The gene *MACCHI-BOU 4/ENHANCER OF PINOID* encodes a NPH3-like protein and reveals similarities between organogenesis and phototropism at the molecular level

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Intercellular transport of the phytohormone auxin is a significant factor for plant organogenesis. To investigate molecular mechanisms by which auxin controls organogenesis, we analyzed the *macchi-bou 4* (*mab4*) mutant identified as an enhancer of *pinoid* (*pid*). Although *mab4* and *pid* single mutants displayed relatively mild cotyledon phenotypes, *pid mab4* double mutants completely lacked cotyledons. We found that *MAB4* was identical to *ENHANCER OF PINOID* (*ENP*), which has been suggested to control PIN1 polarity in cotyledon primordia. *MAB4/ENP* encodes a novel protein, which belongs to the NON-PHOTOTROPIC HYPOCOTYL 3 (*NPH3*) family thought to function as a signal transducer in phototropism and control lateral translocation of auxin. *MAB4/ENP* mRNA was detected in the protodermal cell layer of the embryo and the meristem L1 layer at the site of organ initiation. In the *mab4* embryo, the abundance of PIN1:GFP was severely decreased at the plasma membrane in the protodermal cell layer. In addition, subcellular localization analyses indicated that *MAB4/ENP* resides on a subpopulation of endosomes as well as on unidentified intracellular compartments. These results indicate that *MAB4/ENP* is involved in polar auxin transport in organogenesis.

**KEY WORDS:** Polar auxin transport, Organ development, PIN1, PID, NPH3, Arabidopsis thaliana

**INTRODUCTION**

In higher plants, aerial plant architecture is mainly characterized by the arrangement of leaves and flowers around the stem. Leaves and flowers are formed from the shoot apical meristem (SAM) at well-characterized angles. This pattern of organ development is known as phyllotaxis. A cotyledon is an embryonic leafy organ that is first formed after fertilization, and developed in the apical portion of the embryo from the globular stage onwards. In the case of dicotyledonous plants, the aerial part of the seedling displays bilateral symmetry, as demonstrated by two symmetrically located cotyledons on either side of the SAM. Previous studies have shown that organ positioning is mediated by localized concentrations of the phytohormone auxin during both embryonic and postembryonic development (Benková et al., 2003). Local accumulation of auxin is induced by a directed intercellular transport system from the site of its biosynthesis, referred to as polar auxin transport. In this process, auxin efflux carriers play a key role. They are localized at the plasma membrane with a uniform polarity between cells. This is supposed to permit directional auxin transport through organs and tissues. Recently, genetic analyses and studies in heterologous expression systems indicate that PIN-FORMED (PIN) and P-glycoprotein (PGP) transport proteins function in mediating auxin efflux from cells. In Arabidopsis, mutations of PIN family genes cause phenotypes consistent with impaired polar auxin transport, as pin-formed inflorescences in *pin1*, reduce tropic response in *pin2* and *pin3*, and disrupt the apical-basal axis in *pin7* embryos (Okada et al., 1991; Gälweiler et al., 1998; Luschnig et al., 1998; Chen et al., 1998; Muller et al., 1998; Friml et al., 2002; Friml et al., 2003). Mutations of PGP genes in Arabidopsis, maize and sorghum result in reductions of growth and polar auxin transport (Noh et al., 2001; Noh et al., 2003; Geisler et al., 2003; Geisler et al., 2005; Multani et al., 2003; Lin and Wang, 2005). When expressed in yeast and mammalian cell lines, both PIN and PGP proteins activate the efflux of indole-3-acetic acid, the natural and main auxin, and an artificial auxin (Petrásek et al., 2006; Geisler et al., 2005). PIN-mediated auxin efflux appears to be necessary to establish and maintain the proper auxin distribution, as there are obvious defects of development in *pin* mutants that have not been reported in *pgp* mutants.

In the SAM, PIN1 localizes to the plasma membrane of the epidermis and the vasculature of organ primordia. In the meristem L1 layer, PIN1 localization is preferentially oriented toward the center of incipient organs (Reinhart et al., 2003). In cotyledon development, PIN1 localization is restricted to the apical side of the plasma membrane in the protodermal cells, as well as in the SAM (Steinmann et al., 1999; Benková et al., 2003; Treml et al., 2005). These data indicate that auxin is transported from the site of biosynthesis via the meristem L1 layer into the SAM or the protodermal cell layer in the embryo. Auxin is redistributed and accumulated at the sites where it will promote the initiation of organs. In organogenesis, the mechanism of the regulation of PIN1 polarity and auxin accumulation at the site where a primordium will initiate has remained an important question.

Previous studies have shown that auxin itself modulates the subcellular localization of PIN proteins. PIN proteins constitutively cycle between the plasma membrane and endosomes (Geldner et al., 2001; Geldner et al., 2003). Auxin treatment blocks PIN endocytosis, and promotes PIN accumulation and activity at the plasma membrane in Arabidopsis roots (Paciorek et al., 2005). In pea epicotyls, local auxin...
accumulation leads to rearrangements in polar localization of PIN proteins (Sauer et al., 2006). These data provide a mechanism for the feedback regulation of auxin transport. By genetic studies, several factors were identified as regulators of PIN localization. In Arabidopsis, mutations in GNOM/EMB30, BIG/TIR3/DOC1, PID, and ENHANCER OF PINOID (ENP) genes disrupt the localization of PIN protein. The GNOM/EMB30 gene encodes a GDP-GTP exchange factor for small G-proteins of the ARF class, involved in coat recruitment and cargo-selective vesicle trafficking (Shevall et al., 1994). The gnom/emb30 mutation leads to a disorganized PIN localization (Steinmann et al., 1999). The BIG/TIR3/DOC1 gene encodes a calossin-like protein. In the mutants, treatment with polar auxin transport inhibitors causes mislocalization of PIN1 protein to an unidentified intracellular compartment (Gil et al., 2001). The PID gene encodes a Ser/Thr kinase, involved in polar auxin transport (Christensen et al., 2000; Benjamins et al., 2001). In pid inflorescences, PIN1 polarity is completely reversed in the meristem L1 layer. Inversely, overexpression of PID induces a reverse redistribution of PIN proteins. These results suggest that PID controls PIN polarity (Friml et al., 2004). ENP is also suggested to control PIN1 polarity in concert with PID, as the enp mutation, when combined with the pid mutation, causes a reverse localization of PIN1 protein in the apex of the embryo resulting in a loss of cotyledon primordia (Treml et al., 2005). In addition, blue light-photostimulation has been shown to delocalize PIN1 protein in hypocotyls on the side distal to the light source (Blakeslee et al., 2004). Apparently, this phenomenon is mediated by PHOTOTROPIN 1/Non-PHOTOTROPIC HYPOCOTYL 1 (PHOT1/NPH1), which belongs to the same kinase family as PID, since PIN1 delocalization was not observed in blue light-treatment phot1/nph1 mutant hypocotyls.

In this paper, we report the identification of the macchi-bou 4 (mab4) mutant, which is defective in organogenesis, as a pinoid (pid) enhancer mutant. Whereas mab4 and pid single mutants display minor cotyledon phenotypes, pid mab4 double mutants completely lack cotyledons. We found that MAB4 was identical to ENP, which is suggested to control the PIN1 polarity (Treml et al., 2005). MAB4/ENP encodes a novel protein, which belongs to NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) family supposed to function as a signal transducer in phototropic response and regulate PHOTOTROPIC HYPOCOTYL 3 (NPH3) family expressed in the protodermal cell layer of the embryo and in the meristem L1 layer at the site of organ initiation. In mab4-1 embryos, PIN1:GFP (green fluorescent protein) abundance in the plasma membrane of the protodermal cells was severely reduced. Moreover, in cultured Arabidopsis cells, MAB4/ENP, existed in a fraction of endosomes and unidentified intracellular compartments, partially colocalized with PID. These results demonstrate that MAB4/ENP functions as a regulator of polar auxin transport in organogenesis in concert with PID and imply similarities in molecular mechanisms between organogenesis and phototropicism.

MATERIALS AND METHODS
Plant strains and growth conditions
Arabidopsis thaliana ecotype Columbia (Col) was used as the wild type. The following mutant alleles were used: pin1-201 (Col) (Furutani et al., 2004), pin1-3 [Lansberg erecta (Ler)] (Bennett et al., 1995), pid-15 (Ler) (Treml et al., 2005), pid-2 (Ler) (Christensen et al., 2000), pid-3 (Col) (Bennett et al., 1995). mab4-1 was isolated from the M2 population of Col that had been mutagenized by fast neutron exposure (Lehle Seeds, Round Rock, TX, USA). enp was isolated from the M2 population of Ler mutagenized by ethyl methanesulfonate (Treml et al., 2005). mab4-2 carries a T-DNA insertion at the 5′ untranslated region. This allele was obtained from the Arabidopsis Biological Resource Center (SALK_104491) (Alonso et al., 2003) and was backcrossed three times to wild-type Col before any analysis and construction of pid mab4 double mutants. Plants were grown on soil as previously described (Fukaki et al., 1996), and siliques were collected for analyses of embryo phenotypes and in situ hybridization. Stages of embryogenesis were as defined previously (Jürgens and Mayer, 1994). For analysis of seedling phenotypes, seeds were surface sterilized and germinated on Murashige and Skoog plates, as previously described (Aida et al., 1997).

Alleleism tests
To examine allelism in MAB4 and ENP, pid-3+/mab4-1 seedlings were used to test whether pid-15+/enp+ alleles are identical. The F1 progeny segregated cotyledon-lacking seedlings (2 out of 11 seedlings). Genotypes of the PID locus in the F1 seedlings were confirmed by using PCR primers that detected the pid-3 and pid-15 mutations.

Mapping and cloning of the MAB4/ENP gene
The mab4-1 mutant was crossed with pid-2 (Ler) for mapping of the MAB4/ENP locus. Using the ~300 cotyledonless seedlings in F2 progeny, the MAB4 locus was mapped between the T5N24 CAPS marker and nga1139, a well-known SSLP marker on chromosome 4. Moreover, the MAB4 locus was mapped to the 315-kb region between the F3L17 CAPS marker (one recombinant) and the F10M6 CAPS markers (six recombinants) on chromosome 4 after F2 or F3 analysis of ~1400 F2 plants. The primers used for amplification were: T15N24_F (5′-GATCTGGCCCAT-CATGAGATC-3′) and T15N24_R (5′-GGTCTAAAGATCGGCAAAGC-3′) for T15N24 CAPS marker; F3L17_F (5′-CTTGGTACCGAAGCCCCGC-3′) and F3L17_R (5′-GACTGGCGTATGCAGAAG-3′) for F3L17 CAPS markers; F10M6_F (5′-GGTCTAAAGATCGGCAAAGC-3′) and F10M6_R (5′-TCACGGTTACAGTTACGATG-3′) for F10M6 CAPS markers. The PCR products were digested with EcoRI, HindIII and Dral, respectively.

The 9.4-kb DNA fragment that included the 6.1-kb upstream region of the Arabidopsis thaliana gene and the 0.5-kb downstream region was cloned into the binary vector pBIn19. The construct was transformed into Agrobacterium tumefaciens strain MP90 and transformed into the mab4-1 plants by the floral dip method (Clough and Bent, 1998).

Microscopy
Scanning electron microscopy images were obtained as previously described (Ishida et al., 2000). Fluorescence was imaged by confocal laser-scanning microscopy (FV1000; Olympus, Tokyo, Japan). For confocal microscopy, dissected embryos were mounted in 7% glucose.

In situ hybridization
In situ hybridization was performed as previously described (Takada et al., 2001). Hybridization was performed at 45°C. Templates for transcription of the MAB4/ENP antisense probe were derived from a PCR-amplified 1098 bp fragment corresponding to a region that spanned amino acids 45-410.

Subcellular localization of MAB4/ENP
MAB4/ENP and PID cDNAs were amplified by RT-PCR from the Col wild type. The fragment was subcloned under the control of the cauliflower mosaic virus 35S promoter and the Nos terminator. GFP (S65T) was translationally fused to both ends of the MAB4/ENP protein and the N terminus of PID protein with a triple glycine linker. Monomeric red fluorescent protein (mRFP) (Campbell et al., 2002) was also fused in-frame to the N terminus of MAB4/ENP with a triple glycine linker. 35S::ARABIDOPSIS CATION TRANSPORTER 4 (CAT4) promoter. This construct was transformed into Agrobacterium tumefaciens strain MP90, and transformed into the mab4-1 plants by the floral dip method (Clough and Bent, 1998).
RESULTS
Organogenesis in mab4, a pid enhancer mutant
A wild-type Arabidopsis seedling has two separated cotyledons with bilateral symmetry around the SAM (Fig. 1A). PID is involved in cotyledon development, as evidenced by defects in cotyledon number, separation and position in pid-3 seedlings (Fig. 1B) (Bennett et al., 1995; Benjamins et al., 2001; Furutani et al., 2004). However, its contribution to cotyledon formation is partial as pid-3 (Fig. 1E). Like organogenesis. The seedlings of double mutants completely lacked cotyledon development, as evidenced by defects in cotyledon number, separation and position in pid-3 seedlings (Fig. 1B) (Bennett et al., 1995; Benjamins et al., 2001; Furutani et al., 2004). However, its contribution to cotyledon formation is partial as pid-3 mutants displayed milder defects of cotyledon development than pin1-201 pid-3 double mutants, which lack cotyledons (Fig. 1D) (Furutani et al., 2004). This indicates that two pathways, dependent on PIN1 and PID, might function in cotyledon formation. To identify factors in the pathways, a screening for pid enhancers was carried out. The screening focused on the mutant loci displaying severe defects of cotyledon development when combined with the pid mutation. We identified a pid enhancer, named macchi-bou-4 (mab4) in a screen of fast neutron-mutagenized lines. Seedlings of mab4-1 single mutants exhibited aberrant cotyledon number and cotyledon fusion at a very low frequency (Fig. 1C; Table 1). mab4-1 mutation also caused defects in postembryonic organogenesis. Whereas lateral organ formation was normal in the inflorescence meristem in mab4-1 mutants (Fig. 1G,I,M,N), aberrant initiation of floral organs such as sepals, petals and stamens, and fusion of them within the same whole or sometimes different whorls were observed in the mutant flowers (Fig. 1M,N,Q; Table 2). In addition, they displayed short valves and decreased valve numbers (Fig. 1O,P; Table 2). When combined with the pid-3 mutation, the mab4-1 mutation caused severe defects of organogenesis. The seedlings of double mutants completely lacked cotyledons (Fig. 1E). Like pin1-201 pid-3 double mutants, the pid-3 mab4-1 mutants could not produce any organs in flower meristems (Fig. 1J,K), whereas pid-3 single mutants were able to form floral organs, albeit to a lesser extent than the wild type (Fig. 1H). Recently, it was reported that a combination of mutations in the PID and ENP genes also caused severe defects of organogenesis (Fig. 1F,L) (Tremel et al., 2005). These data demonstrated the possibility that MAB4 is identical to ENP. A series of crosses revealed that the mab4-1 mutation could not complement the enp mutation, indicating that mab4 is allelic to enp. These results showed that MAB4/ENP is involved in organogenesis synergistically with PID.

Molecular cloning of MAB4/ENP
A map-based cloning of the MAB4/ENP gene was carried out by analyzing $>2700$ chromosomes. The MAB4/ENP locus was mapped to the 315-kb region between molecular markers F3L17 and F10M6 on chromosome 4 (Fig. 2A). We sequenced the genomic DNA of the mab4-1 mutant spanning several predicted open reading frames (ORFs) identified in this region, and found a 9-bp deletion, causing a loss of three amino acids (glycine, leucine and tyrosine), in ORF At4g31820 (Fig. 2A,B). The gene was also sequenced in the enp mutant background and a C-to-T nucleotide transition at the Arg-468 codon, which creates a stop codon, was found (Fig. 2A,B). To confirm that this ORF is identical to the MAB4/ENP locus, a 9.4 kb genome fragment containing this ORF was transformed into plants homozygous for mab4-1. Eight kanamycin-resistant transformants were generated, and phenotypes of floral organs in mab4-1 were recovered in almost all lines (seven out of eight; Table 2). Therefore, we concluded that $A_{4g}31820$ is the MAB4/ENP gene. We also obtained another mab4/enp allele, mab4-2, carrying a T-DNA insertion at the 5’ untranslated region of the MAB4/ENP gene, from the Arabidopsis Biological Resource Center (SALK_104491) (Alonso et al., 2003) (Fig. 2A). mab4-2 mutants displayed the same mild defects in cotyledon development and floral organ development and to the same extent as the mab4-1 allele, whereas the enp allele was less potent (Tables 1, 2). When mab4-2 was coupled with a pid-3 mutation, cotyledons and lateral organs were completely absent, nearly identical to pid-3 mab4-1 and pid-15 enp (data not shown).

<table>
<thead>
<tr>
<th>Genotype (ecotype)</th>
<th>Frequency of cotyledon numbers (%)</th>
<th>Total number of seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One*</td>
<td>Two*</td>
</tr>
<tr>
<td>Col</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>mab4-1 (Col)</td>
<td>1.1</td>
<td>98.3</td>
</tr>
<tr>
<td>mab4-2 (Col)</td>
<td>1.4</td>
<td>98.2</td>
</tr>
<tr>
<td>Ler</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>enp (Ler)</td>
<td>0.6</td>
<td>99.2</td>
</tr>
</tbody>
</table>

*Each category gives the frequency of seedlings with the corresponding numbers of separate cotyledons. In the case of fusion, fused organs are counted as one cotyledon.
These results showed that mab4/enp phenotypes are caused by a loss of function of the At4g31820 gene and that both mab4-1 and mab4-2 represent stronger alleles than enp.

The structure of MAB4/ENP

MAB4/ENP encodes a member of the NPH3-family proteins, composed of 571 amino acid residues with a molecular mass of 66.4 kDa (Fig. 2B). The NPH3 family consists of 31 members in Arabidopsis (Fig. 2C) (Kimura and Kagawa, 2006). Among NPH3-family genes, five genes (MAB4/ENP, At4g67440, At4g37590, At2g14820, At2g23050) show high similarity to one another and are less similar to Arabidopsis NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), ROOT PHOTOTROPISM 2 (RPT2), which had been identified previously in Arabidopsis, as their mutations display defects of phototropic response (Fig. 2B,C) (Motchoulski and Liscum, 1999; Sakai et al., 2000). The rice genome consists of at least 26 members of the NPH3 family and two members (Os09g0420900, Os06g0184500) exhibit high similarity to MAB4/ENP and are less similar to COLEOPTILE PHOTOTROPISM 1 (CPT1), the rice NPH3 ortholog, the mutation of which caused a lack of phototropic response in the coleoptile (Fig. 2C) (Haga et al., 2005).

Whereas NPH3, RPT2 and CPT1 contain a BTB/POZ (broad complex, tramtrack, and bric à brac/pox virus and zinc finger) domain at the N-terminal region and a coiled-coil domain at the C terminus, MAB4/ENP contains a BTB/POZ domain at the N-terminal region, but no distinct coiled-coil domain. The BTB/POZ domain has been found in a large number of proteins and is known as a protein-protein interaction motif (Stogios et al., 2005). In addition, in mab4-1, three amino acid residues, G L and Y, from 408 to 410 were deleted (Fig. 2B). Of the three amino acids, tyrosine is a highly conserved amino acid among the NPH3-family proteins. In case of NPH3, the strong nph3-2 allele carries a deletion of this conserved tyrosine residue (Motchoulski and Liscum, 1999). These data indicate that the tyrosine is important for the molecular function of MAB4/ENP and for that of other family members.

The MAB4/ENP gene has an auxin responsive element (AuxRE), TGCTTC (Ballas et al., 1993; Ulmasov et al., 1999), at positions –228 to –223 from the initiation codon and a WUSCHEL (WUS)-binding site, TTAATGG (Lohmann et al., 2001), at position –201 to –195.

MAB4/ENP mRNA expression

In order to investigate the temporal and spatial expression pattern of the MAB4/ENP gene in wild-type development, in situ hybridization analyses were performed. In wild-type embryos, MAB4/ENP expression was detected uniformly in the embryo at the 8-cell stage (Fig. 3A). Differential expression of the MAB4/ENP gene in the embryo began at the 32-cell stage (Fig. 3B). Detection of MAB4/ENP mRNA continued in the protodermal cells but decreased in the inner cells of the embryo. At this stage, the hypophysis is formed at the junction between the embryo proper and the suspensor, continuous with the protodermal cells surrounding the inner cells. However, MAB4/ENP was not expressed in the hypophysis. The differential localization of MAB4/ENP mRNA became more obvious in globular stage embryos (Fig. 3C). As cotyledon primordia develop, MAB4/ENP mRNA in the protodermal cell layer was gradually restricted to the tip of cotyledon primordia and radicles (Fig. 3D,E). In addition, MAB4/ENP was strongly expressed in several inner cells at the tips of the cotyledon primordia (Fig. 3E). From the late-heart stage, MAB4/ENP began to be expressed in the presumptive shoot apical meristem (SAM), and was strongest in the protodermal layer (Fig. 3E,F).

In the postembryonic stage, MAB4/ENP mRNA was detectable in the organ primordia and the SAM. MAB4/ENP was strongly expressed in the lateral regions of young leaf primordia and weakly in vascular tissue (Fig. 3G). In inflorescence meristems, MAB4/ENP mRNA signal was detected in the meristem L1 layer at the site of flower initiation (Fig. 3H). As flower primordia initiated, MAB4/ENP expression was induced in the inner cell layers (Fig. 3H,1). In young flower primordia, MAB4/ENP expression was elevated at the site of sepal induction, and later where the inner floral whorl organs developed (Fig. 3I,K). During the course of ovule development, MAB4/ENP mRNA was found at the tips of the nucellus and the outer integuments and expression was weaker in the inner integuments (Fig. 3L).

MAB4/ENP expression in pin1 pid embryos

Expression analysis of MAB4/ENP reveals that MAB4/ENP expression is induced at the site of incipient organ initiation and that in developing organs it is restricted to the tips of organ primordia, where auxin maxima are established. To determine whether the MAB4/ENP expression pattern depends on auxin distribution, we examined the expression of MAB4/ENP in pin1-3 pid-2 embryos developing in siliques of pin1-3+/ pid-2/pid-2 plants. In these embryos an auxin maximum is not established in the apex. Until the heart stage, we could not detect any defects of MAB4/ENP expression (data not shown). At the heart stage, when the double mutants were confirmed by distinct phenotypes that cotyledon primordia lacked, MAB4/ENP mRNA was usually found in the protodermal cell layer of mutant embryos (Fig. 4A). Later, MAB4/ENP was expressed in the protodermal cell layer of the presumptive SAM (Fig. 4D), as in wild-type embryos. However, MAB4/ENP transcripts were not detectable in inner cells of the double-mutant embryos, whereas in wild type MAB4/ENP is normally expressed in inner cells of tips of cotyledon primordia (compare Fig. 3E and Fig. 4B). Even if rudimentary primordia were occasionally developed in the apex.

Table 2. Floral organ numbers in mab4/enp flowers

<table>
<thead>
<tr>
<th>Genotype (ecotype)</th>
<th>Sepal</th>
<th>Petal</th>
<th>Stamen</th>
<th>Carpel</th>
<th>Total number of flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>4.00</td>
<td>4.00</td>
<td>5.81±0.40</td>
<td>2.00</td>
<td>52</td>
</tr>
<tr>
<td>mab4-1 (Col)</td>
<td>3.77±0.58</td>
<td>4.01±0.49</td>
<td>4.00±0.73</td>
<td>1.84±0.36</td>
<td>90</td>
</tr>
<tr>
<td>mab4-2 (Col)</td>
<td>3.52±0.73</td>
<td>3.98±0.50</td>
<td>4.00±0.77</td>
<td>1.78±0.42</td>
<td>95</td>
</tr>
<tr>
<td>#7-2</td>
<td>4.00</td>
<td>4.00</td>
<td>5.71±0.46</td>
<td>2.00</td>
<td>52</td>
</tr>
<tr>
<td>Ler</td>
<td>4.00</td>
<td>4.00</td>
<td>5.80±0.49</td>
<td>2.00</td>
<td>54</td>
</tr>
<tr>
<td>enp (Ler)</td>
<td>3.79±0.41</td>
<td>3.98±0.13</td>
<td>4.38±0.64</td>
<td>2.00</td>
<td>61</td>
</tr>
</tbody>
</table>

#7-2 is a mab4-1 plant transformed with the MAB4 genome fragment. Data are means derived from analyses of at least five individuals of each genotype. Standard deviations are indicated. In the case of fusion in the same whorl, fused floral organs are counted as one floral organ. In the case of fusion between different whorls, fused organs are counted as if they are separated.
of the double mutants, we could not detect MAB4/ENP transcripts in the inner cells at the tips of these rudimentary primordia (Fig. 4C,D). These results suggest that PIN1 and PID are not essential for the expression of MAB4/ENP in the protodermal layer. However, the expression of MAB4/ENP transcripts in inner cells of cotyledon primordia depend on the activity of PIN1 and PID, possibly through the auxin distribution established by these genes in the apex of embryos.

Expression of PIN1:GFP and DR5rev::GFP in mutant embryos

Phenotypes of pid mab4/enp double mutants were almost identical to those of pin1 pid double mutants, suggesting that MAB4/ENP is involved in the action of auxin on organogenesis. Previously, it was reported that ENP controls PIN1 polarity in concert with PID (Treml et al., 2005). We analyzed the localization of PIN1 and auxin distribution in mutant embryos.

Fig. 2. Identification of the MAB4/ENP gene. (A) Map-based cloning. The MAB4/ENP locus was mapped to lie between the F3L17 and F10M6 markers on chromosome 4. The number of recombinants are indicated under these markers (recombinant chromosomes/analyzed chromosomes). The mab4-1 mutation is a deletion of 9 bp in At4g31820, which loses three amino acids, G,L and Y. The enp mutation is a point mutation, C to T, which creates a stop codon. In mab4-2 mutants, T-DNA is inserted at the 5’ untranslated region of the gene. ARE, auxin-responsive element; WUS, WUS-binding site. (B) The deduced amino acid sequence of MAB4/ENP, shown in comparison with the sequence of NPH3 and RPT2. Asterisks denote residues deleted in the mab4-1 mutant. Black circle indicates the mutated residue in the enp mutant. Gray and black shadings indicate residues identical in the two and three sequences, respectively. (C) Phylogenetic tree of Arabidopsis MAB4/ENP homologs and the two related rice homologs and CPT1. The tree was obtained by the neighbor-joining method using 1000 bootstrap replicates, generated with CLUSTALW. Black circles indicate rice NPH3-like protein.
We used the stronger pid and mab4/enp alleles, pid-3 and mab4-1, instead of the previously used alleles pid-15 and enp (Treml et al., 2005).

In order to investigate the effects of the pid and mab4/enp mutation on auxin action in embryogenesis, we analyzed the expressions of PIN1::GFP and DR5rev::GFP, which enabled us to monitor the auxin response (Friml et al., 2003) in embryos of the pid-3 and mab4-1 single mutant. In wild-type embryos, PIN1::GFP was expressed in the protodermal cells of cotyledon primordia and provascular cells, and was localized on the apical side of cells in the protodermal cell layer of the apex (Fig. 5A,C). DR5rev::GFP was found to be present in the tips of cotyledon primordia (Fig. 5E,G). As compared with wild-type embryos, we found the same defects of PIN1::GFP and DR5rev::GFP expression in embryos homozygous for pid-3 similar to those previously reported for pid-15 embryos (Treml et al., 2005), pid-3 embryos frequently combined the apical and basal localization of PIN1 in the protodermal cell layer of cotyledon primordia (data not shown). The DR5rev::GFP signal was often missing in the apex of pid-3 embryos (data not shown). Interestingly, although no defects in PIN1 localization, per se, were detectable in provascular tissues of mab4-1 embryos, the abundance of PIN1 signal was severely reduced in the protodermal cells. The defects of PIN1 localization in mab4-1 embryos were detectable from the heart stage. At the heart stage, PIN1 expression was reduced especially on the abaxial side of cotyledon primordia of mab4-1 mutants (Fig. 5B). At later stages, PIN1::GFP expression in the tips of cotyledon primordia was severely reduced in mab4-1 embryos (Fig. 5D) compared to the wild type. This effect of the single mab4-1 mutation has not been reported in the previous analysis of ENP. By contrast, no defects of DR5rev::GFP expression could be found. In mab4-1 embryos, expression of DR5rev::GFP was normally detectable in the tips of cotyledon primordia from the heart stage, as in wild-type embryos (Fig. 5E-H).

To examine the combined effects of pid and mab4/enp mutations on polar auxin transport, we carried out expression analyses of PIN1::GFP and DR5rev::GFP in pid-3 mab4-1 embryos. The same results were obtained as previously described in pid-15 enp embryos (Treml et al., 2005). In the apex of pid-3 mab4-1 embryos, PIN1 polarity was shifted to the lateral and basal side of the plasma membrane in the protodermal layer and no DR5rev::GFP expression was detectable (data not shown). Taken together, these results demonstrate that MAB4/ENP regulates PIN1 localization in the protodermal cell layer and is involved in the establishment of auxin maxima in the apex of the embryo in concert with PID.

Subcellular localization of PIN1, PID and MAB4/ENP
Synergistic interactions between MAB4/ENP and PID, detected by the genetic analysis, and the control of PIN1 abundance on the plasma membrane by MAB4/ENP suggest a distinct subcellular distribution of MAB4/ENP and PIN1 or PID. To test this possibility, we confirmed the subcellular distribution of PIN1, PID and MAB4/ENP. First, the subcellular localization of GFP-tagged PIN1 was analyzed in transfected Arabidopsis protoplasts by confocal laser scanning microscopy. GFP-tagged PIN1 was transiently expressed under the control of its own promoter. The functionality of the construct was verified by complementation of the pin1 mutant (Wisniewska et al., 2006). The fluorescence of GFP-tagged PIN1 protein was located in the plasma membrane and distributed in a dot-like pattern throughout the cell (Fig. 6A). Previously, it was shown that PIN proteins cycle between the plasma membrane and endosomes (Geldner et al., 2001; Geldner et al., 2003). To confirm whether PIN cycling is reflected in cultured Arabidopsis cells, Venus- and mRFP-tagged subcellular marker genes under the control of the CaMV 35S promoter were co-introduced with GFP-tagged PIN1, and their subcellular locations were examined. As expected, the fluorescence of GFP-tagged PIN1 overlapped, in part, with the late-endosomal marker ARA6-mRFP (see Fig. S1A in the supplementary material) and the early-endosomal marker mRFP-ARA7 (see Fig. S1B in the supplementary material), but not with the cis-Golgi apparatus marker Venus-SYP31 (see Fig. S1C in the supplementary material) or the trans-Golgi network (TGN) marker Venus-SYP41 (see Fig. S1D in the supplementary material). These results indicate that PIN1 is localized in the plasma membrane and endosomes in cultured Arabidopsis cells as well as in whole plants. To examine the colocalization of MAB4/ENP with PIN1, GFP-tagged PIN1 was co-introduced with mRFP-tagged MAB4/ENP under the control of the CaMV 35S promoter and their subcellular locations were analyzed. The fluorescence of mRFP-tagged MAB4/ENP protein was distributed in a dot-like pattern throughout
control of the CaMV 35S promoter in cultured Arabidopsis did not colocalize with PIN1 in cultured Arabidopsis cells. These results showed that MAB4/ENP is not colocalized with PIN1-GFP, but the two did not merge (Fig. 6B). These results showed that MAB4/ENP is not colocalized with PIN1 in cultured Arabidopsis cells.

Next, GFP-tagged PID at the N terminus was expressed under the control of the CaMV 35S promoter in cultured Arabidopsis cells. The fluorescence of GFP-tagged PID protein was localized to the plasma membrane and was distributed in a dot-like pattern similar to the fluorescence of PIN1-GFP (Fig. 6C). We confirmed the previous report that PID-GFP is mainly localized in the plasma membrane in Arabidopsis root hair cells and tobacco cv Bright Yellow 2 cells (Lee and Cho, 2006). To examine the subcellular localization of MAB4/ENP with PID, GFP-tagged PID was co-introduced with mRFP-tagged MAB4/ENP and their subcellular localizations were analyzed. A fraction of mRFP-MAB4/ENP fluorescence was colocalized with a fraction of the GFP-PID fluorescence (Fig. 6D). These results indicate that MAB4/ENP is in part colocalized with PID in the intracellular compartments.

To identify the intracellular compartment where MAB4/ENP and PID coexist, the subcellular localization of MAB4/ENP and PID was analyzed in detail by using subcellular marker genes. Venus- and mRFP-tagged subcellular marker genes were co-introduced with GFP-tagged MAB4/ENP and PID and their respective subcellular locations were examined. The fluorescence of GFP-tagged MAB4/ENP did not overlap with that of mRFP-ARA7 (Fig. 7B), Venus-SYP31 (Fig. 7C) or Venus-SYP41 (Fig. 7D). A fraction of MAB4/ENP-GFP fluorescence merged with that of the late-endosome marker ARA6-mRFP (Fig. 7A). At the same time, the fluorescence of GFP-tagged PID did not overlap with mRFP-ARA6 (see Fig. S2A in the supplementary material) and Venus-SYP31, but occasionally existed in very close proximity to Venus-SYP31 (see Fig. S2C in the supplementary material). A fraction of GFP-PID fluorescence merged with that of ARA7-mRFP (see Fig. S2B in the supplementary material) and SYP41 (see Fig. S2D in the supplementary material). These results indicate that MAB4/ENP is localized to a subpopulation of late endosomes as well as in unidentified organelles and that PID localizes to the plasma membrane and to a subpopulation of endosomes and the TGN. Furthermore, MAB4/ENP and PID may be colocalized in unidentified intracellular compartments.

**DISCUSSION**

**MAB4/ENP encodes a NPH3-like protein**

In this study, we have isolated a novel gene, MAB4, which is involved in organogenesis in concert with PID. It encodes a NPH3-like protein and is identical to ENP. MAB4/ENP has been shown to regulate cotyledon development through the control of polar auxin transport (Treml et al., 2005). Among 31 members of the Arabidopsis NPH3 family, NPH3 and RPT2 have been identified as signal transducers involved in phototropism. The nph3 mutant has a defect in phototropism of hypocotyls and roots (Liscum and Briggs, 1996). The rpt2 mutant has defects in phototropism of roots, and blue light-induced stomatal opening (Sakai et al., 2000; Inada et al., 2004). Mutation of rice CPT1, an ortholog of NPH3, results in a lack of coleoptile phototropism and severely reduced root phototropism (Haga et al., 2005). In the cpl1 coleoptiles, asymmetrical auxin distribution was not established in response to blue light, suggesting that CPT1 regulates auxin transport in phototropism. These data imply that a part of the NPH3 gene family might control auxin transport. Furthermore, the difference in the subfamily, to which NPH3, RPT2 and MAB4/ENP belong, might reflect their particular roles in development.

**Fig. 5. PIN1:GFP and DR5rev::EGFP in wild-type and mab4/enp embryos.** (A-D) PIN1:GFP localization in wild type (A,C) and mab4-1 embryos (B,D). (E-H) DR5rev::EGFP expression in wild type (E,G) and mab4-1 embryos (F,H). Heart-stage (A,B,E,F) and torpedo-stage embryos (C,D,G,H). White arrowheads in A and C point to PIN1::GFP localization in the plasma membrane. Orange arrowheads in B and D demonstrate the reduction of PIN1::GFP localization in the plasma membrane. Scale bars: 20 µm in A-H.
In the Arabidopsis genome, there are at least four MAB4/ENP-like genes (At2g23050, At4g37590, At5g67440, At2g14820). As mab4/enp single mutants displayed mild defects in organogenesis, it was postulated that these genes function redundantly with MAB4/ENP in organogenesis. T-DNA insertion lines disrupting these loci did not result in any phenotypes in organogenesis (T.K., B.T., R.A.T.-R. and M.T., unpublished). We are currently constructing multiple mutants between these loci and MAB4/ENP.

MAB4/ENP expression discloses a role in organogenesis

MAB4/ENP was expressed in the protodermal cells during early embryogenesis and in the tip region of cotyledon primordia. After germination, it was expressed in the meristem L1 layer at the site of incipient organ initiation and also in the inner cell layers in the tips of organ primordia. These expression patterns demonstrate that MAB4/ENP functions during organ initiation and outgrowth processes. The defects of organogenesis caused by the mab4/enp mutation were identical to those in plants treated with auxin transport inhibitors (Liu et al., 1993; Hadfi et al., 1998; Nemhauser et al., 2000), suggesting that MAB4/ENP is involved in the action of auxin in organogenesis. In the protodermal cell layer of the early embryo and the meristem L1 cell layers at the site of organ initiation, where MAB4/ENP is expressed, PIN1 is localized to the plasma membrane in a polar fashion. The layers are suggested to be an auxin route to the tip of organ primordia, where auxin maxima are established. These data strongly suggest a correlation between MAB4/ENP function and auxin distribution. Indeed, in pid-15 enp embryos PIN1 polarity was completely reversed in the protodermal cell layer and auxin maxima were not established in the apex (Treml et al., 2005). The mab4-1 mutation caused a reduction of PIN1 abundance in the plasma membrane of the protodermal cells of cotyledon primordia, although DR5rev::GFP was normally expressed in the tips of cotyledon primordia (Fig. 5). We speculate that the normal expression of DR5rev::GFP in the mutant is due to other members of the PIN family redundantly functioning to establish auxin distribution. We also suggest that the DR5rev::GFP system could not detect subtle defects of auxin distribution in the mutant.

In the apex of pin1 pid embryos, MAB4/ENP mRNA was not detected in the inner cell layers, whereas it was normally expressed in the protodermal cell layer (Fig. 4). It is possible that MAB4/ENP

**Fig. 6. Spatial relationship between PIN1, PID and MAB4/ENP in cultured Arabidopsis cells.** (A,C) PIN1-GFP was expressed under the control of its own promoter in Arabidopsis protoplasts (A). GFP-PID was expressed under the control of the CaMV 35S promoter in Arabidopsis protoplasts (C). GFP fluorescence images (left) and Nomarski image (right) were taken with an epifluorescence microscope. (B,D) Localization of PIN1-GFP (B), GFP-PID (D) (green; left) and mRFP-MAB4/ENP (red; middle). mRFP-MAB4/ENP and PIN1-GFP or GFP-PID were co-introduced into Arabidopsis protoplasts. Merged image (right) of mRFP-MAB4/ENP, and PIN1-GFP (B) and GFP-PID (D). An arrowhead in B indicates the close proximity of the PIN1-GFP and mRFP-MAB4/ENP fluorescence, which is shown enlarged in the inset. Arrows in D indicate the colocalization of GFP-PID and mRFP-MAB4/ENP fluorescence, which is shown enlarged in the inset. Scale bars: 10 μm in A-D.

**Fig. 7. Subcellular localization of MAB4/ENP.** (A-D) Localization of MAB4/ENP-GFP (green; left) and mRFP or Venus-tagged subcellular marker genes (red; middle). MAB4/ENP-GFP and mRFP- or Venus-subcellular markers were co-introduced under the control of the CaMV 35S promoter into Arabidopsis protoplasts. Merged image (right) of MAB4/ENP-GFP and the late endosome marker ARA6-mRFP (A), the early endosome marker mRFP-ARA7 (B), the cis-Golgi marker Venus-SYP31 (C) and the trans-Golgi-network (TGN) marker Venus-SYP41 (D). Arrows in A indicate colocalization of fluorescence of MAB4/ENP-GFP and ARA6-mRFP. Scale bars: 10 μm in A-D.
expression depends upon the auxin distribution in the inner cells but not in the protodermal cells. In this context, it is interesting to note that the MAB4/ENP gene contains an auxin responsive sequence, AuxRE (Fig. 2) (Ballas et al., 1993; Ulmasov et al., 1999). MAB4/ENP might therefore be able to respond to auxin in the inner cells. However, expression of the MAB4/ENP gene in the protodermal cell layer during early embryogenesis is very similar to that of Arabidopsis thaliana MERISTEM LAYER 1 (ATML1) (Lu et al., 1996). Recently, it was revealed that a small fragment containing known binding sites for homeodomain transcription factors, the ATML1-binding L1 box (Abe et al., 2001) and WUS-binding site (Lohmann et al., 2001), was responsible for the ATML1 expression pattern (Takada and Jürgens, 2007). Although the MAB4/ENP gene has no L1 box, it contains the WUS-binding site (Fig. 2). These data suggest a regulatory mechanism through the WUS-binding site, resulting in a similar expression pattern to ATML1 in early embryogenesis.

**MAB4/ENP regulates localization of PIN1 protein**

MAB4/ENP could control the activity of PIN1 in organogenesis. This idea is supported by the following important results. (1) The mab4/ENP mutation enhanced phenotypes of pid mutants in the same way as does the pin1 mutation. (2) In mab4-I embryos, PIN1 abundance in the plasma membrane was severely reduced. (3) In pid-15 enp embryos, PIN1 polarity was completely reversed (Treml et al., 2005). (4) The MAB4/ENP expression domain overlaps with PIN1 (Reinhardt et al., 2003; Benková et al., 2003; Heisler et al., 2005). What is the action mechanism of MAB4/ENP for the activity of PIN1? Considering that MAB4/ENP is localized in a subpopulation of endosomes and unidentified intracellular compartments, we suggest that MAB4/ENP is involved in trafficking of PIN1 protein to the plasma membrane. In addition, in cultured Arabidopsis cells, MAB4/ENP partially colocalizes with PID, but not with PIN1, suggesting that MAB4/ENP does not modulate PIN1 trafficking in its vicinity, but functions distantly together with PID. Recently, PID protein kinase has been shown to regulate polar auxin transport through the control of PIN localization. Loss-of-function mutants of PID display an apical-to-basal shift in PIN1 polarity at the inflorescence apex. Conversely, ectopic expression of PID induces a basal-to-apical shift of PIN polarity, suggesting that PID functions as a switch that regulates PIN localization (Friml et al., 2004). Lee and Cho (Lee and Cho, 2006) also showed that PID positively regulates auxin efflux through acceleration of PIN trafficking to the plasma membrane using the auxin-sensitive root hair cell system. Taking into consideration the colocalization of PID with PIN1 in the plasma membrane (see Fig. S3 in the supplementary material), we speculate that PID modulates PIN trafficking to the plasma membrane and that MAB4/ENP mediates PID-dependent modulation of PIN trafficking in the intracellular compartment. In this study the subcellular localization analyses were performed in cultured Arabidopsis cells that lack polarity, and additional analyses are necessary in plant cells with polarity in order to uncover the functional interaction between MAB4/ENP, PIN1 and PID.

**An analogy between organogenesis and phototropism**

Based on the structural similarities of the components involved in organogenesis and phototropism, we conclude that both processes share common molecular principles. MAB4/ENP is a member of the NPH3 family. PID, a key regulator of polar auxin transport in organogenesis, is a member of the AGC kinase family (Bögé et al., 2003). PHOTOTROPIN 1/NON-PHOTOTROPIC HYPOCOTYL 1 (PHOT1/NPH1), which functions as a blue light receptor (Huala et al., 1997), also belongs to the same AGC VIII subfamily. PHOT1/NPH1 is a plasma membrane-associated protein and forms a complex with NPH3 and RPT2 supposed to function as the signalsome in phototropism (Motchoulski and Liscum, 1999; Inada et al., 2004). Finally, both processes use PIN proteins to transport auxin such that it is asymmetrically distributed to lateral domains.

In phototropism, asymmetric distribution of auxin is established in a lateral direction on light stimulation (Friml et al., 2002). In this process, PIN-dependent polar auxin transport is suggested to play a significant role. Mutations in the PIN3 gene alter differential growth in response to light stimulation (Friml et al., 2002). Furthermore, upon blue light stimulation PIN1 proteins are asymmetrically localized across Arabidopsis hypocotyls. This response, which is then followed by tropic bending, is apparently controlled by PHOT1/NPH1 since the phot1nph1 mutation blocks the PIN1 delocalization in light-treated plants (Blakeslee et al., 2004). Likewise, mutation of the rice CPT1 gene, an ortholog of NPH3, caused defects in lateral translocation of auxin (Haga et al., 2005). These data suggest that the PHOT1/NPH1-NPH3 signaling complex is involved in the phototropic lateral redistribution of auxin through the control of PIN localization.

The organogenesis also requires the asymmetric distribution of auxin. In cotyledon development, two auxin maxima are established opposite each other at lateral positions in the apex of the embryo (Friml et al., 2003; Treml et al., 2005). At the globular stage, when cotyledon primordia start to develop, PID transcripts are detectable in two domains, each encompassing approximately three-quarters of the embryo (Furutani et al., 2004), while MAB4/ENP is expressed in the protodermal cell layers in the embryo proper (Fig. 3). As the cotyledon primordia develop, PID transcripts accumulate mainly at the boundaries of these primordia and slightly in the regions surrounding their base (Furutani et al., 2004) while the expression domain of MAB4/ENP is restricted to the tips of the cotyledon primordia (Fig. 3). A comparable expression pattern of MAB4/ENP and PID is visible at the site of floral anlagen and floral organ initiation (Fig. 3) (Christensen et al., 2000). Taken together, the expression domains of the PID and MAB4/ENP genes partially and temporarily overlap during embryogenesis. Both genes coordinately control the polar localization of PIN1, which in turn directs the correct formation of auxin maxima at the tips of growing cotyledon primordia (this study) (Treml et al., 2005).

The molecular interaction of PID, MAB4/ENP and PIN1 is less clear. We could show, that the fluorescent protein-tagged PID and MAB4/ENP are partially colocalized in some intracellular compartments when co-introduced in cultured Arabidopsis cells (Fig. 6). These data support the possibility of an interaction between PID and MAB4/ENP. However, when we used the yeast two-hybrid method to confirm a direct interaction between PID and MAB4/ENP, we could not detect an interaction between them (M.F. and M.T., unpublished). Nevertheless, it is possible that another factor could function as an adaptor protein between them to form the complex. Alternatively, the yeast two-hybrid system might be unsuitable for analyses of membrane-associated proteins.

Certainly, significant differences in the detailed molecular interactions of both processes exist as well. This can be inferred, for instance, from the structures of the participating molecules. PID has no LOV (light, oxygen or voltage) domains required for the activation of PHOT1/NPH1 (Briggs and Christie, 2002). MAB4/ENP has no apparent coiled-coil domains, which have been shown to be important for the direct interaction between PHOT1/NPH1 and NPH3 (Motchoulski and Liscum, 1999). It
there should also be considered that both processes take place at different developmental stages and in different cells and tissues. Both will also require quite different, as yet unidentified, molecules. However, the use of similar key components demonstrates an astonishing economy in plants to solve two disparate biological problems. In the end, one major difference is the output of laterally increased auxin concentrations, as one leads to a cotyledon primordium, whereas the other to a tropic bending towards light.

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Supplementary material
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