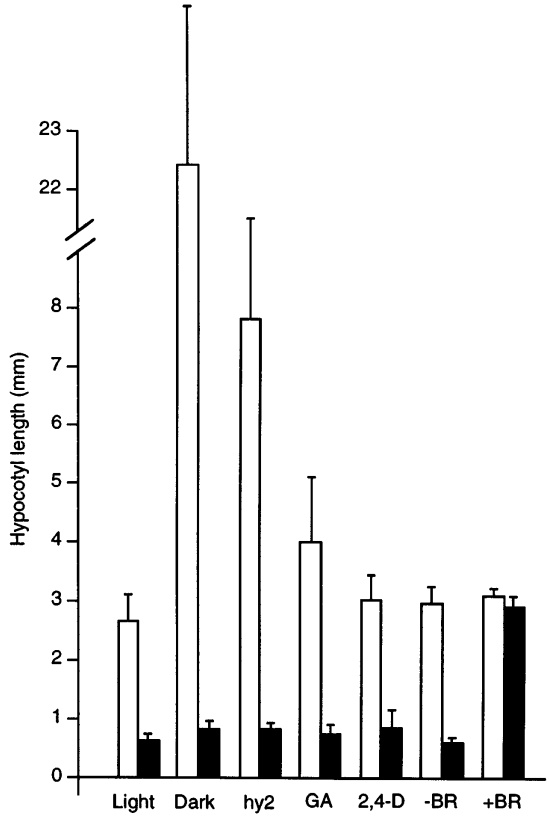

Figure 4. Response to Cell Elongation Signals.

All measurements were performed as described in Methods. BL measurements were performed with *dwf4-3* and the corresponding wild-type control, Enkheim. Open bars indicate the wild type; filled bars indicate *dwf4.* Lines above the bars represent one standard deviation. Light, light-grown control; Dark, dark-grown control; hy2, *DWF4* and *dwf4* plants in a *hy2* background; GA, 10^{-5} M GA; 2,4-D, 10^{-8} M 2,4-D; −BR, liquid-grown controls; +BR, liquid-grown controls with 10^{-6} M BL.

Auxin can also stimulate cell elongation. This effect is especially visible in young seedlings (Klee and Estelle, 1991). The response of wild-type and *dwf4* plants to auxin was tested by growing seedlings for 10 days on vertically oriented plates containing various concentrations of the synthetic auxin 2,4-D. At all concentrations assayed, inhibition of root growth was evident (data not shown). Figure 4 shows that at 10^{-8} M 2,4-D, hypocotyl elongation in wild-type and *dwf4* seedlings was similar to that of the controls. Higher concentrations of auxin were inhibitory for both wild-type and *dwf4* seedlings, and lower concentrations had no effect (data not shown). In view of the inhibition of root growth, it is clear that *dwf4* is not auxin resistant (data not shown); rather, its elongation response is compromised.

As mentioned above, the most obvious exogenous signal for plants is light. Therefore, to investigate whether light-regulated cell elongation is altered in *dwf4*, wild-type and *dwf4* seedlings were grown in the dark, as described in Methods. Figure 4 shows that as expected, wild-type seedlings displayed hypocotyl elongation typical of etiolated growth. By contrast, dark-grown *dwf4* seedlings were only slightly longer than those grown in the light. To assess the relationship between the *dwf4* phenotype and light sensing by *dwf4*, the mutation was crossed into a mutant defective in the *HY2* gene. All *hy* mutants share the common phenotype of an elongated hypocotyl that mimics part of the etiolation response in the light. Specifically, *hy2* is deficient in active phytochrome because chromophore biosynthesis does not take place (Chory et al., 1989a). Figure 4 shows that *dwf4 hy2* double mutants displayed a dwarfed phenotype indistinguishable from that of *dwf4 HY2* (light-grown control); therefore, the elongation block due to the *dwf4* mutation is epistatic to a defect in phytochrome activity.

In the course of our studies, we prepared a genomic library from *dwf4-1*, from which we isolated a clone in which a fragment of T-DNA interrupts a gene encoding a putative cytochrome P450 steroid hydroxylase (data not shown). Because BRs have been shown to elicit elongation in Arabidopsis (Clouse et al., 1993) and because BR-deficient mutants have been recently described (Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996), we tested the effect of BL on Arabidopsis seedlings by germinating seeds in liquid medium containing different amounts of BL. As shown in Figure 4, the *dwf4* hypocotyls were restored to wild-type height by 10^{-6} M BL. This, together with our identification of a disrupted gene encoding a putative BR biosynthetic enzyme, strongly suggests that the phenotype of *dwf4* is specifically due to a defect in BR biosynthesis (see Choe et al., 1998).

The Elongation Defect of *dwf4* Leads to a Light-Regulatory Phenotype

The BR-deficient mutant *det2* was originally identified as defective in regulation by light (Chory et al., 1991). Given the similarity of *det2* and *dwf4* phenotypes and functions and in view of the observation that *dwf4* is epistatic to *hy2*, one can predict that the etiolation response, which includes significant hypocotyl elongation, would not be normal in *dwf4.* To assess to what extent the etiolation response is affected by BR-dependent cell elongation, we grew *dwf4* and wild-type plants on agar under continuous light or in complete darkness, as described in Methods. Figure 5A shows that after 7 days of growth in the light, wild-type seedlings displayed open and expanded cotyledons as well as emerging leaf buds. In contrast, the overall appearance of light-grown *dwf4* seedlings (Figure 5B) is strikingly similar to that of *det2* (Chory et al., 1991). *dwf4* hypocotyls are very short, and the cotyledons are smaller than those of the wild type, displaying

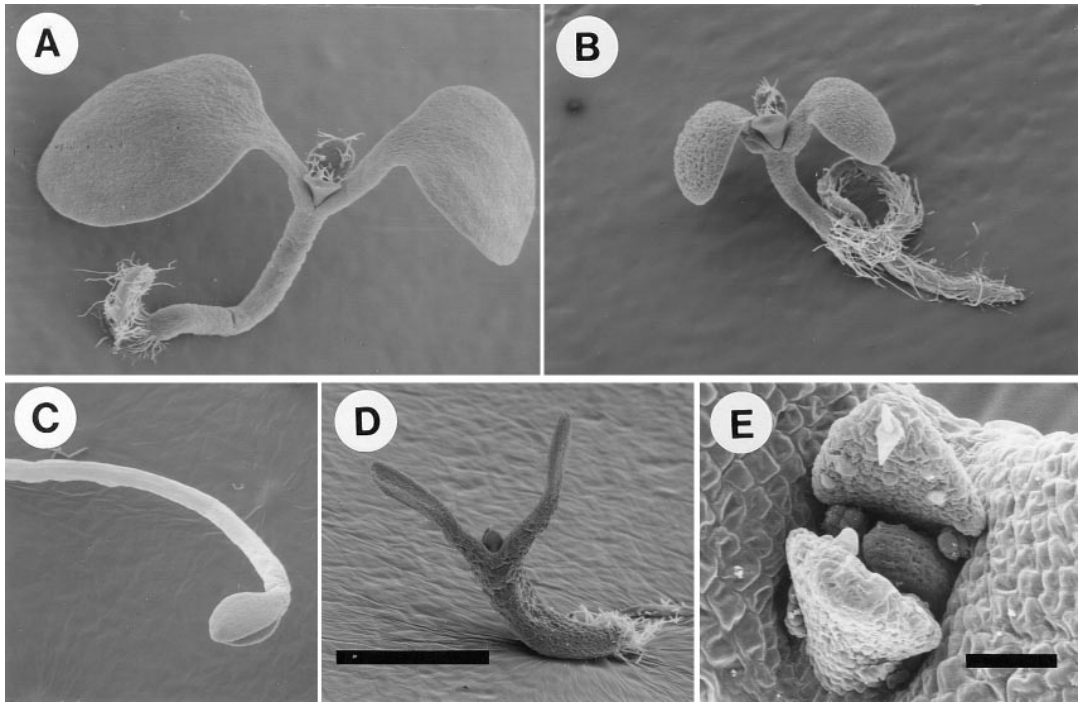

Figure 5. Aberrant Skotomorphogenesis.

(A) Seven-day-old light-grown wild-type plant.
(B) Seven-day-old light-grown *dwf4* plant.
(C) Seven-day-old dark-grown wild-type plant.
(D) Seven-day-old dark-grown *dwf4* plant.
(E) Shoot apex of 7-day-old dark-grown *dwf4* plant. *dwf4* develops true leaves in the dark, as shown by the presence of emerging trichomes.
Bar in **(D)** = 1 mm for **(A)** to **(D)**; bar in **(E)** = 100 μm.

significant epinastic growth. As expected, dark-grown wild-type seedlings have a typical etiolated appearance, with a highly elongated hypocotyl and closed, unexpanded cotyledons (Figure 5C). However, *dwf4* hypocotyls failed to elongate (Figure 5D). That the *dwf4* mutation can abolish the elongation component of the etiolation response is in agreement with the notion that the block in cell elongation in *dwf4* is specifically a BR-dependent process.

In addition to short hypocotyls, dark-grown *dwf4* seedlings displayed partially open cotyledons and leaf primordia, with up to four leaf buds clearly visible (Figures 5D and 5E). This has not been observed with the wild type, although it occurs with certain light-regulatory mutants (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992). *dwf4* leaf development can continue in the darkness for several weeks, resulting in significant expansion of rosette leaves (data not shown). These results indicate that *dwf4* plants can initiate what is normally a photomorphogenic pathway in the absence of light. Although this is often diagnostic of a light-regulatory mutant, wild-type Arabidopsis can perform leaf development and even flowering in complete darkness when grown in liquid culture (Araki and Komeda, 1993).

The cause for this dark-flowering effect is not understood; therefore, the possibility exists that leaf development in dark-grown *dwf4* is related to dark flowering and not to a light-regulatory defect. For example, perhaps the proximity of the *dwf4* shoot apical meristem to the surface of the agar, due to the shortness of the hypocotyls, mimics some effect of submerged culture, such as a high water potential or a high concentration of some nutrient. To test this possibility, wild-type seedlings were grown in complete darkness for 6 weeks in vertically oriented dishes to maximize contact between the seedling and the medium. Wild-type seedlings grown in this fashion displayed open cotyledons and underwent at least partial leaf development (data not shown). In fact, all wild-type seedlings grown along the surface of the agar showed development of an inflorescence with at least one cauline leaf and a terminal flower bud. We conclude, therefore, that the appearance of leaves in dark-grown *dwf4* may be due simply to its short size and the culture conditions.

A number of light-regulatory mutants have been described that undergo photomorphogenesis in the dark at the cellular level. In mutants such as *cop1*, *cop8*, *cop9*, *cop10*, and *cop11*, stomata undergo photomorphogenic maturation (Deng and Quail, 1992; Wei and Deng, 1992; Wei et al., 1994); of these, *cop1* and *cop9* as well as *det1* (Chory et al., 1989b) also initiate differentiation of plastids into chloroplasts. To determine whether *dwf4* plants undergo photomorphogenic cellular differentiation in the dark, we analyzed cotyledons from light- and dark-grown plants by transmission

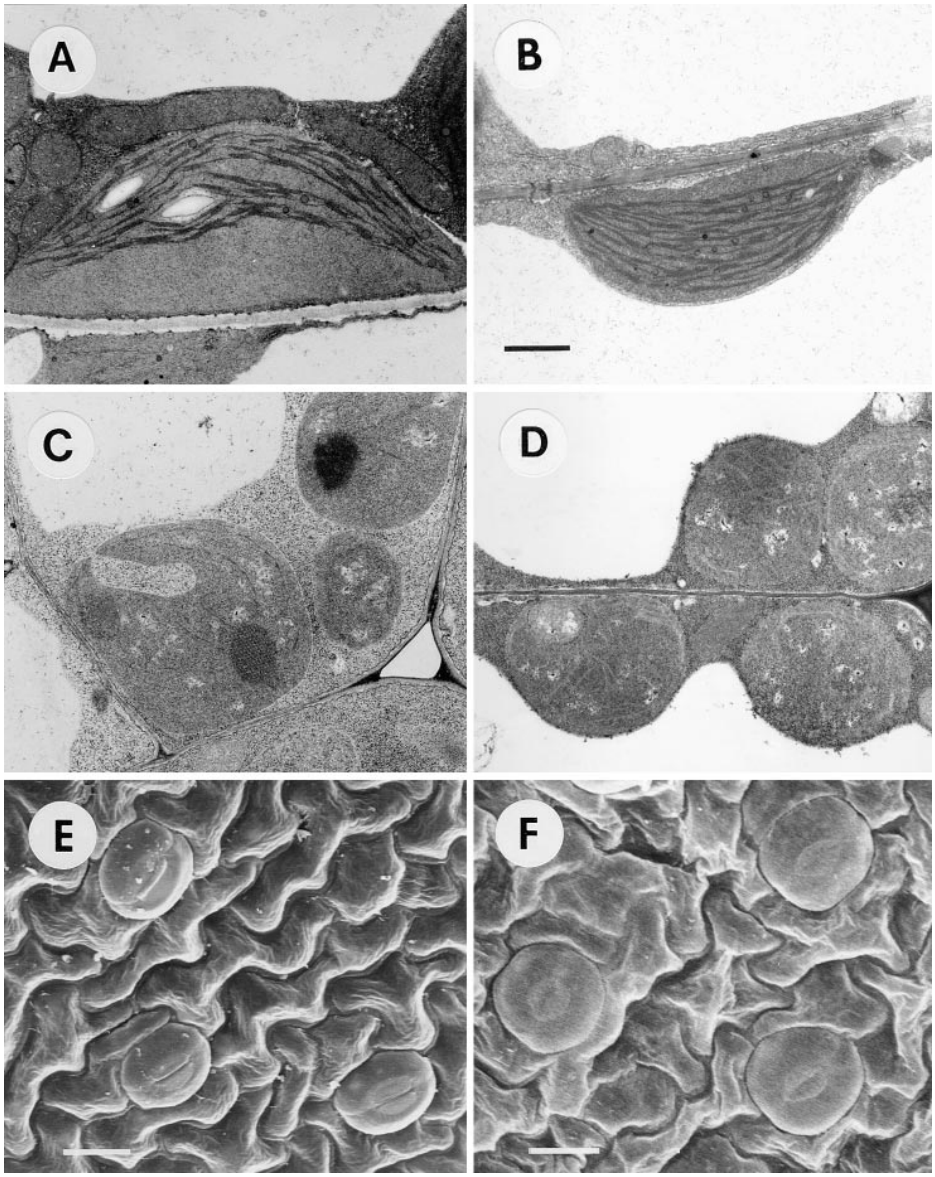

Figure 6. Plastid and Cell Differentiation in Cotyledons of 7-Day-Old Light-Grown and Dark-Grown Wild-Type and *dwf4* Seedlings.

(A) Plastids from a light-grown wild-type plant.
(B) Plastids from a light-grown *dwf4* plant.
(C) Plastids from a dark-grown wild-type plant.
(D) Plastids from a dark-grown *dwf4* plant.
(E) Stomatal guard cells from a dark-grown wild-type plant.
(F) Stomatal guard cells from a dark-grown *dwf4* plant.
Bar in **(B)** = 1 μm for **(A)** to **(D)**; bars in **(E)** and **(F)** = 10 μm.

and scanning electron microscopy; the results are shown in Figure 6. Analysis of plastids in thin sections from 7-day-old dark-grown seedlings showed no difference between the wild type and *dwf4.* Both lines contained normal chloroplasts when grown in the light (Figures 6A and 6B), whereas dark-grown seedlings contained etioplasts, with their characteristic prolamellar body and no significant organization of thylakoids (Figures 6C and 6D). Analysis of stomatal structures on the underside of cotyledons from 7-day-old seedlings indicates that stomatal development was not completed in the dark, because the stomatal opening was occluded in both lines (Figures 6E and 6F). The majority of light-regulatory mutants analyzed to date displayed light-grown morphology in the dark without concomitant chloroplast or stomatal development. As in these mutants, therefore, the *dwf4* mutation uncouples the developmental pathway of seedling morphology from that of light-regulated cellular differentiation.

An additional feature of many light-regulatory mutants is that photomorphogenesis in the dark is accompanied by expression of genes that normally are light induced (Chory et al., 1989b, 1991; Deng et al., 1991; Wei and Deng, 1992; Hou et al., 1993; Wei et al., 1994). To assess whether *dwf4* is able to induce light-regulated transcripts in the dark, we compared the activity of a *CAB* promoter fused to the *Escherichia coli* gene *uidA*, encoding β-glucuronidase (GUS), in light- and dark-grown dwarf and wild-type plants. The *CAB–uidA* fusion in pOCA107-2 (Li et al., 1994) was crossed into *dwf4*, and F_2 dwarf and wild-type plants were grown in the dark or light for 12 days, followed by determination of GUS activity by fluorometry (Gallagher, 1992). The results (data not shown) demonstrate that when grown in the light, both wild-type and *dwf4* seedlings contain GUS activity, which is significantly reduced in both lines when grown in the dark. Moreover, dark-grown *dwf4* seedlings display no GUS activity above the background present in dark-grown wild-type plants. The absence of light-induced gene expression in the dark is a distinguishing feature of certain *cop* and *det* mutants, such as *cop2*, *cop3*, and *det3.* Because we have shown that the defect in cell elongation of *dwf4* is specifically rescued by BRs, even in the presence of light, we conclude that this is not a light-regulatory mutant. That its phenotype is partially deetiolated or constitutively photomorphogenic is a secondary effect of its reduced stature and the growth conditions.

DISCUSSION

We report the isolation and characterization of four alleles of *dwf4. dwf4* plants are reduced in stature but robust and healthy overall (Figure 1). The phenotypic effects of the *dwf4* mutation can be summarized as follows: (1) cell elongation appears to be the primary defect in the mutant; (2) growth is affected in all organs examined; and (3) a deetiolated/constitutively photomorphogenic morphology may be the result of defective cell elongation. The arrest of cell elongation in *dwf4* can be rescued by exogenous application of BL.

The *dwf4* Phenotype

Morphologically, the *dwf4* phenotype can be described as being due to both primary and secondary effects of reduced cell elongation. The primary effect is simply a reduction in the length of individual organs exclusively along their normal growth axis; that is, organ width is not reduced (Table 1). The secondary effects of reduced cell elongation are themselves due to the reduction in organ length. The dark green color of the leaves, for example, may be due exclusively to the existence of a wild-type number of chloroplasts in a significantly smaller cell. Similarly, the sterility of mutants is a consequence of the shortness of the stamens, which fail to deposit their pollen on the stigmatic surface. In addition to the morphological alterations of *dwf4*, mutants display delayed development, the first sign of which occurs at flowering (Table 1). Because rosette leaves are produced continuously during vegetative development, delayed flowering results in *dwf4* rosettes having almost twice the number of leaves observed in the wild type.

A Block in Cell Elongation

Experimental evidence for a block in cell elongation in *dwf4* is first seen from the measurements shown in Figure 2. The rate of growth was significantly reduced in agar-grown *dwf4* seedlings, which ceased to grow when their hypocotyl length was $<20\%$ of the final wild-type length. Because all of the cells in a hypocotyl before the initiation of leaf development are present in the embryo, the initial growth of seedlings is due exclusively to cell expansion, which therefore must be reduced in *dwf4.* A similar situation applies to soil-grown plants. Five weeks after germination, well after plants had bolted, *dwf4* plants were shorter than wild-type plants (Figure 1 and Table 1). Although the mutants continued growing for several weeks more than did the wild type, they remained shorter through senescence. That cell elongation is the direct cause of this decreased growth is shown by measurements of cell length both in 7-day-old hypocotyls (Figures 3A and 3C) and in 5-week-old stems (Figures 3B and 3D). Not only is the reduction in cell length in good agreement with the reduction in organ length, but insofar as could be determined, there is no difference in the number of cells between *dwf4* and wild-type plants.

Organ growth by cell elongation in plants occurs as part of normal development in response to a variety of input signals. Mutants that are defective in these signaling pathways invariably fail to elongate normally in response to the appropriate stimuli. A mutant with a block at a step that is common to several individual pathways would therefore be

expected to have defective responses to all of the corresponding signals. *dwf4* appears to be such a mutant. Figure 4 shows that elongation induced by the *hy2* mutation is blocked in a *dwf4 hy2* double mutant. Not surprisingly, in view of this result, *dwf4* also failed to display hypocotyl elongation as a response to growth in complete darkness (Figure 5). In addition, *dwf4* was capable of perceiving GA, but its response was severely compromised. This mutant could also respond to the inhibitory effects of auxin but was incapable of auxin-stimulated elongation. It was only exogenous BL that fully restored wild-type length to *dwf4* hypocotyls (Choe et al., 1998).

Because *dwf4* failed to respond to at least three independent signaling pathways but responded fully to only one, the most likely explanation for the dwarf phenotype is therefore that a fully functional BR system is required for a full response to GA, auxin, and deetiolation. From the perspective of cellular economy, it may be advantageous that the downstream elements involved in cell elongation are shared among at least some of the signaling pathways that evoke this response. The interaction of various pathways at a common step provides the plant with a potential point for the integration of signals produced by diverse independent stimuli. Our results indicate that BRs act at this downstream step.

Abnormal Skotomorphogenesis as a Consequence of the Dwarf Growth Habit

When *dwf4* is grown in the light, its morphology is similar to that of various *cop* and *det* mutants, with multiple short stems, short rounded leaves, loss of fertility due to reduced stamen length, and delayed development (see Figures 1 and 4). Dark-grown *dwf4* seedlings possess short hypocotyls, open cotyledons, and developing leaves (Figure 5). Therefore, it is tempting to speculate that this mutant may be defective in the control of light-regulated processes. On the other hand, because a dark-flowering phenotype has been demonstrated for liquid-grown Arabidopsis (Araki and Komeda, 1993), and given that agar medium is mostly water, it is especially significant that it is the dwarf seedlings, whose apical meristems are very close to the agar surface, that display a light-grown phenotype in the dark. Furthermore, because wild-type seedlings grown along the surface of the agar reproduce the dark-flowering phenotype, it is possible that the apparent light-regulatory defect of dwarf seedlings is a dark-flowering response. This possibility is strengthened by the observation (data not shown) that wild-type seedlings (ecotype Wassilewskija [Ws-2]) grown in the dark on horizontally oriented plates occasionally bend down and touch the agar surface, and these seedlings invariably produce leaves. In addition, of the eight *DWF* loci identified in this laboratory, only the shortest mutants displayed open cotyledons and leaf bud development; in the case of *dwf1* (Feldmann et al., 1989), this aberrant skotomorphogenesis is confined to the most severely affected alleles (B. Dilkes and K.A. Feldmann, data not shown).

In addition to the presence of a short hypocotyl and at least partially open cotyledons in the dark, *cop1* (Deng and Quail, 1992), *det1* (Chory et al., 1989b), and *det3* (Cabrera y Poch et al., 1993) have been shown to initiate leaf formation in the dark. In mutants such as *cop1*, *cop8*, *cop9*, *cop10*, and *cop11*, stomata undergo photomorphogenic maturation (Deng and Quail, 1992; Wei and Deng, 1992; Wei et al., 1994); of these, *cop1* and *cop9* as well as *det1* (Chory et al., 1989b) also initiate differentiation of plastids into chloroplasts. *dwf4* displayed, in addition to a light-grown dwarf phenotype, a dark-growth phenotype of short hypocotyls, open cotyledons, and developing leaves; however, in contrast with the light-regulatory defect seen with whole plants, the cellular differentiation phenotype was unaffected (Figure 6). In dark-grown dwarf seedlings, stomata did not complete their development, and differentiation of chloroplasts was not observed. The absence of a cellular light-regulatory phenotype in *dwf4* is similar to that of a number of photomorphogenic mutants, such as *det2*, *det3*, *cop2*, *cop3*, and *cop4* (Chory et al., 1991; Cabrera y Poch et al., 1993; Hou et al., 1993).

In view of the dark-flowering phenotype on agar and the absence of a light-regulatory defect in differentiating cells, we conclude that at least in the case of *dwf4*, aberrant skotomorphogenesis may be a consequence of a dwarf growth habit rather than dwarfism being part of a defect in the control of light-regulated processes. This effect may also explain the light-regulatory phenotype found in other mutants with severely reduced height, such as *axr2* (Timpte et al., 1992; see below), and strong alleles of *dwf1*, both of which are also rescued by exogenous BRs (Szekeres et al., 1996).

BRs Interact with Multiple Signaling Pathways

Because a dwarflike phenotype is the only invariant trait of all known light-regulatory mutants, it follows that the primary defect in *dwf4* mutants is identical to the minimal *cop*/*det*/*fus* phenotype. It is possible, then, that the dwarf phenotype in all of the above mutants is due to defective cell elongation, which therefore may not be exclusively related to regulation by light. GA-deficient mutants also display a variable set of traits with different degrees of severity. But as with the photomorphogenic mutants, the similarity in phenotype between *dwf4* and the *ga* mutants is confined to reduced cell elongation. The evidence is compelling for BL involvement at or near a downstream control point where multiple signaling pathways interact. First, our results (Figure 4) show that BL is required for cell elongation as a response to darkness as well as GA and auxin. In addition, previous studies (Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996) and this work show that BR can compensate for the cell elongation defect of mutants as diverse as *det2*, *cpd*, *dwf4*, *det1*, *cop1*, and *dwf1.* This places BRs downstream of

all the cellular functions affected in these mutants. Finally, at least one of the BR biosynthetic genes has been shown to be modulated by light, cytokinins, and the carbon source (Szekeres et al., 1996).

Mutations in *axr2* result in a dwarf growth habit and a dark-grown phenotype with short hypocotyl and open cotyledons (Timpte et al., 1992). In addition, *axr2* mutants are resistant to auxin, ethylene, and abscisic acid and have defective root and shoot gravitropism. The dwarf phenotype in *axr2* mutants has been shown to be due to reduced cell elongation and is rescued by BL (Szekeres et al., 1996). This suggests that at least one of the multiple hormone signaling pathways affected in *axr2* involves a BR-dependent step. Mutations at another locus, *acaulis1*, also have a significant reduction in cell elongation, but the defect is confined to inflorescence stems and leaves (Tsukaya et al., 1993). Flowers are fully fertile and mature into normal-sized siliques with normal seed set. There is no change in hypocotyl length. If BRs are directly involved in this apparently organ-specific signaling pathway, it may be due to organ-specific responsiveness to individual BR species.

With regard to the mechanism of action of BRs, at the moment one can only speculate that the target may be a component of the cell expansion machinery. Perhaps steroid signaling initiates a series of events leading to cell wall loosening. Future studies of cell wall composition together with analysis of genetic interactions between BRs and cell wall mutants (Reiter et al., 1993) may provide information about the role of these compounds in cell elongation.

METHODS

Plant Growth Conditions

The *dwf4-1* and *dwf4-2* mutations are in the *Arabidopsis thaliana* ecotype Wassilewskija (Ws-2) background; the *dwf4-3* and *dwf4-4* mutations are in the Enkheim (En-2) background. The conditions used for plant growth were essentially as described previously (Feldmann, 1991; Forsthoefel et al., 1992), except that agar-solidified medium contained 0.5% sucrose. Seedlings up to 2 weeks of age (6 weeks of age for dark-growth experiments) were grown on agar-solidified medium; older plants were grown in potting soil. Germination of seeds for dark growth experiments was induced by overnight exposure of the seeds to light immediately after removing the plates from incubation at 4°C.

Analytical Methods

Protoplasts were obtained by overnight incubation of sliced leaves in 0.1% cellulysin, 0.1% driselase, 0.1% macerase (Calbiochem, San Diego, CA) in 125 mM Mes, pH 5.8, 0.5 M mannitol, and 7 mM $CaCl_2$ (Galbraith et al., 1992). Immediately before observation, chloroplasts were stained with a solution of 1.5% KI and 1% I_2. Measurements were performed as described for tissue sections (see below), and plane areas were calculated according to the formula $A = \pi r^2$.

Chlorophyll determinations were performed from 2-week-old soil-grown plants. Green tissue was weighed, frozen in liquid nitrogen, and extracted in dim light with 80% acetone in the presence of a mixture of equal parts sand, $NaHCO_3$, and Na_2SO_4. After brief centrifugation, the supernatant was collected and the extraction was repeated twice, pooling the supernatants from each sample. Chlorophylls *a* and *b* were measured spectrophotometrically, as described in Chory et al. (1991).

Growth Signal Response Measurements

Gibberellic acid (GA) response was assayed on plants grown individually in 5.7-cm pots. Once inflorescences reached 1 to 2 mm in height, they were sprayed weekly with 1 mM GA_3 (Sigma). Control plants were sprayed with water. One week after the third spraying, plants were collected, and the length of the main stem was measured between the top of the rosette and the base of the most distal pedicel; 13 to 18 plants of each line were measured per treatment. Auxin response was tested by growing seedlings for 10 days under 16 hr of light on vertically oriented agar plates containing various concentrations of 2,4-D (Gibco, Grand Island, NY). Genetic interaction with the *hy2* mutation was tested by growing seedlings under continuous light for 7 days. Brassinolide (BL) response was determined in liquid culture, as described by Clouse et al. (1993), except that three or four seedlings were grown in each well of a 24-well culture plate for 7 days. Measurements were taken for 10 to 20 seedlings for each genotype and condition, under a dissection microscope fitted with an ocular micrometer.

Microscopy

Tissues were fixed in 2% glutaraldehyde and 0.05 M sodium cacodylate, pH 6.9, for 2 hr at room temperature or overnight at 4°C, followed by three washes in buffer. For light microscopy, 1% safranin was included in the first wash, and embedding was performed in Paraplast Plus (Oxford Labware, St. Louis, MO). Ten-millimeter sections from five individual plants per line were analyzed and photographed, and cell measurements were taken using a ruler on 5 × 7 inch prints. A print of a hemocytometer grid at the same final magnification was used for calibration. At least 25 cells were measured per sample, with a minimum of 150 cells per line. For electron microscopy, the tissues were treated after fixation with 1% tannic acid in buffer for 30 min, washed three times, and postfixed in 1% OsO_4 in buffer for 2 hr, followed by five washes and dehydration through an ethanol series. Samples for transmission electron microscopy were embedded in Spurr's resin. Sections (90 nm) were stained with saturated uranyl acetate followed by Reynolds's lead citrate (Reynolds, 1963) and examined in a JEOL (Tokyo, Japan) 100-CX instrument. For scanning electron microscopy, samples were transferred to freon 113, critical point dried, and sputter-coated with 30 to 50 nm of gold. Analysis was performed in a microscope (ISI model DS130; Topcon, Inc., Paramus, NJ) with an accelerating voltage of 15 kV.

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