BIG: a calossin-like protein required for polar auxin transport in *Arabidopsis*

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Polar auxin transport is crucial for the regulation of auxin action and required for some light-regulated responses during plant development. We have found that two mutants of *Arabidopsis*—*doc1*, which displays altered expression of light-regulated genes, and *tir3*, known for its reduced auxin transport—have similar defects and define mutations in a single gene that we have renamed BIG. BIG is very similar to the *Drosophila* gene *Calossin/Pushover*, a member of a gene family also present in *Caenorhabditis elegans* and human genomes. The protein encoded by BIG is extraordinary in size, 560 kD, and contains several putative Zn-finger domains. Expression-profiling experiments indicate that altered expression of multiple light-regulated genes in *doc1* mutants can be suppressed by elevated levels of auxin caused by overexpression of an auxin biosynthetic gene, suggesting that normal auxin distribution is required to maintain low-level expression of these genes in the dark. Double mutants of *tir3* with the auxin mutants *pin1*, *pid*, and *axr1* display severe defects in auxin-dependent growth of the inflorescence. Chemical inhibitors of auxin transport change the intracellular localization of the auxin efflux carrier PIN1 in *doc1/tir3* mutants, supporting the idea that BIG is required for normal auxin efflux.

[Key Words: Auxin transport; light signaling; *Arabidopsis*]

Received April 19, 2001; revised version accepted June 4, 2001.

Regulation of plant growth and development by genetic and environmental signals relies on tightly controlled spatial and temporal distribution of plant hormones. Changes in the level of the auxin, indole-3-acetic acid (IAA), during light-regulated growth responses have been described in several systems (Jones et al. 1991; Behringer and Davies 1992). However, until recently, evidence directly linking light and auxin signal transduction pathways has been lacking. Several gene families encoding primary-response auxin-inducible transcripts have been characterized, including the Aux/IAA genes, the SAUR genes, and the GH3-like genes (Abel and Theologis 1996). In *Arabidopsis*, gain-of-function mutations in the Aux/IAA genes *SHY2/IAA3, AXR2/IAA7*, and *AXR3/ IAA17* promote characteristics of photomorphogenetic development such as short hypocotyls and development of leaves in etiolated seedlings (Tian and Reed 1999; Nagpal et al. 2000). Interestingly, some Aux/IAA proteins may be direct targets of phytochromes because they are phosphorylated by phytochrome A in vitro (Cor-}

lon-Carmona et al. 2000]. The phenotypes caused by mutations in the auxin-responsive GH3-like genes, *FIN219* and *DFL1*, also suggest a role for auxin in light-regulated development. Mutations in *FIN219* result in reduced far-red light–mediated inhibition of hypocotyl elongation and induction of light-regulated genes (Hsieh et al. 2000), whereas mutations in the *DFL1* gene result in a short hypocotyl phenotype in light-grown seedlings that increases in severity with increasing light fluence (Nakazawa et al. 2001). The *NPH4/ARF7* locus encodes a member of the ARF family of proteins, thought to function as transcriptional activators of auxin-regulated genes. Mutations in this gene cause impaired hypocotyl phototropism and other differential growth responses associated with changes in auxin response (Stowe-Evans et al. 1998; Harper et al. 2000).

In aerial parts of the adult plant, IAA is transported basipetally from its site of synthesis at the shoot apex toward the roots in a process referred to as polar auxin transport (Lomax et al. 1995). In roots, auxin moves in the opposite direction in different cell types, acropetally through the root stele and basipetally through epidermal cells (Tsurumi and Ohwaki 1978; Meuwly and Pilet 1991). Disruption of auxin transport affects critical processes such as embryo development, vascular differentiation, stem elongation, flower and root development,
apical dominance, and tropic responses [Lomax et al. 1995].

Physiological studies have indicated that the polar auxin transport system requires the activity of specific auxin influx and efflux carriers located on the plasma membrane of transporting cells. These carriers act to move auxin through files of cells by successively transporting auxin into and out of adjacent cells in the file. Net auxin movement is polar because the efflux carriers are asymmetrically localized in transporting cells [Lomax et al. 1995]. Chemical inhibitors referred to as phytohormones, such as N-1-naphthylphthalamic acid (NPA), specifically inhibit the efflux component of polar auxin transport [Katekar and Geissler 1977; Hertel et al. 1983], apparently by binding to a plasma membrane–associated protein called the NPA-binding protein [NBP, Muday et al. 1993; Bernasconi et al. 1996]. The identity of this protein is not known, but some experiments suggest that it is distinct from the efflux carrier and may act to regulate auxin transport [Morris et al. 1991]. A role of intracellular protein trafficking in the regulation of auxin transport has been proposed, because treatments of tobacco cultured cells with the inhibitor of vesicular transport brefeldin A (BFA) are able to block the auxin efflux system [Delbarre et al. 1998].

Much of our current knowledge of the components of polar auxin transport comes from genetic studies in Arabidopsis. The pin1 mutants have reduced auxin transport and a phenotype similar to plants treated with auxin transport inhibitors, including alterations in vascular development and the formation of pin-like structures instead of flower buds [Okada et al. 1991; Galweiler et al. 1998]. PIN1 encodes a membrane protein that likely functions as an auxin efflux carrier expressed in vascular tissues [Galweiler et al. 1998]. A second related protein, EIR1/AGR1/AtPIN2, performs a similar function in the epidermal and cortical cells in the meristematic and elongation zone of the root [Chen et al. 1998; Luschneg et al. 1998; Muller et al. 1998]. Immunolocalization studies have shown that PIN2 is asymmetrically localized at the periclinal side of the carrier cells, and a model has been proposed that explains directional auxin fluxes in the root [Muller et al. 1998]. Pid1 mutants are similar to pin1 in appearance and are also deficient in auxin transport [Okada et al. 1991]. Pid1 encodes a serine-threonine protein kinase, suggesting a signaling or regulatory function [Christensen et al. 2000]. The influx carrier, an amino acid permease-like protein, is encoded by the AUX1 gene [Bennett et al. 1996; Marchant et al. 1999]. Roots of the aux1 mutant are defective in gravitropic responses and resistant to exogenous auxin [Pickett et al. 1990].

tir3 [transport inhibitor response 3] mutants were isolated in a screen for resistance to the inhibitory effects of NPA on root elongation [Ruegger et al. 1997]. The mutants have a pleotropic phenotype, including decreased inflorescence height, decreased petiole and root length, fewer and shorter siliques, and reduced apical dominance. In addition, tir3 plants display reduced lateral root production, a process that depends on polar auxin transport from the shoot [Reed et al. 1998]. These defects are associated with decreased polar auxin transport in the inflorescence and a reduction in the number of NPA-binding sites. These results suggest that the NBP has a positive role in polar auxin transport and that TIR3 either encodes the NBP or is required for its expression, localization, or stabilization.

An interesting link has been established between polar auxin transport and light responses in hypocotyl growth in Arabidopsis. Steinleder and coworkers [1999] have reported that the light-regulated ATHB-2 gene acts as a negative regulator of shade-induced hypocotyl elongation, and they found this response to be altered in the auxin response mutant axr1-2. A role in this response for auxin transport was supported by experiments in which treatments with NPA caused reduced hypocotyl elongation in response to low red light to far-red light (R:FR) ratios [Steindler et al. 1999]. These results are consistent with experiments performed with Arabidopsis seedlings in which auxin transport was blocked with NPA, suggesting that auxin transport is required for phytochrome-regulated hypocotyl elongation in light-grown plants [Jensen et al. 1998]. Additional data indicating a strong connection between light and auxin transport include reported changes in auxin transport rate and intensity induced by dim red light treatments in cucumber seedlings [Shinkle et al. 1998] and differential growth mediated by lateral transport of auxin triggered by phototropic responses in pea and tobacco [Li et al. 1991].

At the molecular level, one of the best-studied plant responses to light is the transcriptional regulation of nuclear genes encoding photosynthetic proteins, including the chlorophyll a/b-binding proteins of photosystem II (CAB, Terzaghi and Cashmore 1995). CAB genes are expressed at high levels in the light in green plants and at low levels in dark-grown seedlings. The DOC1 gene [dark overexpression of CAB] was identified as a putative component of light signal transduction pathways regulating this response because mutations in the locus lead to overexpression of CAB genes in the dark [Li et al. 1994]. Unlike more pleiotropic mutants with increased CAB mRNA levels in the dark, such as shy2-1 and the det/cop/fus class [Kim et al. 1998; Hardtke and Deng 2000], doc1 seedlings have etiolated morphology when germinated in the dark. Thus, the phenotype of dark-grown doc1 plants suggests that the expression of some light-regulated genes could be genetically separated from the morphological changes that occur during the transition from etiolated to photosynthetic growth.

In this report, we show that doc1 mutants are allelic to tir3 mutants. We describe the cloning of the gene identified by these mutants that we have renamed BIG, owing to its large size. The BIG gene is predicted to encode an enormous protein with significant identity to the Drosophila protein Calossin/Pushover. To better understand the connection between the light- and auxin-related phenotypes, we have performed expression-profiling experiments. These results reveal that altered expression of light-regulated genes in etiolated doc1 seedlings can be suppressed by increasing endogenous auxin levels. In addition, we have shown that NPA has an unusual
effect on PIN1 localization in the doc1/tir3 mutants. We propose that the reduction in levels of NBP in the doc1/tir3 mutants affects cellular localization or function of auxin efflux carriers.

Results

Isolation of new doc1 alleles

To further characterize the DOC1 locus, we isolated new doc1 alleles using two approaches. In the first approach, a doc1 ap3-1 double mutant in the Columbia background was constructed. The ap3-1 mutant contains a temperature-sensitive allele of the apetala3 homeotic gene, which is male sterile at the nonpermissive temperature (Sablowski and Meyerowitz 1998). This feature was used to facilitate crosses of γ-ray–mutagenized pollen from DOC1 plants of the Landsberg erecta [La-er] accession onto the doc1 ap3-1 mutant. We screened ~2000 M1 lines for the doc1 visible phenotype and identified doc1-2, a line that contained Landsberg DNA at PCR markers <0.2 cm from doc1. The progeny of this line consisted of plants with reduced height and increased numbers of inflorescences. Our second approach to isolate new doc1 alleles was to screen transferred-DNA [T-DNA] mutagenized Arabidopsis lines for the morphological phenotype of doc1. During this screen we identified the doc1-3 mutant and confirmed that it was a new doc1 allele by complementation analysis.

The tir3-1 and doc1-1 mutants define mutations in a single gene and have similar defects in auxin transport and CAB gene expression

The Arabidopsis tir3 mutations map to the same region of chromosome 3 as the doc1 mutations and cause very similar morphological defects (Li et al. 1994; Ruegger et al. 1997). Although tir3 and doc1 mutants were isolated in very different mutant screens, complementation tests indicated that tir3-1 and doc1-1 were alleles (data not shown). To determine if each mutation conferred a similar set of defects, we performed auxin transport and CAB gene expression studies on the doc1-1 and tir3-1 alleles, respectively [Fig. 1]. As for tir3-1, doc1-1 stem segments transported approximately one third the amount of IAA compared with that of wild type during an 18-h period, indicating that doc1-1 has an auxin transport defect [Fig. 1B]. Likewise, an analysis of CAB2 gene expression in light- and dark-grown tir3-1 seedlings showed that CAB2 mRNA was more abundant in the dark-grown seedlings compared with wild-type seedlings [Fig. 1A]. Thus, the tir3 and doc1 mutations appear to have similar, if not identical, effects on these diverse aspects of plant growth and development.

Expression profiling of doc1 and doc1 yucca1 mutants

To further examine the effects of the doc1/tir3 mutations on light-regulated gene expression, we performed expression-profiling experiments using the commercially available Arabidopsis Affymetrix GeneChip. These chips monitor the expression of >8200 genes. Of these, >5000 genes gave signals that were significantly above the background in both dark-grown wild-type and doc1-1 seedlings. Any genes that gave a signal below or near the background were assigned as absent on the chip and were not subjected to further analysis. Among 77 genes expressed at least threefold higher in dark-grown doc1-1 than in wild type, we identified many known light-regulated genes, including components of the photosynthetic machinery [Fig. 2B], nitrate reductase, and genes encoding enzymes participating in flavonoid biosynthesis [Table 1]. Interestingly, multiple enzymes for the latter pathway were overexpressed, suggesting increased production of anthocyanins and related ultraviolet-protective pigments. When the doc1-1 mutant was grown in the light, transcripts for the CAB genes, light-regulated genes, and flavonoid biosynthetic genes that were overexpressed in dark-grown doc1-1 accumulated to similar levels in the mutant compared with the wild type [Li et al. 1994; data not shown]. There were also 22 genes, of no particular classification, that had at least a threefold decrease in expression level in doc1-1. Genes with predicted functions with altered expression in dark-grown doc1-1 are shown in Table 1. Because doc1-1 was originally isolated as a mutant with elevated expression of CAB genes in the dark and was later found to be allelic to tir3, we suspected that the derepression of CAB ex-
that type affecting all parts of the seedling, we hypothesized wild type (Zhao et al. 2001). Because been shown to produce at least 50% more free IAA than related genes in dark-grown seedlings analysed by microarray.

Both sets of oligos for the gene (GenBank X56062). Both sets of oligos may also pull out other homologous genes such as experimentsshownhere.Oligosforboth

psaE1(A)

and

psaE1(B)

were designed based on the mRNA sequence of the photosystem-I subunit IV precursor (GenBank AJ245908). Both sets of oligos were designed based on the mRNA sequence of the photosystem-1 subunit IV precursor (GenBank AJ245908). Both sets of oligos were designed based on the mRNA sequence of the CAB gene (GenBank AL049665), and

CAB(B)

oligos were based on the mRNA sequence of the CAB gene (GenBank X56062). Both sets of oligos for the CAB gene may also pull out other homologous genes such as CAB2 in the experiments shown here. Oligos for both

psaE1(A)

and

psaE1(B)

were designed based on the mRNA sequence of the photosystem-1 subunit IV precursor (GenBank AJ245908). Both sets of

psaE1

oligos were designed for detecting the same gene, but whereas some rules for oligo design were dropped for the

psaE1(B)

Microarray experiments were performed using instructions provided by Affymetrix.

expression might be the result of altered auxin distribution. To test this hypothesis, we made use of a recently identified auxin overproducing mutant called

yucca.

The

YUCCA

gene encodes a flavin monooxygenase-like enzyme, and its overexpression in the

yucca

mutant has been shown to produce at least 50% more free IAA than wild type (Zhao et al. 2001). Because

yucca

has a phenotype affecting all parts of the seedling, we hypothesized that

yucca

would suppress
doc-1

if defects in auxin distribution were responsible for the observed

doc1

phenotype. As expected, the dark-grown

yucca doc1-1

double mutant looked similar to the

yucca

mutant (Fig. 2A), and light-regulated genes were not overexpressed (Fig. 2B). Of the 99 genes with altered expression in dark-grown

doc1

mutants, 73 genes were restored to wild-type levels by overexpression of

YUCCA

(Table 1). These results suggest that altered levels of expression of light-regulated genes in dark-grown

doc1

are due to lower concentrations of auxin in some cells, resulting from defective polar auxin transport. The hypothesis that auxin needs to be transported to specific target cells to repress the expression of light-regulated genes is consistent with the fact that exogenous auxin failed to suppress the altered

CAB

gene expression in dark-grown

doc1

seedlings (data not shown). In the light, the aerial phenotype of the double-mutant

yucca doc1-1

included long hypocotyls and epinastic cotyledons similar to the

yucca

mutant. However, the double mutant displayed defective lateral root formation like the

doc1-1

mutant.

Interactions of

tir3

with mutations affecting auxin response and transport

mation between adjacent flowers, so the flowers had an extremely bunched appearance. The phenotypes of these double mutants suggest that the combination of

doc1

with either

pin1

or

pid1

results in a severe phenotype. Therefore, the combination of

tir3-1

with

axr1-12

also results in a severe phenotype. AXR1 encodes a subunit of a heterodimeric RUB-activating enzyme that functions in the RUB conjugation pathway. The RUB (related to ubiquitin) protein is conjugated to the cullin component of SCF-type ubiquitin protein ligases (E3). RUB conjugation appears to be particularly important for function of SCF

TIR1

, an E3 implicated in auxin response (Leyser et al. 1993; del Pozo et al. 1998; Gray et al. 1999). Thus, the

axr1

mutants are deficient in all aspects of auxin response. The

tir3-1 axr1-12

double mutant is an extreme dwarf with a phenotype much more severe than either single mutant (Fig. 3G,H). Like the

tir3-1 pin1

plants, growth of the primary inflorescence in these double mutants arrested prematurely, in this case at ~4 cm of height. Secondary inflorescences continued to elongate, indicative of a loss of apical dominance. In addition, there was very little internode elongation between adjacent flowers, so the flowers had an extremely bunched appearance. The phenotypes of these double mutants suggest that the combination of

tir3-1

with either

pin1

, pid1

, or

axr1

results in a severe defect.
Table 1. mRNAs with altered expression in dark-grown doc1-1

**A. Suppressed by yuca**

| Accession | Gene product description                                      | doc1  | yuca  | doc1 - yuca |
|-----------|--------------------------------------------------------------|-------|-------|-------------|
| M20308    | chalcone synthase                                            | 16.1  | -1.6  | 21.7        |
| AC006585  | phytocyanin/early nodulin-like protein                       | -11.3 | 1.8   | -22.2       |
| AF064064  | flavanone 3-hydroxylase                                      | -6.6  | -1.3  | -7.9        |
| AF134125  | Lhcb2 protein                                                | 6.4   | 1.0   | 7.4         |
| AC006580  | NAM (no apical meristem)-like protein                       | -5.5  | -2.7  | -8.2        |
| AF1060864 | coumarate:CoA ligase 2                                       | 5.0   | -1.3  | 6.3         |
| AL049171  | caffeoyl-CoA O-methyltransferase                             | -5.0  | -3.2  | -8.2        |
| AC002334  | mitochonrial chaperonin HSP60                                 | 4.9   | 1.8   | 6.7         |
| AC002062  | metalloendoproteinase                                        | -4.9  | -2.8  | -7.7        |
| Y14251    | GST11                                                       | 4.8   | -1.0  | 5.8         |
| AC002340  | cytochrome P450                                              | -4.5  | -1.6  | -6.1        |
| AF062590  | peroxisomal 3-keto-acyl-CoA thiolase                         | -4.4  | -1.2  | -5.6        |
| L40031    | 5'-adenosyl-L-methionine-trans-calfolyl-Coenzyme A 3-O-methyltransferase | -4.4  | -1.3  | -5.7        |
| L34693    | thiamin-like protein                                         | 4.3   | 1.5   | 5.8         |
| AF069495  | cytochrome P450 (CYP79B2)                                    | 4.2   | 1.5   | 5.7         |
| AJ245908  | photosystem I subunit IV precursor (psaE1)                   | 4.1   | 1.7   | 5.8         |
| AL021635  | cytochrome P450                                              | 4.0   | -2.2  | 6.2         |
| X65541    | carbonic anhydrase                                           | 3.9   | -4.1  | -8.0        |
| U69604    | 5'-adenylsulfate reductase                                   | 3.7   | -10.2 | -13.9       |
| U70616    | ADP glucose pyrophosphorylase small subunit                  | 3.6   | 1.6   | 5.2         |
| AL021713  | potassium channel protein KAT2                               | -3.5  | -1.4  | -4.9        |
| AC002392  | thioredoxin-like proteins                                    | 3.4   | 2.5   | 6.9         |
| AC005819  | cytochrome b5                                                 | 3.3   | -1.4  | -4.7        |
| U75201    | germin-like protein (GLP1)                                   | 3.2   | -2.2  | -5.4        |
| AL078470  | glycine-rich cell wall structural protein                    | 3.2   | 1.0   | 4.2         |
| AC006920  | putative xyleglucan-fucosyltransferase                       | 3.2   | 1.3   | 4.5         |
| AJ245632  | photosystem I subunit VI precursor (psaH2)                   | 3.2   | 1.5   | 4.7         |
| AL049655  | chlorophyll a/b-binding protein                              | 3.2   | 1.7   | 4.9         |
| AF134132  | early light-inducible protein (Elip)                         | 3.2   | 2.1   | 5.3         |
| AC003033  | putative glucanase                                           | 3.1   | 1.5   | 4.6         |
| M86358    | chalcone isomerase                                           | -3.1  | -1.1  | -4.2        |
| Z97342    | disease resistance PPR5-like                                 | -3.1  | -1.6  | -4.7        |
| AC002343  | cellulose synthase                                           | 3.0   | -1.0  | 4.0         |
| X56062    | chlorophyll a/B-binding protein                              | 3.0   | 1.6   | 4.6         |
| X13434    | nitrate reductase                                             | 3.0   | 2.2   | 5.2         |
| U84259    | flavonol synthase                                            | 3.0   | -2.7  | -5.7        |
| AC004122  | branched-chain amino acid aminotransferase                   | -6.1  | 1.6   | -7.7        |
| AC003981  | L-allethreonin aldolase homolog                              | -6.1  | 1.5   | -7.6        |
| AC002391  | auxin-responsive GH3-like protein                            | -5.3  | 8.0   | 13.3        |
| Z97341    | cyanohydrin lyase-lyase protein                               | -4.4  | 2.9   | -7.3        |
| AF020109  | anthranilate N-benzoyltransferase-like protein                | -4.2  | 1.2   | -5.4        |
| AL022197  | putative cytochrome b561                                     | -3.8  | 2.0   | -5.8        |
| Z97335    | selenium-binding protein-like                                | -3.8  | 2.1   | -5.9        |
| AC002560  | putative 1-aminoacylopropane-1-carboxylate oxidase           | -3.7  | -7.2  | -10.9       |
| A71596    | berberine bridge enzyme-like                                 | -3.7  | -2.1  | -5.8        |
| AF077955  | branched-chain alpha keto-acid dehydrogenase E1 alpha subunit | -3.5  | 1.5   | -5.0        |
| AF055372  | At4 protein                                                 | -3.3  | -1.2  | -4.5        |
| L25657    | cor75 protein                                               | -3.3  | -2.1  | -5.4        |
| X8263     | beta-glucosidase-like                                        | -3.3  | -3.7  | -7.0        |
| AC007576  | putative mandelonitrile lyase                                | -3.1  | -2.4  | -5.5        |
| U81369    | MADS box protein (AGL3)                                     | -3.0  | -1.5  | -4.5        |

**B. Not suppressed by yuca**

| Accession | Gene product description                                      | doc1  | yuca  | doc1 - yuca |
|-----------|--------------------------------------------------------------|-------|-------|-------------|
| AC006068  | similar to ch-TOG protein from Homo sapiens                  | -16.4 | -21.6 | -38.0       |
| U81294    | germin-like protein (GLP9)                                   | -16.3 | -3.6  | -19.9       |
| AC002343  | putative cellulose synthase                                  | 9.8   | 2.3   | 7.5         |
| AC007212  | putative peroxidase                                          | -9.3  | -11.6 | -21.0       |
| AC002131  | extensin-like protein                                         | -8.8  | -31.8 | -40.6       |
| AL035396  | pollen-specific protein precursor like                       | -7.2  | -6.6  | -13.8       |
| AL035396  | putative glycerol-3-phosphate permease                       | 6.3   | 10.9  | 17.2        |
| AC003727  | putative para-aminobenzoate synthase and glutamine amidotransferase | -6.0  | -5.6  | -11.6       |
| AC006081  | 50S ribosomal protein L4                                     | -5.8  | 8.2   | 14.0        |
| AR005929  | putative squalene epoxidase                                  | 4.0   | 4.7   | 8.7         |
| AC004625  | putative peroxidase                                          | 3.5   | -1.8  | 5.3         |
| AF069442  | putative leucine-rich repeat protein                         | -3.5  | -2.3  | -5.8        |
| Z99595    | putative peroxidase                                          | 3.3   | -1.9  | 5.2         |
| U94998    | non-symbiotic hemoglobin                                     | -9.3  | -1.5  | -10.8       |
| AC007210  | putative MADS-box protein ANR1                               | -5.5  | -4.0  | -9.5        |
| AC002341  | putative cysteine protease                                   | -4.6  | -1.2  | -5.8        |
| AC004218  | nodulin-like protein                                         | -4.1  | -4.0  | -8.1        |
| AL035327  | peptide transporter-like protein                              | -3.8  | 1.2   | -2.6        |

- Indicates that the expression level in wild type is very low and the signals are near or below background. The fold changes of those genes are only approximate.
inauxin-dependent growth of the inflorescence, presumably associated with a reduction in internode elongation and the cessation of meristem activity shortly after the floral transition.

Identification of the DOC1/TIR3 gene

To investigate the molecular mechanism of DOC1/TIR3 action, we cloned the gene by a combination of chromosome walking and T-DNA tagging. First, we performed an extensive fine-mapping of the doc1 locus by identifying recombinant chromosomes from a mapping cross between doc1-1 (Col-0) and La-er using cleaved amplified polymorphic sequence (CAPS) and single sequence length polymorphism (SSLP) markers [Konieczny and Ausubel 1993; Bell and Ecker 1994]. DOC1 was mapped between the SSLP marker nga32 and the CAPS marker 17D8LE on the top of chromosome 3. A set of overlapping yeast artificial chromosomes (YACs) spanning the region between both markers had been identified previously [Bartel and Fink 1995]. YAC ends from yUP6B and yUP2D2, and the COL2 gene [Ledger et al. 1996] that maps between them, were isolated and subsequently used as probes to screen cosmids and bacterial artificial chromosome (BAC) libraries. Multiple cosmids and BACs overlapping the region between markers nga32 and 17D8LE were identified [Fig. 4A]. Fragments from BACs F9O20, F18H18, and F26L9 were mobilized into a binary vector and together with cosmids clones were used to generate a complete contig.

Southern blot analyses using BAC F18H18 as a probe indicated that the doc1-3 allele contains a T-DNA insertion of ~4 kb. DNA probes derived from this BAC were used in Southern blot analyses to further delimit the T-DNA insertion site to a 2-kb genomic region defined by the 6B2RE YAC end [Fig. 4A]. Sequencing of T-DNA flanking sequences revealed that the insertion had disrupted an open reading frame (ORF) spanning 17,331 bp of genomic DNA represented as incomplete fragments in several clones of our contig. Given the remarkably large size of this gene [for Arabidopsis], we have renamed it BIG. The putative start codon [Net Start score, 0.770] was located immediately downstream of a DNA region of 257 bp with 60% identity to the 5’/H11032 region of an Arabidopsis L-isoaspartylmethyltransferase gene [Mudgett and Clarke 1996], including a putative TATA box (TATTAAT) at position −208 with respect to the putative start codon. BAC sequences from the putative gene were used to screen a cDNA library and to perform BLAST searches of the dbEST database. Several expressed sequence tags (ESTs) from Arabidopsis identical to regions of the putative transcript were present in GenBank. EST accession Z47688 included a poly[A] tail that indicated that mRNAs for this gene include a 3’/H11032 untranslated region (3’UTR) of 165 bases. Overlapping cDNA clones and RT–PCR products were assembled to reconstruct a transcript 15.4 kb long consisting of 14 exons [Fig. 4B].

To confirm that the BIG gene was DOC1/TIR3, we identified the specific mutations in five doc1 and tir3 alleles [Fig. 4B]. The T-DNA insertion in doc1-3 was localized before position +7228 with respect to the putative start codon. The mutation in doc1-1 was a base pair change from G to A at position +5513 that causes an amino acid change from Cys to Tyr. The doc1-2 and tir3-1 mutations were also found to be single base pair changes [from C at position +8872 to T and from T at position +7331 to A, respectively] likely to affect pre-mRNA splicing as they disrupt the acceptor site of intron 7 and the donor site of intron 4, respectively. The tir3-2 mutation was a small deletion from position +8874 to +8883 (CTTCAGGTTC) that causes a frameshift of the ORF, causing a stop codon after seven amino acids. Southern analysis using fragments of the gene as

Figure 3. The tir3 mutation confers a severe phenotype in combination with auxin transport and response mutants. Plants were photographed after 45 d of growth in potting soil (see Materials and Methods). All bars represent 3 cm. (A) wild type; (B) tir3-1; (C) pin-formed1-1; (D) tir3-1 pin-formed1-1; (E) pinoid1-3; (F) tir3-1 pinoid1-3 (insert: a primary inflorescence dissected from a tir3-pid1 plant; the arrow points to a terminal pin-shaped organ); (G) from left to right: wild type, tir3-1, axr1-12, and tir3-1 axr1-12; (H) larger image of tir3-1 axr1-12 plant showing severe reduction in elongation between flowers.
probes suggested that BIG is a single copy gene [data not shown]. This was later confirmed as BLAST searches to the complete Arabidopsis genome failed to identify any DNA or protein sequence similar to the BIG gene.

BIG encodes a putative member of the Calossin/Pushover family of proteins

The predicted BIG protein is 5077 amino acids long [MW 566 kD, pH 5.7] and very similar in size and features to the Drosophila protein Calossin/Pushover (CalO; GenBank no. AF007931; Xu et al. 1998). CalO was first identified because it binds to calmodulin in vitro [Xu et al. 1998]. Mutations in the pushover [push] locus cause defects in behavior, altered synaptic transmission at the neuromuscular junctions, male sterility, and defective achiasmic segregation of chromosomes in female meiosis [Richards et al. 1996; Sekelsky et al. 1999]. There are very similar predicted proteins of unknown function present in the human and Caenorhabditis elegans genomes (GenBank accession nos. AB007931 and AF003140, respectively). In addition, numerous ESTs from several animals and plants encoding putative proteins highly similar to these proteins have been identified, suggesting that BIG belongs to a class of proteins ubiquitously present in the plant and animal kingdoms.

Gapped alignment indicated that BIG and CalO are 28% identical at the amino acid level over >3000 amino acids at their C terminus, with a BLAST score of 885 and an E-value of 0.00 (Fig. 4C; Altschul et al. 1997). However, the similarity between these proteins is not uniform. Rather, it is particularly high (>60% identity) in several stretches 30 to 50 amino acids long, including two cysteine-rich domains that resemble Zinc fingers. The first cysteine-rich domain (CRD-1) at amino acid position +1564 has similarity to a putative Zinc finger domain present in some ubiquitin ligases (Fig. 4C; Pfam accession PF02207; E value = 2.3e-13). This domain
seems to be important for BIG function because the doc1-1 mutation is a substitution of one of the conserved Cys to Tyr [Fig. 4C]. The second conserved cysteine-rich domain (CRD-2) at position +3442 is highly conserved among the Calossin family of proteins [Fig. 4C] and is similar to Zn-finger domains in eukaryotic transcription factors. A third cysteine-rich domain at position +2597 belongs to the ZZ domain family but is not conserved in the Calossin proteins [Fig. 4C; Pfam accession no. PF00569; E = 1.6e-07]. Proteins containing this domain include proteins that function in the ubiquitin pathway such as HERC2 and p62 [GenBank T14346; Vadalumudi and Shin 1998], transcriptional coactivators such as the CREB-binding protein (CBP) and ADA2 [Chrivita et al. 1993; Marcus et al. 1994], and Dystrophyn-related proteins [Roberts and Bobrow 1998].

Expression of BIG

Doc1/tir3 mutations cause reduced growth in most plant organs, suggesting that BIG is expressed ubiquitously during development [Li et al. 1994; Ruegger et al. 1997]. However, several putative components of the polar auxin transport machinery are expressed preferentially in particular organs or cell types. To test if the BIG gene was expressed in specific tissues, we performed quantitative RT–PCR analyses to monitor changes in mRNA accumulation. We compared BIG mRNA levels in roots, rosette leaves, inflorescence stems, and flowers. As shown in Figure 5A, BIG mRNA accumulated to similar levels in different organs of wild-type Arabidopsis. Because doc1/tir3 mutants display increased accumulation of CAB mRNA in the dark, BIG mRNA levels were compared between light-grown and dark-grown seedlings.

Similarly, because of the effects of the doc1/tir3 mutations on auxin transport, we monitored BIG mRNA levels after seedlings were treated with 50 μM of the synthetic auxin 2,4-D. In addition, we also tested induction of BIG with 1 μM brassinolide, a steroid hormone known to act synergistically in several responses controlled by auxin [Kim et al. 2000]. In all cases, BIG mRNA levels remained unchanged [Fig. 5B]. As a control for the auxin treatments, we checked and detected elevated mRNA levels of the auxin-inducible gene SAUR-AC1 [Fig. 5B; Gil et al. 1994]. Interestingly, this auxin-induced transcript served as a double control, because it was also induced by brassinolide treatment. The results of these experiments are consistent with BIG being ubiquitously and constitutively expressed throughout the plant.

Localization of PIN1 in tir3 and doc1 seedlings

Because the mutant phenotype suggests a key role for BIG in polar auxin transport [Ruegger et al. 1997], the cellular localization of the putative auxin efflux carrier PIN1 [Galweiler et al. 1998] in tir3-1 and doc1-3 was determined. Using affinity-purified anti-PIN1 antibody, whole mount immunolocalization experiments on Arabidopsis seedlings were performed. In wild-type roots, PIN1 is localized at the bottom [acropetal] side of stele cells, in some cases accompanied by a weaker signal in the endodermis [Friml et al., in prep.]. The polarity of PIN1 localization was partially disrupted by treatment of seedlings with polar auxin transport inhibitors like NPA [Geldner et al., in prep.]. In untreated tir3 or doc1 seedlings, no difference in PIN1 localization was detected compared with that of wild type [data not shown]. However, in mutants treated with NPA, >50% of roots [doc1: 52%, n = 83; tir3: 61%, n = 165], showed mislocalization of PIN1 protein to an unidentified intracellular compartment [Fig. 6]. This phenomenon was never observed in wild-type roots [n = 167] treated with NPA.

Discussion

Mutations in the BIG gene were identified in two independent screens and originally characterized as mutants affecting photomorphogenesis and auxin transport. In this report, we present evidence indicating that the defect in light-regulated gene expression first identified in the doc1 mutant is caused by an alteration in auxin distribution in dark-grown seedlings. BIG was identified as a putative component of the polar auxin transport machinery because tir3 mutations promote resistance to inhibition of root growth by phytotropins, decrease IAA movement through inflorescence stems, and result in a reduction in the number of NPA-binding sites in microsomal fractions. Moreover, many of the morphological defects observed in the mutants are consistent with a defect in some aspect of auxin physiology. In this study, we provide several additional lines of evidence indicating the involvement of BIG in polar auxin transport. These include synergistic effects of the tir3 mutation
tissue is overlaid with the green immunofluorescence channel, color. The tissue autofluorescence indicated by red color of stele signals of the FITC-conjugated antibody are indicated by green was imaged by laser-confocal microscopy. Indirectly visualized a FITC-conjugated secondary antibody. Fluorescent staining was performed using primary antibodies raised against PIN1 and ment with 150 µM NPA for 3 h. Immunocytochemical analysis suppression of some mutations that alter auxin signaling or transport, with 150 µM NPA for 3 h. Immunocytochemical analysis was performed using primary antibodies raised against PIN1 and a FITC-conjugated secondary antibody. Fluorescent staining was imaged by laser-confocal microscopy. Indirectly visualized signals of the FITC-conjugated antibody are indicated by green color. The tissue autofluorescence indicated by red color of stele tissue is overlaid with the green immunofluorescence channel, facilitating the visualization of PIN1.

with mutations that alter auxin signaling or transport, suppression of some doc1 defects by elevated levels of endogenous auxin, and effects of doc1/tir3 mutations on the intracellular localization of the auxin efflux carrier PIN1.

A number of additional mutations have been isolated that affect both auxin physiology and photomorphogenesis. Mutations in the shy2, axr2, and axr3 loci cause photomorphogenesis in dark-grown seedlings, including reduced hypocotyl length and formation of leaves (Tian and Reed 1999, Nagpal et al. 2000). However, unlike these signaling mutants, the doc1/tir3 defects in auxin transport have little effect on seedling morphology and hypocotyl elongation in the dark (Li et al. 1994) but result in decreased hypocotyl elongation in the light. This is consistent with results from Jensen et al. (1998) that indicate that in Arabidopsis, polar auxin transport is important for hypocotyl elongation in the light but not in the dark. On the other hand, our results indicate that overexpression of the auxin biosynthetic gene YUCCA in the doc1-1 mutant suppresses the aberrant expression of light-regulated genes in the dark. This result indicates a novel and unexpected role for auxin in the repression of light-regulated genes in dark-grown seedlings and suggests that polar auxin transport is important in etiolated seedlings.

The tir3-1 mutation shows a strong synergistic interaction with mutations that affect either auxin response or transport, consistent with the proposed role for BIG in auxin transport. The phenotype of the tir3 pin1 double mutant includes a drastic reduction in inflorescence growth and premature arrest of this growth, suggesting that BIG and PIN1 have overlapping functions in the inflorescence. Because BIG is expressed throughout the plant, it will be interesting to determine if combination of tir3 with mutations in other members of the PIN family will result in additional synergistic effects. Multiple growth responses are also compromised in the auxin-signaling mutant axr1-12. The tir3-1 axr1-12 double mutant also had a very severe phenotype as expected for plants with defects in both auxin transport and response. One of the most interesting aspects of all three double-mutant phenotypes was the early arrest of inflorescence elongation. This observation suggests that auxin levels and/or response are limiting for inflorescence elongation and organ formation.

Any model for BIG function must explain the contradictory responses of the doc1/tir3 mutants to NPA. On the one hand, the tir3 mutants were isolated because they are resistant to the growth-inhibiting properties of NPA. In contrast, the doc1/tir3 mutants appear to be hypersensitive to NPA with respect to effects on PIN1 localization. However, this paradox can be resolved by considering the effects of NPA on root elongation and the basis for tir3 resistance to the compound. Evidence from a number of studies now indicates that NPA inhibits root elongation by promoting accumulation of IAA to inhibitor levels in the root tip [Ruegger et al. 1997; Sabeti et al. 1999; Casimiro et al. 2001]. As the tir3 mutant is deficient in auxin transport in the shoot, it follows that less auxin is transported down into the mutant root system. As a consequence, NPA-mediated accumulation of IAA in the root tip is reduced, leading to less inhibition of root elongation. This view is also consistent with the decrease in lateral root formation in tir3 seedlings, a process that depends on IAA from the shoot system. The key element of this model is that decreased inhibition of root elongation in tir3 is not primarily due to a decrease in the number of NPA-binding sites but rather to a decrease in auxin transport into the root system.

In contrast, the effects of NPA on PIN1 localization are likely to be related to a decrease in NPA-binding sites. A reduction of NPA-binding sites is also evident in the tir3-1 mutant [Ruegger et al. 1997], suggesting that BIG encodes or is required for expression, localization, or stabilization of the NBP. Because none of the doc1/tir3 mutations are proven null alleles, the additive effects of doc1/tir3 mutations and NPA-treatments on PIN1 localization are not conclusive in defining a role for BIG in normal NBP microsomal localization. Recent results have suggested that NPA does not have a specific role in auxin transport. Rather, NPA appears to affect trafficking of a number of unrelated proteins to the plasma membrane [Geldner et al., in prep.]. The fact that auxin transport is particularly sensitive to NPA and related compounds may be a reflection of the importance of correct polar localization of the efflux carriers to the auxin transport process. Consistent with this, intracellular vesicle transport is clearly required for polar auxin transport [Delbarre et al. 1998; Steinmann et al. 1999]. In Droso phila, CalO affects synaptic transmission at the neuromuscular junctions, with specific defects in neurotransmitter release evoked by nerve stimulation. This
process is dependent on the synaptic vesicle cycle (Sudhof 1995). Thus, it is possible that BIG and CalO participate in intracellular vesicle transport. There is also a well-established role for changes of intracellular Ca$^{2+}$ levels in this process.

Calossins are among the biggest proteins in eukaryotic cells. The Drosophila CalO protein was first identified by screening a retinal expression library for calmodulin-binding proteins (Xu et al. 1998). At this point it is not known if BIG also binds calmodulin, but it is possible that this protein participates in Ca$^{2+}$/calmodulin regulation of auxin transport because Ca$^{2+}$ and calmodulin have been implicated in auxin transport responses (Lomas 1995). Although the precise biochemical role of BIG and other calossin-like proteins in cellular metabolism awaits further study, the presence of a number of protein domains suggests a few general possibilities. The three Zn-finger domains are likely to participate in protein-protein interactions, suggesting that BIG is likely to interact with a number of other proteins. Among the Zn-finger domains, the CDR-1 motif is very well conserved in calossins and several ubiquitin ligases represented by the Drosophila protein hyperplastic discs (Hy; Mansfield et al. 1994). This domain is critical for BIG function because the doc1-1 mutation replaces one of the putative Zn-binding cysteines. The function and binding partners of the CDR-1 motif are not known. Interestingly, among the proteins similar to BIG at another Zn-binding domain, the ZZ domain, there is a family of very large proteins that includes dystrophin. This protein is associated with the plasma membrane and the cytoskeleton in postsynaptic neuronal complexes and plays a role in the maintenance of membrane-associated complexes at points of intercellular contact (Roberts and Bobrow 1998). Moreover, the ZZ domain has been found to interact with calmodulin (Anderson et al. 1996). In the future, it will be important to determine the cellular localization of BIG and the identity of interacting proteins. Clearly, BIG and other calossins play a critical role in some aspect of cellular function in both plants and animals.

Materials and methods

Plant materials and growth conditions

For all experiments described, unless specified otherwise, the Arabidopsis wild type is ecotype Columbia. For mutant screens to identify new doc1 alleles, plants were grown on 0.5x MS media plates and transferred to soil under conditions described previously (Li et al. 1994). To generate male sterile doc1-1 ap3-1 mutants, plants were grown initially in the greenhouse and then transferred to 28°C before bolting. For microarray experiments, wild-type and mutant plants (doc1-1, doc1-1 yucca, and yucca) were grown in 0.5x MS media without sugar in the dark at 22°C for 4 d before they were harvested for RNA preparation. For immunolocalization experiments, seeds were surface sterilized for 4 d before they were harvested for RNA preparation. For the generation of the doc1-2 mutant, La-er pollen was γ-ray mutagenized by exposing inflorescence sections to 17 to 20 Krads in a 60Co irradiator. Irradiated flowers were used for manual fertilization of doc1-1 ap3-1 mutants. The doc1-3 allele was identified by its visible phenotype among a collection of transgenic plants containing T-DNA insertions (by-products of several projects) compiled by Dr. Jianming Li in Dr. Joanne Chory’s laboratory at the Salk Institute. To determine alleleism between tir3-1 and doc1-1, these mutants were crossed, and F1 seeds were plated on nutrient media containing 5 µM of the auxin transport inhibitor 2-carboxyphenyl-3-phenylpropionate-1,2-dione [CPD] to assay resistance to inhibition of root growth. F1 seedlings (25/25) were resistant to CPD. The seedlings were transferred to soil for later confirmation of a mutant aerial phenotype.

Identification of new alleles of doc1 and genetic analysis

A homozygous doc1-1 line was crossed to La-er, and the resulting F1 plants were self-pollinated to generate F2 plants segregating the doc1-1 mutation. DNA for SSLP and CAPS analysis from doc1 F2 plants was prepared as described previously (Dellaporta et al. 1983). To place the doc1 locus in the physical map between markers nga32 and 17D8LE, 1091 chromosomes were scored for recombination events. Standard methods for YAC manipulation and generation of end probes were as described (Gibson and Somerville 1992). Cosmid library (Olszewski et al. 1994) was hybridized using YAC ends as described previously (Dellaporta et al. 1983). To place the doc1 locus in the physical map between markers nga32 and 17D8LE, 1091 chromosomes were scored for recombination events. Standard methods for YAC manipulation and generation of end probes were as described (Gibson and Somerville 1992). Cosmid library (Olszewski et al. 1998) and BAC filters (provided by Dr. Joe Ecker, University of Pennsylvania) were hybridized using YAC ends as described (Auszbel et al. 1994; http://http.tamu.edu:8000/~creel/BACVEC.html). The dCAPS marker (Neff et al. 1998) COL2 required oligonucleotides 5′-GGCCATAGGAGTTTAGAAGG-3′ and 5′-CAT CACAAGTGATGAGTTTAGAACGG-3′ as primers for PCR amplification followed by digestion with the restriction enzyme BcgI.

DNA and RNA analysis

DNA from yeast and plant tissues was isolated according to standard protocols (Ausubel et al. 1994). Total RNA was puri-
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Acknowledgments

We thank Kim Hanson and Melissa McCarthy for technical support, and Adan Colon-Carmona, Jianming Li, and Karin Schumacher for their help in generating and identifying the *doc1*-3 T-DNA line. Seeds of *ap3-1* and a cosmid library were supplied from DFG to K.P., and by a Marsden grant of New Zealand to K.P., and by grants from the Department of Energy (DE-FG03-89ER13993) and the National Science Foundation (MCB96-31390) to J.C., by grants from the Department of Energy (DE-FG02-98ER20313) and the National Institutes of Health (GM43644) to M.E., by a grant from DAAD to J.F., by a grant from DFG to K.P., and by a Marsden grant of New Zealand to J.P. and K.S. J.C. is an Associate Investigator of the Howard Hughes Medical Institute (HHMI), and Y.Z. is a HHMI fellow of the Life Sciences Research Foundation.

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*Genes Dev.* 2001, 15:

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