The *Arabidopsis det3* mutant reveals a central role for the vacuolar H⁺–ATPase in plant growth and development

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In all multicellular organisms growth and morphogenesis must be coordinated, but for higher plants, this is of particular importance because the timing of organogenesis is not fixed but occurs in response to environmental constraints. One particularly dramatic developmental juncture is the response of dicotyledonous seedlings to light. The *det3* mutant of *Arabidopsis* develops morphologically as a light-grown plant even when it is grown in the dark. In addition, it shows organ-specific defects in cell elongation and has a reduced response to brassinosteroids (BRs). We have isolated the *DET3* gene by positional cloning and provide functional and biochemical evidence that it encodes subunit C of the vacuolar H⁺–ATPase (V-ATPase). We show that the hypocotyl elongation defect in the *det3* mutant is conditional and provide evidence that this is due to an alternative mechanism of V-ATPase assembly. Together with the expression pattern of the *DET3* gene revealed by GFP fluorescence, our data provide in vivo evidence for a role for the V-ATPase in the control of cell elongation and in the regulation of meristem activity.

During the development of multicellular organisms, an intricate coordination of cell division and cell enlargement is necessary to achieve both morphogenesis and growth. In contrast to our rapidly growing knowledge of pattern formation and morphogenesis in a variety of model organisms, relatively little is known about the mechanisms that control cell and organ growth and integrate it with morphogenesis. Because plants are sessile, such mechanisms are of pivotal importance as their postembryonic development takes place under a multitude of environmental constraints, including the quality and quantity of light and the availability of water and nutrients. To compensate for their lack of mobility, plants have achieved a unique plasticity of development, which allows them to adapt to their environment. Both the initiation of organs by the apical meristems, and their subsequent growth through further cell divisions and cell expansion, continue throughout the plant life cycle. Therefore, growth and morphogenesis are not only coordinated with each other, but must provide the flexibility for adaptation to suboptimal environmental conditions.

One of the most striking examples for developmental plasticity in response to an environmental cue is found during early seedling development. When dicotyledenous seedlings germinate in the absence of light, morphogenesis is inhibited and growth is achieved mostly by organ-specific cell expansion. Hypocotyl cells elongate ≥100-fold of their embryonic length to position the shoot apical meristem into an environment providing light necessary to establish photoautotrophic growth. The closed cotyledons and the formation of the apical hook protect the largely inactive shoot apical meristem. Once this so-called etiolated seedling reaches the light, however, it switches to the photomorphogenetic program in which new organs develop and growth is achieved by both cell division and cell expansion in these newly initiating organs (for review, see Kendrick and Kronenberg 1994). In the deetiolating seedling, the rate of hypocotyl elongation is inhibited while cotyledons unfold and expand and primary leaves are initiated by the shoot apical meristem. Moreover, genes necessary for photoautotrophic growth are expressed and the photosynthetic machinery, absent from etiolated seedlings, is installed.

Light triggers this developmental switch; however, it is well known that in particular the hypocotyl growth response is mediated by the action of plant hormones. Physiological studies have shown that gibberellins, auxin, and brassinosteroids (BRs) have a stimulatory

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function, whereas ethylene, abscisic acid, and cytokinins have inhibitory effects on hypocotyl elongation (Davies 1995). How light might interact with these hormone signal transduction pathways is not understood.

Because of the dramatic differences in the body plan of light- and dark-grown seedlings, early seedling development is a striking example for developmental plasticity that is also particularly amenable to genetic dissection of the underlying regulatory mechanisms. In *Arabidopsis*, genetic screens based on the described differences in seedling morphology have identified >40 mutants, which fall into two phenotypic classes. Light-insensitive mutants (~20 loci), identified based on their inability to restrict hypocotyl cell expansion in response to light of different wavelengths, comprise the first phenotypic class. The second class of mutations in an additional 20 genes affects the entire morphogenetic program, resulting in initiation of deetiolation in the absence of light. When grown in darkness, these mutants show several features of light-grown seedlings, such as a short hypocotyl, expanded cotyledons, developing leaves, expression of light-regulated genes, and chloroplast development. In one subclass consisting of 10 genes (the *COP/ DET1/FUS* genes), mutations result in seedling lethality, suggesting that these gene products play an essential role in both light and dark development of *Arabidopsis* (Deng and Quail 1999). Their exclusively recessive nature identifies them as negative regulators and the molecular analysis reveals that they are nuclear proteins, although their precise mechanism of action is not known (for review, see Fankhauser and Chory 1997; Deng and Quail 1999).

The second subclass of deetiolated mutants has revealed that BRs play a key role in the control of photomorphogenesis. Mutants affected in either the BR biosynthesis (Li et al. 1996; Szekeres et al. 1996) or response pathways (Clouse et al. 1996; Kauschmann et al. 1996; Li and Chory 1997b) show a deetiolated phenotype when grown in the dark and are characteristic dark-green dwarfs with reduced male fertility, reduced apical dominance, and delayed senescence when grown in the light.

The *det3* mutant (Cabrera y Poch et al. 1993) is unique among the deetiolated mutants as it uncouples the morphological and molecular aspects of deetiolation and combines features of both subclasses. After prolonged growth in the dark, *det3* seedlings do not only have a short hypocotyl, expanded cotyledons, and numerous leaves, they even undergo the transition to the reproductive phase and form flower buds (Fig. 1). In contrast to other deetiolated mutants, the morphological changes are not accompanied by a derepression of light-specific genes or signs of chloroplast development. When grown in the light, an organ-specific reduction of cell elongation leads to adult *det3* plants with reduced stature and apical dominance. Moreover, it has been reported that *det3*, again unlike most other members of the deetiolated class of mutants, does not show hypocotyl elongation in response to BRs (Szekeres et al. 1996), indicating a possible role for DET3 either as a component of a branch of BR signal transduction controlling deetiolation

Figure 1. Phenotype of the *det3* mutant. Col-0 (*left*) and homozygous *det3* mutant plants (*right*) were grown for 5 weeks in the dark in the presence of 1% sucrose (*A*) or in the light on soil (*B*).

or as a downstream target of BR signaling. Here we show that the phenotype of the *det3* mutant is caused by a weak mutation in the gene for subunit C of the vacuolar H$^+$–ATPase (V-ATPase) and provide evidence that this ubiquitous eukaryotic enzyme complex plays an important role in the control of growth and morphogenesis of *Arabidopsis* seedlings.

Results

The hypocotyl elongation defect of det3 *is conditional*

The dwarf stature of *det3* can to a large extent can be ascribed to a reduction in cell expansion (data not shown), which most strongly affects cells of the hypocotyl, petioles, and inflorescence stems (Fig. 1). Previously, it was reported that *det3* hypocotyls do not respond to applications of BRs (Szekeres et al. 1996); however, in our hands *det3* did not show complete insensitivity. The *det2* mutant is deficient in BR biosynthesis and can be rescued by application of brassinolide (BL), the most active BR. We constructed a *det2–det3* double mutant to analyze the effect of the *det3* mutation in a BR-deficient background. As shown in Figure 2A, dark-grown *det2* seedlings were rescued to wild-type stature by application of 1 μM BL. *det3* hypocotyls, in contrast, only partially elongated in response to BL applications and dark-grown *det2–det3* double mutants behaved like the *det3* single mutant, that is, BL failed to fully restore hypocotyl growth. Thus, the *det3* mutation reduces the ability of etiolated seedlings to respond to BRs.

The inability of *det3* seedlings to respond to BRs might be explained by a general defect in cell expansion. To test the ability of *det3* hypocotyls to respond to a different growth stimulus (gravity), we grew seedlings upside down. When wild-type seedlings were grown in the dark on inverted plates with the growth medium facing down, the negative gravitropic growth response led to a strong curvature of the hypocotyl achieved by asymmetric cell expansion (Fig. 2B). To our surprise we found that after 5
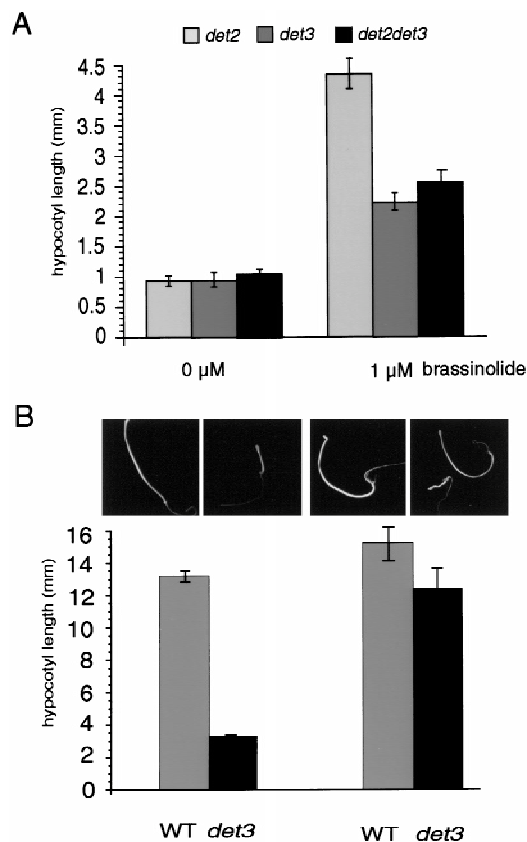
Figure 2. The hypocotyl elongation defect of *det3* is conditional. (*A*) The *det3* mutation reduces the ability of seedlings to respond to BL. Seedlings of *det2-1*, *det3*, and the *det2–det3* double mutant were grown in the dark on plates containing either no or 1 μM BL. Seedlings of *det2-1*, *det3*, and the *det2–det3* double mutant were grown in the dark on plates containing either no or 1μM BL. Hypocotyl length was measured after 4 days. Bars represent standard errors (*n* = 40). (*B*) Seedlings of Col-0 and *det3* were grown in the dark on plates, which were either incubated in the normal orientation (*left*) or inverted by 180° (*right*). Hypocotyl length was measured after 5 days. Bars represent standard errors (*n* = 40).

days of growth under such conditions a majority of *det3* seedlings achieved almost normal hypocotyl length (Fig. 2B). Taken together, our results suggest that the *det3* mutation leads to a conditional defect in hypocotyl elongation.

During the course of these studies, we noted that in comparison to both wild type and other mutants with similar phenotypes, dark-grown *det3* hypocotyls show a highly irregular surface structure and variations in diameter within individual seedlings. Microscopic analysis (Fig. 3a–d) showed the presence of collapsed individual epidermal and cortical cells that seemed to have elongated normally initially. Expanding neighboring cells then seem to compress these cells leading to the irregular surface structure. Collapsed cells were observed when seedlings were grown in either the presence or absence of 1% sucrose in the growth medium, but in the presence of sucrose their number was increased. Staining

with iodine revealed that *det3* under these conditions accumulated high levels of starch in its hypocotyl cells (Fig. 3e,f). These observations suggest that the *det3* mutation leads to a defect in the execution of the actual growth response rather than in the signaling pathways initiating it.

Positional cloning of the DET3 gene

Recombination breakpoint analysis of homozygous *det3* plants derived from the cross *det3* (Col) × *DET3* (L*er*) placed the *DET3* gene between markers NCC1 and m219 on the top arm of chromosome 1, an interval shown to be contained within a single YAC clone, CIC12A9 (see Material and Methods). Using hybridization data for CIC12A9 provided by the *Arabidopsis thaliana* Genome Center, we established a BAC contig anchored by the
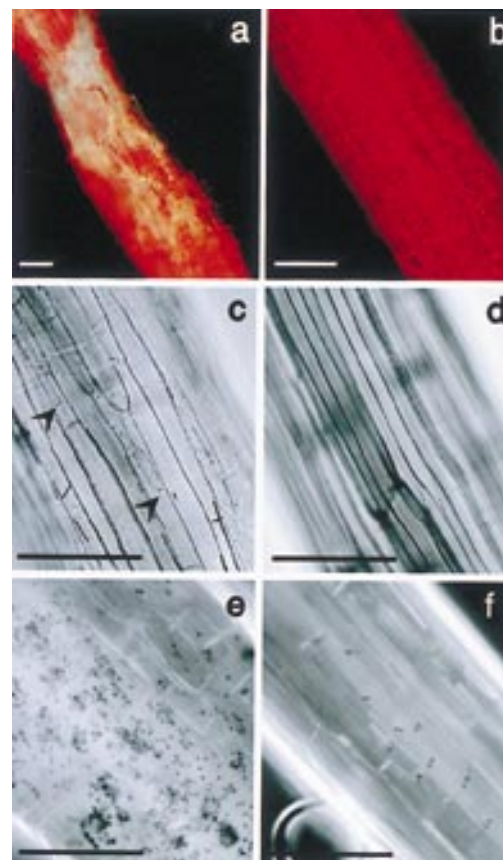

Figure 3. *det3* shows altered hypocotyl morphology. In comparison to wild-type seedlings (*b*), light-grown seedlings of *det3* (*a*) show an irregular surface structure. In addition to the red chlorophyll autofluorescence, *det3* seedlings show green fluorescence likely to be caused by the presence of dead cells. Collapsed cells are found in the epidermal layer of dark-grown *det3* seedlings (*c*, marked by arrowheads) but were never found in wild-type seedlings (*d*). Staining of dark-grown seedlings grown on medium containing 1% sucrose with I/KI solution reveals high levels of starch accumulation in *det3* seedlings (*e*) in comparison with wild type (*f*). Bars, 50 μM.

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NCC1-containing BAC, T15N14. Random subfragments from this contig and BAC end fragments amplified by thermal asymmetric interlaced PCR (TAIL–PCR) were used to generate new polymorphic markers. Two such markers, 8L18e and 22N2d (Fig. 4A), derived from two overlapping BACs were separated from *det3* by only one recombination event. Therefore, we subcloned both BACs into a binary plasmid vector and identified a contig of six overlapping plasmid subclones between the two flanking markers.

These six plasmids were used to transform *det3* plants. Two overlapping subclones, 22N2TH5 and 22N2TH3, rescued the mutant phenotype, thereby localizing the *DET3* gene to a region of 6 kb (Fig. 4B). Only one of three cDNA clones, 2–4, found to hybridize to both plasmids was fully included in both of them. To our surprise, sequence analysis of three independent full-length RT–PCR products derived from the only available mutant allele *det3-1* did not uncover any changes with respect to

Figure 4. Positional cloning of *DET3*. (*A*) Recombination analysis placed the *DET3* gene in close linkage to marker NCC1 on chromosome 1 (6 recombinant chromosomes among 1658 analyzed). Establishing a BAC contig for this region and fine mapping using the two markers 8L18.e (only 1 of the 6 NCC1 recombinants) and 22N2.d (1 of 18 recombinants for m219) placed *DET3* on the two overlapping BACs T13M17 and T22N2. Six plasmid subclones covered the region of interest and were used to transform *det3*. (*B*) Rescue of *det3* by plasmids 22N2TH3 and 22N2TH5. The photographs represent 3:1 segregating T_2 progenies of individual wild-type-looking transformants obtained with 22N2TH5 and 22N2TH3. (*C*) The *det3-1* mutation destroys a branchpoint consensus sequence in the first of 10 introns. RNA gel blot analysis using 5µg of total RNA from 4-day-old light-grown seedlings detected a reduction of the *DET3* mRNA in *det3*. Using 18S rRNA for normalization, the reduction was determined to be twofold in three independent experiments.

the wild-type sequence of clone 2-4. Therefore, we analyzed the corresponding genomic sequence from both mutant and wild type. In the first of 10 introns, we found a $T \rightarrow A$ mutation 32 bp upstream of the putative 3′ splice site (Fig. 4C). Both the surrounding sequence CTAAT and the distance from the 3′ splice junction indicate that this mutation destroys a branchpoint consensus sequence (Simpson et al. 1996).

RNA gel blots revealed that the *det3-1* mutation caused a reduction of the transcript to ~50% of the wild-type level (Fig. 4C). A second identical sequence matching the branchpoint consensus was found only 10 bp upstream of the mutation and is likely to be responsible for the fact that this intron gets spliced out eventually, explaining the presence of unaltered cDNAs derived from *det3*. Low-stringency Southern hybridizations showed that cDNA 2-4 was derived from a single copy gene indicating that the detected message is derived from the *DET3* gene. Final confirmation that this gene is indeed *DET3* was obtained by expressing the 2-4 cDNA under the control of the strong and ubiquitously expressed cauliflower mosaic virus 35S promoter. When *det3* mutants were transformed with this construct T_1 plants showed a wild-type phenotype (data not shown).

DET3 *encodes subunit C of the V-ATPase*

The *DET3* gene is predicted to encode a hydrophilic protein of 377 amino acids with a molecular mass of 43 kD. Database searches revealed that the deduced amino acid sequence had between 30% and 40% identity with amino acid sequences for subunit C of V-ATPases from a variety of eukaryotic species (Fig. 5A,B). Beyond the similarity to the V-ATPase subunit C, database searches did not identify other similar proteins or conserved motifs or domains.

The V-ATPases constitute a family of highly conserved ATP-dependent proton pumps responsible for acidification of endomembrane compartments in eukaryotic cells. They are multimeric protein complexes composed of the peripheral cytoplasmic V_1 sector responsible for ATP hydrolysis consisting of subunits A–H and the V_0 membrane sector responsible for proton translocation and consisting of subunits a, c, and d (Forgac 1999). In *Saccharomyces cerevisiae*, the V-ATPase subunit C is encoded by the *VMA5* gene, which upon disruption, renders cells unable to grow on medium buffered to neutral pH (Beltran et al. 1992).

To prove that *DET3* indeed encodes the *Arabidopsis* ortholog of the subunit C, we tried to rescue a *vma5* mutant (White and Johnson 1997) by expressing the *DET3* cDNA under the control of two different yeast promoters. Neither of the two constructs was able to rescue the *vma5* phenotype (data not shown). Therefore, we performed the reciprocal experiment in which we expressed the *VMA5* gene in plants. We found that the growth of homozygous *det3* mutants transformed with a construct carrying the *VMA5* gene under the control of the 35S promoter was restored to wild type (Fig. 5C).

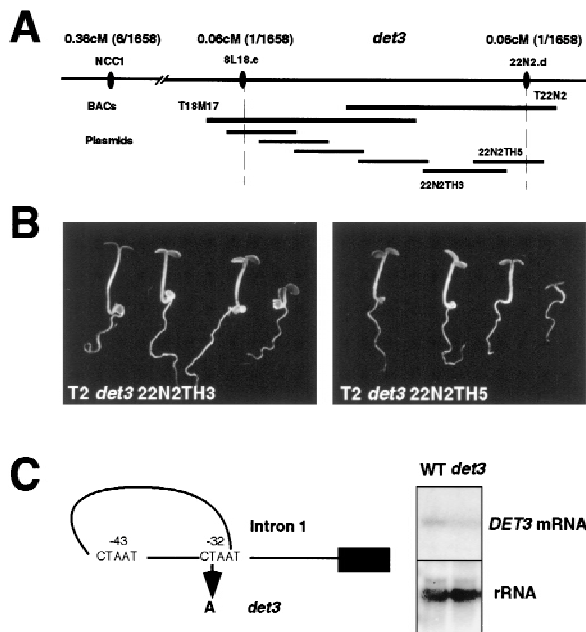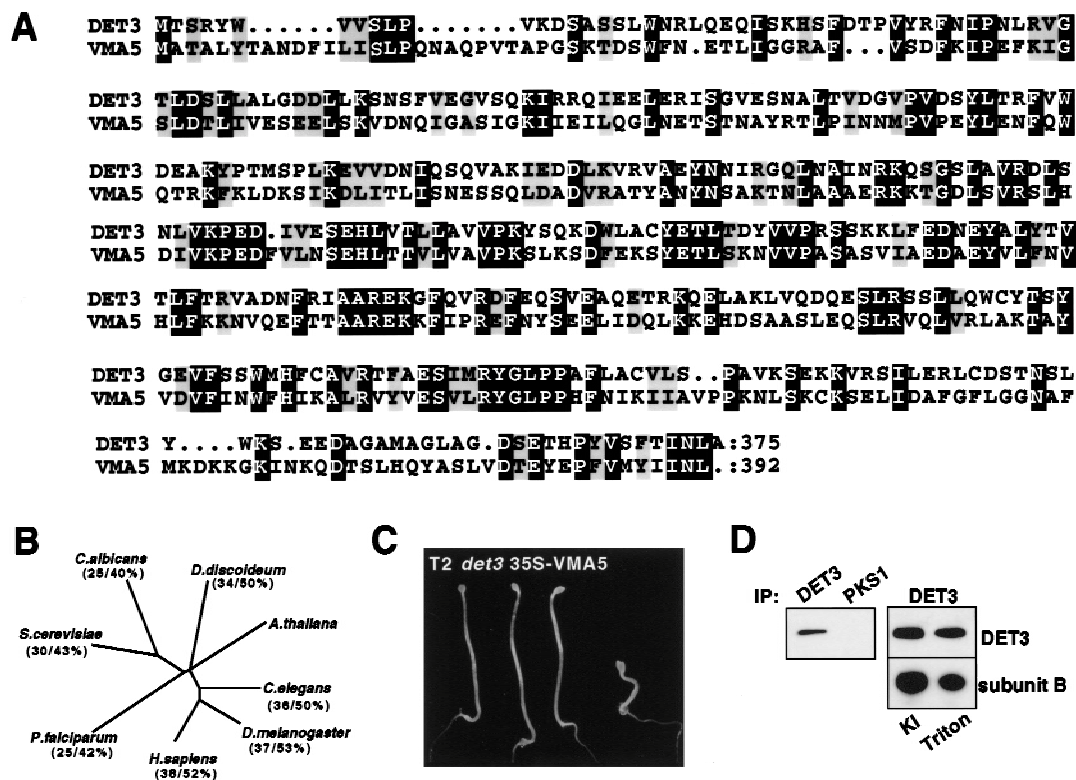Biochemical evidence that *DET3* encodes the V-ATPase

Figure 5. *DET3* encodes subunit C of the vascuolar H⁺–ATPase. (*A*) Alignment of the amino acid sequence deduced for *DET3* and the *VMA5* gene encoding subunit C of the vascuolar H⁺–ATPase in *S. cerevisiae*. Identical residues are boxed in black, conserved residues are boxed in gray. (*B*) Phylogenetic tree for V-ATPase subunit C from the following species: *Candida albicans* (gnl/Stanford_5476/ C.albicans_Con4-2572 Candida albicans unfinished fragment of complete genome), *Caenorhabditis elegans* (PID g4579712), *Drosophila melanogaster* (PID g2245679), *Dictyostelium discoideum* (PID g1718089), *Homo sapiens* (PID g340188), *Plasmodium falciparum* (gnl/pf1/Sanger_ContigID_00715 Plasmodium falciparum 3D7 unfinished sequence from chromosome 1), and *Saccharomyces cerevisiae* (PID g549206). Values for percent identity and percent similarity are shown in parentheses. (*C*) Functional complementation of *det3* by expression of *VMA5*. Shown are seedlings representing the segregating T_2 of a T_1 plant expressing VMA5 under the control of the 35S promoter. (*D*) Immunoprecipitates obtained with an antibody against DET3 contain subunit B. The DET3 antibody was covalently bound to protein A and used for immunoprecipitation. PKS1 antibody coupled to protein A was used as a negative control and the antibodies against DET3 and subunit B (mAB2E7) were used for detection on immunoblots. Immunoprecipitates were performed on microsomal protein that had been treated either with KI or Triton (see Materials and Methods) to dissociate the two subcomplexes V_1 and V_0.

subunit C was obtained by immunoprecipitation. After affinity purification, a polyclonal antiserum raised against purified recombinant His-tagged DET3 was coupled covalently to immobilized protein A. The resulting matrix was used to immunoprecipitate DET3 from microsomal protein extracts solubilized under conditions that dissociate the two V-ATPase subcomplexes V_1 and V_0 while not dissociating the individual V_1 subunits. The precipitates were subjected to immunoblot analysis using the DET3 antiserum,, as well as a monoclonal antibody (2E7) against subunit B (Ward and Sze 1992) (Fig. 5B), and a polyclonal antiserum against subunit A (Kim et al. 1999) (data not shown). We detected all three proteins in the immunoprecipitate and concluded that DET3 is indeed the V_1-associated subunit C of the V-ATPase from *Arabidopsis*.

Expression and cellular localization of DET3

We expressed a carboxy-terminal fusion between *DET3* genomic sequence and the green fluorescent protein (GFP) under the control of the *DET3* promoter, allowing us to analyze both expression pattern and cellular localization of the DET3 protein by fluorescence microscopy. Expression of the DET3–GFP fusion protein rescues the *det3* phenotype indicating that it is functional (data not shown). In addition, we used immunoblots to show that the expression levels for endogenous DET3 and for DET3–GFP were comparable (data not shown). High levels of fluorescence were found in the apical hook region of 2-day-old seedlings (Fig. 6a), in developing petioles (Fig. 6b), and in root-tips (Fig. 6c,d), all of which consist of cells about to enter a phase of rapid cell elongation in both light-grown and etiolated seedlings. In more mature organs, highest levels of fluorescence were found in the vascular tissues (Fig. 6c). In very young cells that do not have a central vacuole, fluorescence outlined the individual cells indicating that the DET3–GFP fusion is associated with the plasma membrane (Fig. 6c,d). The
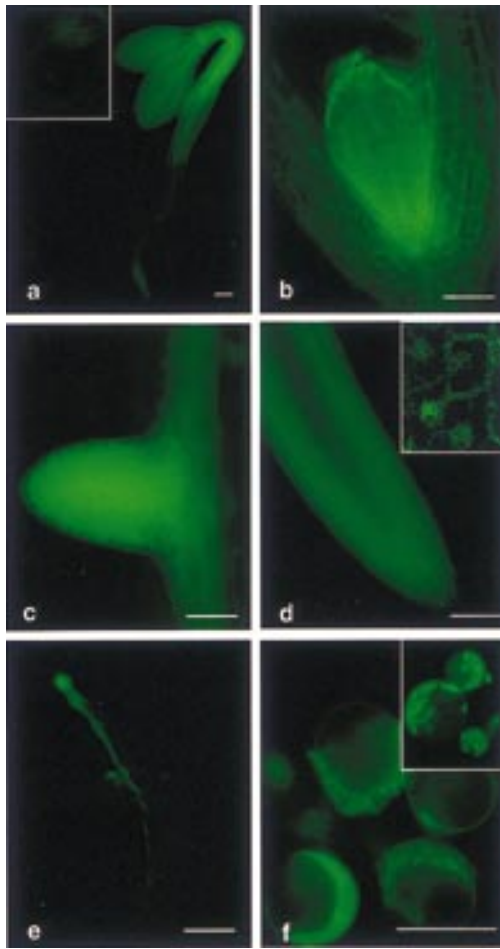
Figure 6. Expression pattern and cellular localization of DET3. A DET3–GFP fusion protein was expressed under the control of the *DET3* promoter and GFP was imaged in living cells. Original gray scale images were enhanced using green pseudocolor. Shown here are images of (*a*) 2-day-old dark-grown seedlings (negative control in upper left corner), (*b*) developing primary leaves in a 7-day-old light-grown seedling, (*c*) lateral root emerging from the vascular cylinder of the primary root, (*d*) primary root tip with cells showing perinuclear fluorescence indicative of ER localization, (*e*) tip-growing pollen tube, and (*f*) leaf mesophyll protoplasts. Note that in two of the protoplasts the plasma membrane is ruptured and the vacuolar membrane is exposed. Chlorophyll autofluorescence is also shown in green pseudocolor (negative control in upper left corner). Bars, 50 µM.

same cells also showed high fluorescence in the perinuclear region indicating endoplasmic reticulum (ER) localization. In tip-growing cells, such as root hairs (data not shown) and pollen tubes (Fig. 6e), highest fluorescence was found in the vesicle-rich tip region. In protoplasts derived from more mature cells with large central vacuoles high levels of fluorescence coincided with the vacuolar membrane (Fig. 6f). Although high levels of GFP were associated with membraneous structures, we also detected fluorescence, which appeared to be cytoplasmic. However, at this resolution we cannot distinguish between fluorescence derived from small cytoplasmic vesicles versus truly cytoplasmic-localized DET3–GFP. Although we have shown that expression of the DET3–GFP fusion is able to rescue the *det3* phenotype, we cannot exclude that only a subfraction of the detected protein is functional. However, our observations concerning its cellular localization are in good agreement with results obtained by other groups using immunocytochemistry (Herman et al. 1994) and fractionation studies (Rouquie et al. 1998).

det3 *shows a conditional lack of V-ATPase assembly and activity that correlates with the conditional cell expansion response*

Analysis of the yeast *vma5* mutant indicates that the C subunit functions in the assembly of the cytoplasmic V_1 subcomplex with the membrane integral V_0 subcomplex (Beltran et al. 1992). On the basis of this assumption, we reasoned that *det3* mutants should not only have reduced levels of subunit C but should also have reduced membrane-associated levels of other V_1 subunits. Therefore, we compared the levels of subunits C and B, representing V_1, and of subunit c, the core subunit of V_0. Immunoblots of microsomal proteins isolated from 5-day-old etiolated wild-type and *det3* seedlings probed with the respective antibodies showed comparable levels of subunit c, whereas both V_1 subunits were significantly reduced (Fig. 7A).

When we performed a similar experiment with proteins derived from gravi-stimulated seedlings grown on inverted plates, we saw the expected reduction of DET3 in the microsomal fraction, but found wild-type levels of subunit B in this fraction. This provides evidence that V_1V_0 assembly can be achieved by different mechanisms not necessarily involving the C subunit. To correlate the level of membrane-associated V_1 with V-ATPase activity, we used a colorimetric Pi release assay (Benett et al. 1988) to measure ATP hydrolysis by microsomes prepared from 5-day-old dark-grown seedlings. These studies show that under normal growth orientation, *det3* had ~40% of wild-type activity, whereas the increased hypocotyl elongation seen in *det3* seedlings grown on inverted plates was accompanied by an increase in V-ATPase activity to ~70% of wild type (Fig. 7B).

Discussion

det3 *reveals an important role for the V-ATPase in both growth and morphogenesis*

We have isolated the *Arabidopsis DET3* gene by positional cloning and have shown by functional complementation and by immunoprecipitation that it encodes subunit C of the V-ATPase. V-ATPases play a central role in eukaryotic cells because of their primary function in the acidification of endomembrane compartments. A variety of membrane and protein trafficking processes like receptor-mediated recycling (Johnson et al. 1993), endo- and exocytotic processes (Palokangas et al. 1994),
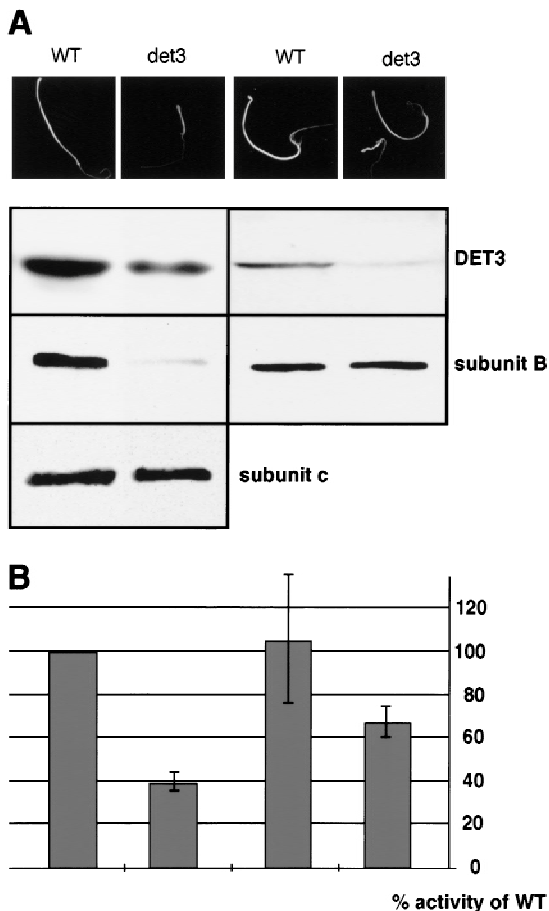
Figure 7. *det3* shows a conditional lack of V-ATPase assembly and activity. (*A*) Immunoblots with microsomal protein prepared from Col-0 and *det3* grown in the normal orientation (*left*) or in the inverted orientation (*right*) were probed with antibodies against DET3, subunit B (representing the V_1 subcomplex), and subunit c (representing V_0). (*B*) V-ATPase activity of microsomal fractions used in *A* was determined using a colorimetric P_i release assay. Results in percent of wild-type activity were averaged for three independent measurements. Bars represent standard errors.

and the affinity of the KDEL receptor for ER localized proteins (Wilson et al. 1993) are pH dependent. Furthermore, in plants, which use protons almost exclusively as their coupling ions, secondary active transport of solutes across endomembranes is energized mainly by the activity of the V-ATPase (Sze et al. 1999).

Much of our current knowledge about the structure and function of V-ATPases originates from the study of the *vma* mutants of *S. cerevisiae* in which genes for individual subunits have been inactivated (Stevens and Forgac 1997). The *vma* mutants show conditional lethality only when grown on medium buffered to neutral pH and on high extracellular calcium concentrations (Kane et al. 1992; Ho et al. 1993).

Unlike yeast, the in vivo analysis of V-ATPase function in multicellular eukaryotes has been hindered so far by the fact that null mutations identified in genes en-

coding V-ATPase subunits in *Drosophila* (Davies et al. 1996; Guo et al. 1996a,b) and *Neurospora* (Ferea and Bowman 1996) cause lethality. We have shown that the severe phenotype of the *det3* mutant is due to a weak allele causing only a twofold reduction in mRNA and protein levels for subunit C of the V-ATPase. Together with the fact that despite using multiple mutagens we failed to identify additional mutant alleles of *DET3* (K. Schumacher and J. Chory, unpubl.), it seems likely that a complete loss-of-function of this gene would also cause lethality in *Arabidopsis*. Despite the severity of its phenotype, however, the *det3* mutant is viable and fertile and allows us for the first time to study the effects of reduced V-ATPase function in a multicellular eukaryote.

The *det3* mutant is defective in both cell expansion and morphogenesis. Plant cell expansion requires coordination between changes in cell wall properties, synthesis and transport of new membrane and wall materials, and maintenance of osmotic potential. The influx of water is the driving force for cell expansion, reducing the osmotic potential, which is in turn reestablished by solute uptake into the cytoplasm and into the often large central vacuoles. Because the V-ATPase together with the H^+–pyrophosphatase drives solute uptake into the vacuole, it has long been assumed that V-ATPase function is important for cell expansion (Taiz and Zeiger 1991). Indeed, several lines of circumstantial evidence exist such as coincident peaks in cell elongation and V-ATPase activity in rapidly elongating developing cotton fibers (Smart et al. 1998); however, the only direct evidence for a role for the V-ATPase in cell expansion has come from the analysis of transgenic carrot lines in which cell expansion is reduced by antisense inhibition of subunit A (Gogarten et al. 1992).

Here, we show that *det3* mutants have reduced cell expansion; however, this reduction in expansion of cells in the hypocotyl of *det3* could either be caused by a reduced solute uptake or by a reduced membrane flow. Cells in the hypocotyl of *det3* initially appear to expand normally, which is suggestive that the effect of reduced V-ATPase activity is in the osmotic machinery bringing cell expansion to a halt. In addition, the accumulation of starch is evidence that there is reduced solute uptake into the vacuole. This reduced vacuolar uptake might lead to a higher cytosolic sugar concentration, which, in turn, is compensated for by the accumulation of starch in amyloplasts.

In addition to a reduction in cell expansion, the *det3* mutant fails to arrest its shoot apical meristem when seedlings are grown in the dark, and the increased activity of the lateral meristems in the axils of rosette and cauline leaves leads to a strongly reduced apical dominance. It has been shown that increased meristem activity in dark-grown plants can be influenced by the availability of sucrose (Araki and Komeda 1993; Roldan et al. 1997). As such, it is possible that the failure of *det3* to arrest its shoot apical meristem in the dark is simply due to its altered cellular carbohydrate distribution. On the other hand it is known that V-ATPase function is important for protein targeting and ion homeostasis, and

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therefore, it is conceivable that a lack of V-ATPase activity interferes with signal transduction pathways controlling meristem activity. Finally, although we have shown that DET3 is a subunit of the V-ATPase, we cannot exclude that it has additional functions independent of its role as a V-ATPase subunit.

The det3 mutant does not show a general defect in cell expansion

The phenotype of *det3* can be described to a large extent as the result of a reduction in cell expansion strongly affecting the hypocotyl, petioles, and inflorescence stems, whereas cell expansion in other organs, such as leaf blades, cauline leaves, flowers, siliques, and roots, is less affected. To understand why the reduced cell expansion in *det3* mutants is more dramatic in certain organs and is conditional in the case of the hypocotyl, several facts revealed by the molecular analysis have to be considered. First, the *det3* mutation leads only to a twofold reduction in expression of an otherwise fully functional protein and it is conceivable that this is sufficient for near-normal growth and development in some organs. In contrast, in very rapidly growing cells the full V-ATPase activity could be required not only to maintain cell expansion but also to provide vital cellular functions. Although *DET3* appears to be a single-copy gene, we cannot exclude functional redundancy due to the presence of the H^+–pyrophosphatase, a second proton pump specific to plants and phototrophic bacteria (Rea and Poole 1993). For instance, in *det3* roots the H^+–pyrophosphatase, which is highly active under anoxic conditions (Carystinos et al. 1995), could be responsible for the lack of a strong root growth phenotype. Finally, we have shown that the hypocotyl elongation defect is conditional and most likely due to an increased V-ATPase assembly that is at least partially independent of the presence of DET3. As such alternative assembly mechanisms might be active in cells less severely affected.

det3 reveals that the V-ATPase can be assembled by more than one mechanism

Although cell- and organ-specific variations in the composition of V-ATPases have been described (Forgac 1999), subunit C has been identified as a ubiquitous component. Subunit C or at least a protein of the corresponding molecular weight has been identified as part of the V-ATPase purified from a variety of different organisms and organelles (e.g., see Xie and Stone 1988; Parry et al. 1989; Ward and Sze 1992) and stoichiometry measurements of the coated vesicle enzyme indicate that it is present in a 1:1 ratio per complex (Arai et al. 1988). Its precise molecular function is unknown, but based on the analysis of the *vma5* mutant of *S. cerevisiae* (Ho et al. 1993) it has been assumed that it is necessary for the assembly of V_1 and V_0. On the other hand, in vitro reconstitution experiments have shown that a less stable and less active V-ATPase complex can be assembled in

the absence of subunit C (Puopolo et al. 1992), indicating that C might play a role in stabilization and regulation of V-ATPase activity rather than being essential for assembly. The increased assembly that we observe in *det3* under certain conditions suggests that subunit C is not essential for assembly under all conditions. However, it is possible that the assembly efficiency of subunit C is enhanced under certain conditions. Independent of the actual mechanism, our data provide the first in vivo evidence for a conditional variation in the stoichiometry of the V-ATPase within a single organism and points to the existence of independent assembly mechanisms. This is particularly interesting as it has been shown in yeast that disassembly and reassembly provide a fast and efficient way to regulate V-ATPase activity according to environmental cues (Parra and Kane 1998).

Is the V-ATPase a target for hormonal control of cell expansion?

Elongation of hypocotyl cells is under hormonal control and our data show that DET3 is necessary for BR-induced cell elongation, whereas the gravitropic growth response, in which auxin is the most likely signal (Davies 1995), is much less affected by the reduction of DET3 protein. Having shown a tight correlation between V-ATPase activity and cell elongation and having identified a conditional assembly mechanism, we suggest a model in which the V-ATPase activity is regulated differentially by multiple phytohormones through different modes of assembly. For instance, regulated assembly of the V-ATPase by BR signal transduction acting through DET3 might be a rapid and efficient way to initiate cell expansion, which of course has to be coordinated with changes in cell wall properties and changes in gene expression necessary to sustain this growth response. In support of this hypothesis, we have found that the regulatory subunit H of the V-ATPase interacts with and is phosphorylated in vitro by the putative BR receptor BRI1 (J. Li and J. Chory, unpubl.). Alternatively, the reduced BR sensitivity of *det3* could also be explained by mistargeting of BRI1 (Li and Chory 1997b) or changes in second messenger systems caused by a reduction in V-ATPase activity. Further physiological and genetic analysis of the *det3* mutant is necessary to confirm the role of the V-ATPase as a downstream target of hormone signal transduction pathways leading to cell expansion and hopefully will provide insight into additional functions of the V-ATPase in plant growth and morphogenesis.

Materials and methods

Plant materials and growth conditions

A. thaliana ecotype Columbia (Col-0), the *det3-1* mutant in a Col-0 background (Cabrera y Poch et al. 1993) and the *det2-1* mutant (Chory et al. 1991) were used in this study. Ecotype Landsberg carrying the *erecta* mutation (L*er*) was used for mapping purposes. Seed sterilization, seedling growth media, and plant growth conditions were as described (Li and Chory 1997a).

Genetic analysis

To generate a mapping population, homozygous *det3* mutants were pollinated with L*er* pollen. The resulting F$_1$ plants were self-pollinated to generate F$_2$ plants segregating the *det3* mutation. To obtain *det2–det3* double mutants, homozygous *det2-1* plants were pollinated with pollen from a homozygous *det3* mutant. The resulting F$_1$ plants were self-pollinated to generate a segregating population. The *det2–det3* double mutant was identified among F$_3$ progenies derived from self-pollinated F$_2$ plants with a *det3* phenotype that showed a 3:1 segregation for a *det2* phenotype.

DNA and RNA analysis

Plant genomic DNA was isolated as described in (Li and Chory 1997a). BAC DNA was isolated using the Qiagen-Midi-Kit following a protocol by the manufacturer (Qiagen Inc., Chatsworth, CA). RNA was isolated according to a standard protocol (Ausubel et al. 1994). Total RNA (2 µg) was used to obtain cDNA by oligo(dT)-primed reverse transcription using Superscript II reverse transcriptase (Boehringer Mannheim, Indianapolis, IN).

Mapping of det3

To map the *det3* mutation, DNA from 829 F2 *det3* mutants was isolated and used for SSLP (Bell and Ecker 1994), CAPS (Konieczny and Ausubel 1993), or dCAPS (Neff et al. 1998) analysis. After analysis of 1658 chromosomes *det3* was mapped to a region flanked by the CAPS markers NCC1 and m219. YAC clone CIC12A9, which contains both flanking markers, was identified by the *A. thaliana* Genome Center and had been used as a probe in the hybridization of filters of a BAC library (http://genome.bio.upenn.edu/physical-mapping/ BAC_data/allhybs/allframe.html). The corresponding BAC clones from the TAMU library (http://genome-www.stanford.edu/ Arabidopsis/ww/Vol2/choi.html) were obtained from the ABRC (http://aims.cps.msu.edu/aims/) and were used for restriction and Southern analysis to establish a BAC contig anchored by BAC T15N14 containing NCC1. To create new markers in this region, random subfragments or BAC end fragments that were generated by TAIL-PCR (Liu and Whittier 1995) were subcloned into pBLUESCRIPT and subjected to sequence analysis. The respective sequences were then amplified from L*er* and mismatches between the two ecotypes were used to create either CAPS or dCAPS markers.

BAC subcloning

DNA of two BAC clones, T13M7 and T22N2, covering the region between the two closest flanking markers was subjected to partial digestion with the two enzymes *Sau*3AI and *Tsp*509I. After gel purification, fragments >12 kb were ligated into the binary plasmid vector pPZP221 (Hajdukiewicz et al. 1994) that was digested with *Bam*HI or *Eco*RI, respectively. Individual subclones were picked and grown in microtiter plates. Using the two flanking markers and additional random subfragments as probes for colony hybridizations, we established a contig of six subclones that covered the region of interest.

Plant transformation

Plasmids that were used to generate transgenic plants were introduced into the *Agrobacterium* strain GV3101. Homozygous *det3* mutants or Col-0 plants were used for in planta transformation using a protocol modified after (Bechtold and Pelletier 1998; Clough and Bent 1998).

Sequence analysis of DET3

Sequencing reactions were performed on an ABI310 sequencer and primary sequencing data were analyzed using the AutoAssembler and SequenceNavigator software (PE Applied Biosystems Inc, Foster City, CA). Database searches were performed using the BLAST program (Altschul et al. 1990). Multiple sequence alignments were obtained using the CLUSTAL X program (Thompson et al. 1997) and the phylogenetic tree was created with a bootstrap value of 1000. The original cDNA clone, 2-4, was isolated from a cDNA library described in (Schindler et al. 1992) and sequence comparisons indicated that it most likely was a full-length clone. The corresponding sequence was amplified from *det3* RNA in three independent RT–PCR reactions. To determine the genomic sequence, primers derived from the *DET3* cDNA sequence were used to amplify the corresponding genomic region from 22N2TH5 and from genomic DNA of Col-0 and *det3*. Sequence analysis of three independent PCR products of Col-0 and *det3* detected a single mismatch that was confirmed further by using the *Mse*I site that this mismatch creates.

Plasmids

To determine whether the putative *DET3* cDNA rescues the *det3* phenotype, it was cloned into the binary plasmid vector CHF3 (C. Fankhauser and J. Chory, unpubl.) which is based on pPZP221 (Hajdukiewicz et al. 1994) and carries the cauliflower mosaic virus 35S promoter and the pea ribulose 1,5-bisphosphate carboxylase terminator. The same vector was used to express the *VMA5* gene (PID g549206) that was amplified from *S. cerevisiae* genomic DNA by PCR. For expression in *Escherichia coli* the *DET3* cDNA was cloned into pET28c (Novagen, Inc., Madison, WI). The green fluorescent protein used for expression analysis was obtained by introducing the S65T mutation (Reichel et al. 1996) into a non-ER localized version of GFP5 (GenBank accession no. U87974; Siemering et al. 1996). The *DET3* promoter region is defined by the presence of an ORF 500 bp upstream of the *DET3* start codon. A genomic fragment including this region was cloned into pPZP221 in a way that allowed a fusion of the last exon of *DET3* with the GFP coding sequence.

Protein analysis

Microsomal membrane fractions were prepared from 5-day-old dark-grown seedlings of *det3* and Col-0. Tissue was homogenized with an equal volume of homogenization buffer [0.35 M sucrose, 70 mM Tri-HCl (pH 8), 10% (vol/vol) glycerol, 3 mM Na$_2$EDTA, 0.15% (wt/vol) BSA, 1.5% (vol/vol) PVP-40, 4 mM DTT, 1 mM Pefablock; Boehringer Mannheim]. The homogenate was filtered through three layers of Miracloth and centrifuged at 15,000g for 15 min at 4°C. The supernatant was filtered through Miracloth again and then centrifuged at 100,000g for 1 hr at 4°C. The microsomal pellet was resuspended in resuspension buffer [0.35 M sucrose, 10 mM Tris-MES (pH 7), 2 mM DTT, 1 mM Pefablock]. For immunoprecipitation, solubilization was achieved by adding an equal volume of resuspension buffer containing 20% (vol/vol) glycerol and 10% (vol/vol) Triton X-100 and incubation on ice for 1 hr. To dissociate the V$_1$ subcomplex from the microsomal membranes an equal volume of resuspension buffer containing 0.2 M KI, 10 mM MgSO$_4$, and 10 mM Mg ATP was added and incubated at 4°C for 1 hr.

The *DET3* cDNA was cloned into pET28c (Novagen, Inc.,

Madison, WI) providing an amino-terminal HIS-tag that allowed affinity purification using Ni-NTA agarose (Qiagen Inc., Chatsworth, CA). The purified protein was used to raise a polyclonal antiserum in rabbits. After affinity purification, the antiserum was covalently attached to immobilized recombinant protein A beads using the rProtein A IgG Plus Orientation Kit (Pierce, Rockford, IL). After immunoprecipitation the beads were incubated at 95°C in SDS sample buffer to dissociate the precipitates from the beads. PKS1 antibody (Fankhauser et al. 1999) coupled to rProtein A was used as a negative control. After separation from the beads samples were run on SDS-PAGE gels and were subjected to immunoblotting. The DET3 antiserum and mAB 2E7 (Ward and Sze 1992) were used at a 1:2000 dilution to detect the immunoprecipitates.

To compare levels of different V-ATPase subunits, 5 µg of microsomal protein per lane were separated by SDS-PAGE and subjected to immunoblotting. The DET3 antiserum and mAB2E7 were used as described and a polyclonal antiserum raised against synthetic peptides corresponding to the amino-terminal domain (MSTTFSGDETA) and the carboxy-terminal domain (SSRAGQSRAE) of subunit c from cotton was used at a dilution of 1:1000.

Enzyme activity measurements

ATPase activity was measured colorimetrically as P_i release (Ames 1966; Benett et al. 1988). V-ATPase activity of microsomal fractions was measured as NO_3^- inhibited, Cl^- stimulated, and vanadate insensitive ATPase activity in the presence of 3 mM Tris ATP, 3 mM $MgSO_4$, 30 mM Tris-MES (pH 7), 1 mM NaN_3, 0.1 mM Na molybdate, 0.5 mM Na vanadate, and 0.01% lysophosphatidylcholine. NO_3^- inhibited activity $(A + N)$ was measured in the presence of 50 mM KNO_3 and Cl^- stimulated activity $(A - N)$ was measured in the presence of 50 mM KCl. The values for $(A - N)$ minus $(A + N)$ in nmol/min per mg protein were determined in three independent measurements and the value for wildtype grown in normal orientation was set to 100%.

Fluorescence microscopy

Transgenic plants expressing gDET3–GFP were examined using an Olympus BX-60 microscope equipped with a mercury lamp and a filter set suited for GFP excitation/emission (470 nm/525 nm). GFP fluorescence was visualized through Uplan Fl objectives and digitized using a Photometrics Quantix CCD camera (Photometrics, Tucson, AZ). Images were processed using the IPLab Spectrum software (Signal Analytics Corp., Vienna, VA) and the Adobe Photoshop software (Adobe Systems, Mountain View, CA).

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Note added in proof

The sequence data for the *DET3* gene reported in this paper have been submitted to the GenBank data library.

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The *Arabidopsis det3* mutant reveals a central role for the vacuolar H$^+$ −ATPase in plant growth and development

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