

The *ANGUSTIFOLIA* gene of *Arabidopsis*, a plant *CtBP* gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation

Gyung-Tae Kim¹, Keiko Shoda^{2,3},
Tomohiko Tsuge^{3,4}, Kiu-Hyung Cho¹,
Hiroyuki Uchimiya³, Ryusuke Yokoyama⁵,
Kazuhiko Nishitani⁵ and
Hirokazu Tsukaya^{1,6,7}

¹National Institute for Basic Biology/Center for Integrative Bioscience, 38 Nishigounaka, Myodaiji-cho, Okazaki 444-8585, ³Institute for Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, ⁵Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, 980-77 and ⁷Form and Function, PRESTO, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi 332-0012 and School of Advanced Sciences, the Graduate University for Advanced Studies, Shonan Village, Hayama, Kanagawa 240-0193, Japan

²Present address: Molecular Membrane Biology Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

⁴Present address: Osborn Memorial Laboratory, Department of Molecular, Cellular and Developmental Biology, Yale University, 165 Prospect Street, New Haven, CT 6520-8104, USA

⁶Corresponding author
e-mail: tsukaya@nibb.ac.jp

We previously showed that the *ANGUSTIFOLIA* (*AN*) gene regulates the width of leaves of *Arabidopsis thaliana*, by controlling the polar elongation of leaf cells. In the present study, we found that the abnormal arrangement of cortical microtubules (MTs) in *an* leaf cells appeared to account entirely for the abnormal shape of the cells. It suggested that the *AN* gene might regulate the polarity of cell growth by controlling the arrangement of cortical MTs. We cloned the *AN* gene using a map-based strategy and identified it as the first member of the *CtBP* family to be found in plants. Wild-type *AN* cDNA reversed the narrow-leaved phenotype and the abnormal arrangement of cortical MTs of the *an-1* mutation. In the animal kingdom, *CtBPs* self-associate and act as co-repressors of transcription. The *AN* protein can also self-associate in the yeast two-hybrid system. Furthermore, microarray analysis suggested that the *AN* gene might regulate the expression of certain genes, e.g. the gene involved in formation of cell walls, *MER15*. A discussion of the molecular mechanisms involved in the leaf shape regulation is presented based on our observations.

Keywords: *ANGUSTIFOLIA*/cortical microtubules/*CtBP*/leaf morphogenesis/*MER15*

Introduction

Plants are composed of three basic organs, namely, leaves, stems and roots. The floral organs, such as petals, stamens and carpels, can be considered to be modified leaves. Thus, the leaf is a key organ for a full understanding of plant

morphogenesis. As is the case for animal embryos, the development of leaf primordia is dependent on the establishment of several polarities (for review see Tsukaya, 1998, 2002). Using the techniques of developmental genetics to unravel the details of leaf morphogenesis (Tsukaya, 1995) we previously showed that two genes are responsible in *Arabidopsis* for the polarity-specific expansion of leaves (Tsukaya *et al.*, 1994; Tsuge *et al.*, 1996). They are the *ANGUSTIFOLIA* (*AN*) gene, which regulates polar elongation in the leaf-width direction, and the *ROTUNDIFOLIA3* (*ROT3*) gene, which regulates polar elongation in the leaf-length direction. We cloned the *ROT3* gene by T-DNA tagging and showed that the *ROT3* gene encodes a novel cytochrome P450 (Kim *et al.*, 1998a, 1999). The phenotype of *an* mutant plants is similar to that of *rot3* plants, but the defects in the polarity-dependent elongation of leaf cells affect leaf expansion in different directions (Tsuge *et al.*, 1996). The defect in both cases is limited to leaves and to floral organs, which are modified leaves. The product and function of the *AN* gene remains to be determined.

The polarity-dependent expansion of plant cells is generally associated with the organization of cortical microtubules (MTs) that are arranged beneath the plasma membrane (Cyr, 1994; Shibaoka, 1994). In *Arabidopsis*, disturbance by mutation of the organization of MTs can cause dwarfism (Traas *et al.*, 1995; McClinton and Sung, 1997), and the abnormal arrangement of cortical MTs has been noted in mutants with altered polar development of roots (Aeschbacher *et al.*, 1995; Hauser *et al.*, 1995) and shoots (Furutani *et al.*, 2000). Several *Arabidopsis* mutants with abnormal amounts of cellulose microfibrils have also been reported (Potikha and Delmer, 1995). However, the role of the regulation of the arrangement of MTs in the genetic control of leaf-cell expansion remains to be clarified.

We show here that a defect in the arrangement of cortical MTs in leaf cells is associated with the *an* mutation and is reversed by *AN* cDNA, which we cloned in the present study. The abnormal pattern of cortical MTs in *an* leaf cells can provide a complete explanation for the polarity-dependent alteration in the shape of the leaf cells and, therefore, it can also explain the change in shape of the leaves. We discuss the results of our analysis in terms of possible roles of the *AN* gene, the first member of the family of *CtBP* genes to be identified in plants.

Results

The orientation of cortical MTs in the an mutant is abnormal

The regulation of polar changes in cell morphology, which is disrupted in leaf cells of *Arabidopsis* plants with the *an* mutation (Figure 1) should be closely related to the

regulation of the orientation of cellulose microfibrils which is governed, in turn, by the arrangement of cortical MTs. Therefore, we analyzed the orientation of cortical

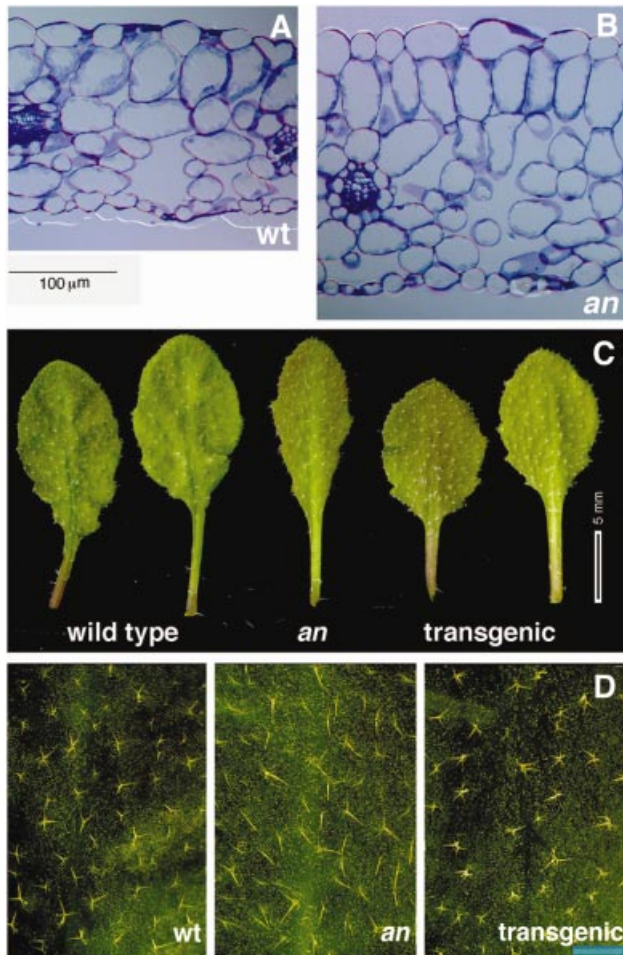


Fig. 1. Cross sections and gross morphology of the fifth rosette leaves of a wild-type (wt) plant, an *an-1* mutant plant and a transgenic *an-1* mutant that expressed the wild-type *AN* gene included in the TAC clone 1A13. (A and B) Cross sections of the fifth rosette leaves of the wt (A) and the *an-1* mutant (B). Bar, 100 µm. Note the abnormal shape of the palisade cells in the *an-1* mutant leaf. (C and D) The fifth rosette leaves of wt, *an-1* and a transgenic *an-1* plant that harbored 1A13 (C), and magnified views of the same leaves (D). Bars, 5 mm (C) and 1 mm (D).

MTs in leaf cells of *an* and *rot3* mutant plants by immunohistochemical staining and by monitoring the expression of the *TUBULIN6::Green Fluorescent Protein* (*TUB6::GFP*) gene for the TUB6–GFP fusion protein. We analyzed the orientation of MTs in epidermal cells and in the subepidermal layer of cells (palisade cells) on the adaxial side of the leaf. As shown in Figure 2, we found that the cortical MTs in epidermal cells of the *an* mutant were more regularly aligned parallel to the leaf-width direction than in those of the wild type. This pattern of MTs can explain the morphological phenotype of the *an* epidermal cells, which protrude less extensively than wild-type cells in the leaf-width direction (Figure 2; Tsuge *et al.*, 1996). We next focused on the quantitative characterization of the palisade cells because of the simple rod-like shape of normal cells. We considered that the palisade cells represented lamina cells since the shapes of epidermal cells and spongy cells are irregular, as a consequence of multidirectional expansion, and this irregularity hampers detection of polarity-specific changes. We found no difference in numbers of MTs between wild-type (wt) and mutant plants when we examined the MTs immunohistochemically and by monitoring expression of the *TUB6::GFP* fusion gene (data not shown). Analysis by RT–PCR on one of the endogenous genes for tubulin, *TUB4*, indicated that there were no significant differences among the levels of expression of the transcript of this gene also (see Figure 7).

We examined the orientation of the cortical MTs by analyzing the angle (θ) between the cortical MTs and the plane that was parallel to the paradermal plane, on both transverse and longitudinal sections of the palisade layer, as shown in Figure 3A. The mean value of θ was close to 45° in wt plants at the developmental stage examined (just after stage I; Tsuge *et al.*, 1996), regardless of the direction within the leaf (longitudinal sections, $43.2 \pm 22.4^\circ$; transverse sections, $48.5 \pm 18.6^\circ$; Figure 3). In contrast, θ was significantly reduced (level of significance, 1%, Student's *t*-test) in transverse sections of the *an* mutant (*an*, $29.7 \pm 20.6^\circ$; wt, $48.5 \pm 18.6^\circ$), but it was not significantly reduced (level of significance, 1%) in longitudinal sections (*an*, $39.3 \pm 22.5^\circ$; wt, $43.2 \pm 22.4^\circ$; Figure 3). Thus, θ was significantly smaller in the transverse sections of the *an* mutant than in the wild type. In contrast, θ was very slightly larger in longitudinal

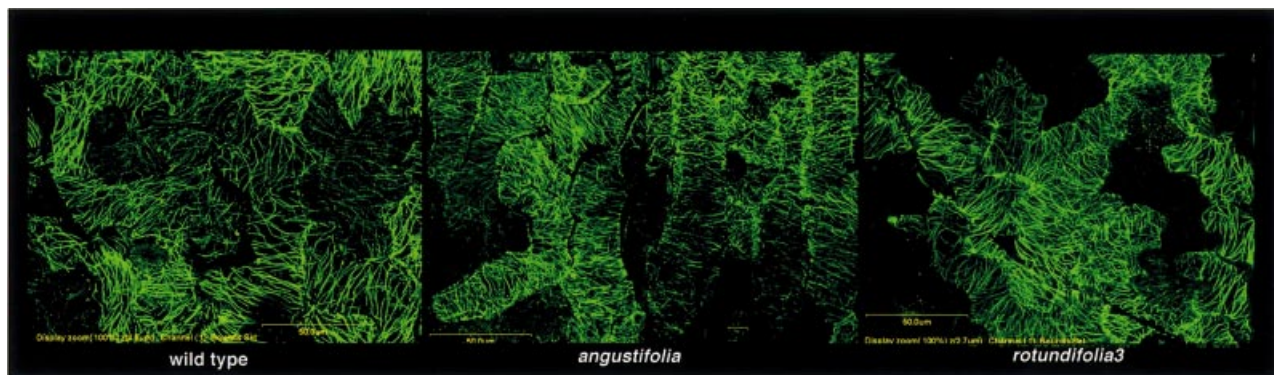


Fig. 2. Confocal images of the arrangement of cortical MTs in the leaf epidermis. The adaxial epidermis is shown of wt, *an-1* mutant and *rot3-1* mutant leaves, from left to right. MTs are visible as fluorescence from the GFP–TUB6 fusion protein. Bars, 50 µm.

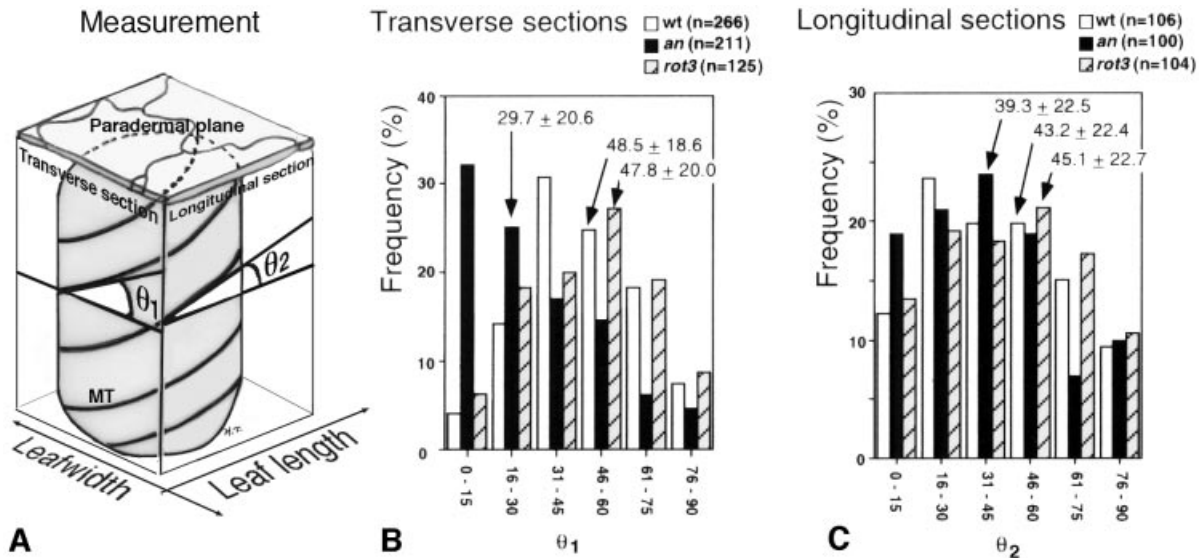


Fig. 3. Arrangement of cortical MTs in leaf palisade cells in the subepidermal layer of wt, *an* and *rot3* leaves. (A) Strategy for measurement of the angle θ between cortical MTs in a palisade cell and the paradermal plane (plane of the epidermis). The orientation of the clearest array of MTs was measured for each palisade cell. (B and C) Distribution of θ on longitudinal sections (B) and on transverse sections (C). *n*, number of palisade cells examined. The numbers indicated by arrows are means \pm SD of the value of θ of the indicated strains.

sections of the *rot3* mutant than in the wild type (*rot3*, $45.1 \pm 22.7^\circ$; wt, $43.2 \pm 22.4^\circ$). However, there was no significant difference in the orientation of the cortical MTs between the wild type and the *rot3* mutant both in longitudinal sections and in transverse sections. Thus, the regulation of cell elongation by the *ROT3* gene seemed to be independent of the regulation of the orientation of cortical MTs.

No abnormalities in the arrangement of cortical MTs were apparent in cells of internodal stems, leaf petioles and primary roots of the *an* mutant (Table I). The organ-specific abnormal arrangement of cortical MTs in the *an* mutant corresponded to the organ-specific defect in the polarity-specific elongation of cells in this mutant (Tsuge *et al.*, 1996). In the *rot3* mutant, no abnormalities were recognized in the orientation of cortical MTs in the cells of the internodal stems, the petioles or the roots (Table I).

Molecular cloning of the AN gene

The *AN* locus was first identified by Rédei (1962) in a study of plants derived from mutagenized seeds of ecotype Landsberg and it was mapped to the upper end of the first chromosome (Lee-Chen and Steinitz-Sears, 1967; Koornneef *et al.*, 1983). Using Cleaved Amplified Polymorphic Sequence (CAPS; Research Genetics, Huntsville, AL) markers from this region, we found that the *AN* locus was strongly linked to the marker *PVV4*. No recombination between *AN* and *PVV4* was recognized after five back-crosses with the Columbia wild type. In addition, we calculated that the *NCCI* locus, which has been mapped to a site ~ 10.4 cM below the *PVV4* locus, was located 13.6 ± 2.7 cM from the *AN* locus (data from 144 wt and 57 *an* plants). Thus, we estimated that the *AN* locus was located close to the *PVV4* locus. Using this new information, we screened genomic clones using the *PVV4*-containing genomic clone E3-pART (a kind gift from Dr T. Golden, University of Rochester, NY) as the probe. We

Table I. Orientation of cortical MTs in cells in the leaf petiole, stem and root of wild-type and mutant plants

	Stem ^a	Leaf petiole ^b	Root ^c
wt	4.9 ± 4.1 (<i>n</i> = 27)	15.6 ± 10.8 (<i>n</i> = 38)	6.6 ± 4.6 (<i>n</i> = 69)
<i>an</i>	5.4 ± 3.6 (<i>n</i> = 70)	20.5 ± 12.1 (<i>n</i> = 56)	6.1 ± 4.2 (<i>n</i> = 60)
<i>rot3</i>	5.3 ± 3.6 (<i>n</i> = 74)	14.0 ± 11.9 (<i>n</i> = 66)	6.3 ± 5.5 (<i>n</i> = 50)

Results shown are mean angles \pm SD between cortical MTs and the radial axis (*n*, number of cells examined). All data for mutants shown here are not significantly different from those for the wild type (level of significance, 1%; Student's *t*-test).

^aThe first internodes of type II metamers of 5–7 mm in length were examined.

^bThe epidermis of petioles of fully matured fifth leaves was examined.

^cPrimary roots of seedlings 3–7 days after sowing were used for analysis, and cells at the growth stage prior to elongation were examined.

obtained three positive genomic TAC clones (TAC = transformation-competent artificial chromosome; Liu *et al.*, 1999; kindly supplied by Dr D. Shibata, Mitsui Plant Biotechnology Research Institute, Tsukuba, Japan) that covered this region, namely, 1A13, 2E23 and 9N19, and we examined the ability of these clones to complement the *an* mutation. The 4.5 kb long E3-pART clone did not complement the *an* mutation, but one of the TAC clones, 1A13, clearly complemented the mutation in transgenic plants (Figure 1C and D). Not only narrow leaves but also trichomes with fewer branches (Tsuge *et al.*, 1996) were restored in five independent primary transformants that harbored the 1A13 clone (Figure 1C and D). Thus, we concluded that the *AN* gene was included within the 45 kb long genomic fragment of the 1A13 clone.

To identify the open reading frame (ORF) that actually encodes the AN protein, we performed RFLP analysis of wild-type and *an* mutant genomes using the 1A13 clone as the probe. We found one RFLP fragment that differed between the wild-type genome and the *an* genome and contained an ORF (data not shown). The sequence of this

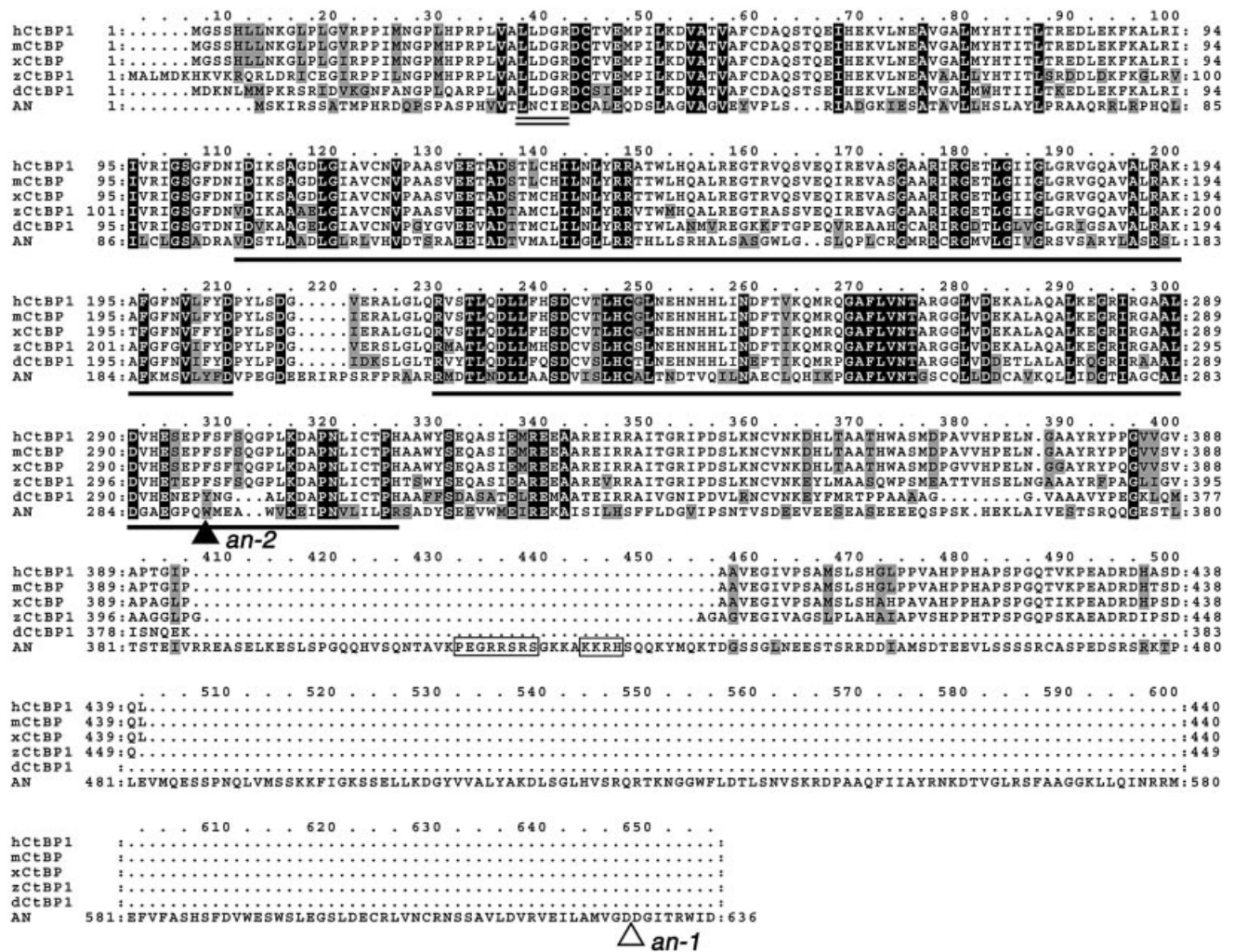


Fig. 4. Alignment of the amino acid sequences of the AN protein of *Arabidopsis* and various animal CtBPs (h, human; m, mouse; x, *Xenopus*; z, zebrafish; d, *Drosophila*). Identical amino acids in all sequences are indicated by black boxes and similar amino acids are indicated by gray boxes. The underlined regions are regions of homology between CtBPs and 2-Hacid_DH. Double-underlined regions are Rb-binding motifs (see text). The open and the filled triangle indicate the site of the translocation in the *an-1* allele and the site of the mutation in the *an-2* allele, respectively.

ORF suggested that it encoded a dehydrogenase-like protein that was also encoded within a 40 kb genomic clone that had previously been sequenced by Terryn *et al.* (1998). Our sequence of this ORF matched that of EST clone YAY141 (DDBJ/EMBL/GenBank accession No. Z48386) which had been partially sequenced. Sequencing and northern blotting analysis of the YAY141 clone revealed that this EST clone included full-length cDNA. Comparison of genomic sequences with the cDNA revealed the presence of six introns and seven exons in the genomic sequence. Using this cDNA as a probe, we performed Southern blotting analysis, under high-stringency conditions, of genomic DNA digested with several restriction enzymes. In each case, we obtained only one genomic fragment (data not shown). The cDNA encoded a predicted protein of 636 amino acids with a molecular mass of 70 kDa.

To identify the mutation in the *an* allele, we constructed a genomic DNA library of the *an-1* mutant, which had originally been isolated after X-ray mutagenesis. We isolated three independent genomic DNA clones that

included the coding sequence of the putative AN gene and sequenced the clones derived from the *an-1* mutant. In each of the three independent genomic clones, we identified an insertion caused by translocation of a genomic fragment at the putative AN locus. A genomic DNA fragment of ~3 kbp, which was located at a site 6 kbp from the AN gene on the same chromosome, was translocated to a site 22 bp upstream of the termination codon in the 3' sequence of the AN gene (Figure 4). Furthermore, sequence analysis of the *an-2* mutant allele (a kind gift from Dr T.Hattori, Mie University, Tsu, Japan), which was isolated after mutagenesis with ethyl methanesulfonate (EMS), had a nucleotide transition from G to A, which resulted in the introduction of a termination codon at position 291 (which replaced tryptophan in the wt protein) in the conserved D-isomer-specific 2-hydroxyacid dehydrogenase (2-Hacid_DH motif) (see below and Figure 4). These results confirmed that the ORF that we had identified as the putative AN locus did indeed encode the AN protein. Complementation analysis of the *an* phenotype by AN cDNA is described below.

The AN gene encodes a homolog of human CtBP

A homology search using the sequence of the AN gene in the WHICH database revealed strong sequence similarity between the product of the AN gene and a carboxy-terminal binding protein (CtBP), a human protein named for its ability to interact with C-terminal sequences of adenovirus E1A protein (Schaeper *et al.*, 1995; Figure 4). Members of the CtBP family have been found in several animal species. The AN protein exhibited strong homology at the amino acid level to zebrafish CtBP (32%), human CtBP (31%) and other animal CtBPs. There is a strongly conserved region in the N-terminal amino acid sequences of AN and animal CtBPs (~47% homology). This common conserved sequence resembles the 2-Hacid_DH motif at its N-terminus (Boyd *et al.*, 1993; Turner and Crossley, 1998) as indicated by the underlined amino acids at positions 96–306 in Figure 4. It has been suggested that this motif is involved not only in energy metabolism but also in interactions with various proteins (Arthur *et al.*, 1991; Singh and Green, 1993). Furthermore, we found the retinoblastoma-binding (Rb-binding) motif LxCxE (Lee *et al.*, 1998) at the N-terminus of AN (double underlining in Figure 4). A putative NLS (NLS = nuclear localization signal) motif, PEGRRSR and KKRH, was found in the region between positions 412–427 in the AN protein (Figure 4). No obvious similarities to animal CtBPs and other proteins were evident in the C-terminal region of AN.

To examine the possible evolutionary history of CtBP genes, we compared the sequence of AN to available sequences of animal CtBPs. The phylogenetic tree revealed that common CtBPs can be divided into at least two subfamilies (Figure 5), i.e. one for plants and one for animals. We identified plant homologs of CtBPs by examining plant sequences similar to the sequence of the product of the AN gene, namely, sequences from Japanese morning glory (*Ipomoea nil* L., Convolvulaceae, Asteridae, Magnoliophyta; unpublished data from Dr E.Nitasata of Kyushu University, Fukuoka, Japan) and from liverwort (*Marchantia polymorpha* L., Marchantiaceae, Marchantiidae, Bryophyta; unpublished data from Dr H.Takano of Kumamoto University, Kumamoto, Japan). These polypeptides and the product of the AN gene clearly formed a monophyletic clade, suggesting the evolutionary conservation of AN homologs in the genomes of all land plants (Figure 5).

Patterns of expression of the AN gene and cellular localization of the AN protein

We examined levels of AN mRNA by northern blotting and RT-PCR analysis. Northern blotting analysis showed that the AN gene was expressed throughout all organs examined in wild-type plants, namely, in cotyledons, leaves, roots, stems and floral buds (Figure 6A). We also examined the expression of two mutant *an* alleles by RT-PCR to determine the effects of these mutations on the accumulation of *an* mRNA. Wild-type levels of AN-type mRNA were detected in seedlings of both *an-1* and *an-2* mutant plants (Figure 6B), suggesting that these mutations alter the function of the AN protein and not the synthesis or stability of the AN transcript.

The presence of a putative NLS motif in the AN protein suggested that AN might be localized in nuclei. To

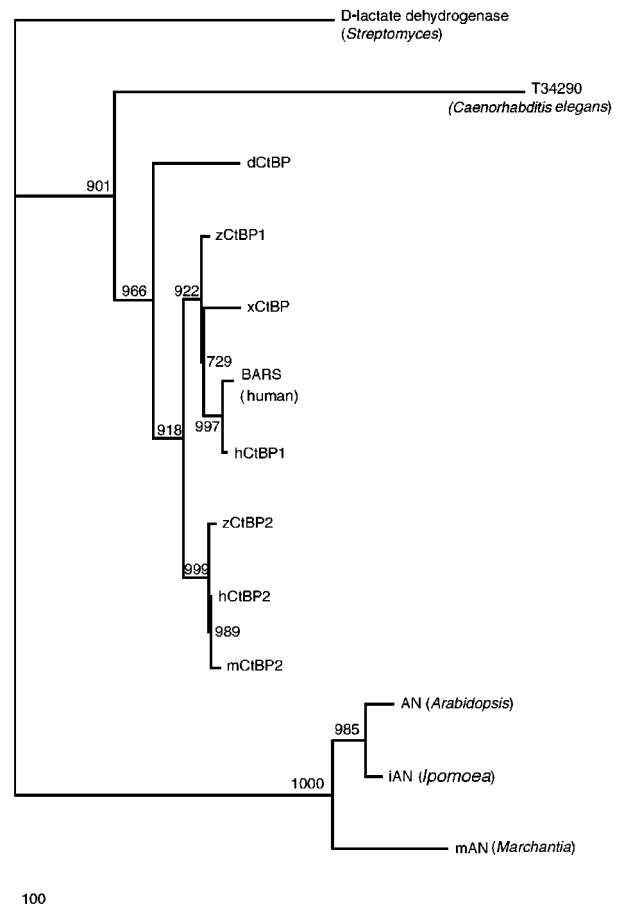


Fig. 5. Phylogenetic tree generated from the deduced amino acid sequences of animal CtBPs and other related proteins, as indicated. The tree was constructed with 1000 samplings of bootstrap values. The conserved region of the 2-Hacid_DH motif at the N-terminus, shown in Figure 4, was used to construct the tree. Branch lengths are proportional to the number of amino acid substitutions, and the scale bar indicates 100 substitutions.

determine the localization of this protein, we fused the AN gene to a gene for green fluorescent protein (*sGFP::S65T*) under the control of the constitutively expressed 35S RNA promoter of cauliflower mosaic virus (CaMV). The fusion gene was expressed in both wild-type and *an-1* mutant plants. Using a fluorescence microscope, we detected green fluorescence within the nuclei of cells in stable *Arabidopsis* transformants (Figure 7A). However, we detected the fusion protein not only within nuclei but also in the cytoplasm when the fusion gene was transiently expressed in onion epidermal cells (Figure 7C). Transgenic *an-1* plants developed wild-type leaves and trichomes, suggesting that the expression of AN cDNA could complement the *an* mutation, with restoration of the normal arrangement of cortical MTs (Figure 9).

Control of cell shape in leaves by the AN gene

In an attempt to demonstrate genetic complementation of the *an* mutation by the ORF that we had identified and to clarify the function of AN, we transformed wild-type and *an-1* mutant plants with the AN::*GFP* gene that was under the control of the CaMV 35S promoter. The *an-1*

phenotype was reversed by expression of the transgene (Figure 8A and B).

To determine the cellular basis of the phenotypes of the transgenic plants, we performed an anatomical analysis of the fifth leaves from wild-type plants, *an-1* mutant plants, and transgenic *an-1* plants that expressed the *AN* gene and had a wild-type phenotype. The palisade cells of the *an-1* mutant were narrower (Figures 1B and 8D) than those of the wild type (Figures 1A, 8C and F), but those of the transgenic *an-1* plants that expressed the *AN* gene were similar to those of the wild type (Figure 8E and G). The arrangement of cortical MTs in epidermal cells of the transgenic *an-1* plants was also normal, resembling that in the wild-type plants (Figure 9). The cortical MTs in epidermal cells of transgenic plants were not mostly oriented parallel to the leaf-width direction, unlike those in the epidermal cells of the *an* mutant. This result suggests that recovery from the *an*-type phenotype upon expression of the *AN* transgene might be attributable to normalization of the arrangement of cortical MTs.

AN can self-associate in yeast cells

The CtBP of *Drosophila* (dCtBP; Poortinga *et al.*, 1998) and of mouse (mCtBP2; Turner and Crossley, 1998) self-associate in a yeast two-hybrid system. It has been suggested that the dimerization of CtBP is important for linkage between a DNA-binding protein and other kinds of regulatory protein (for review see Turner and Crossley,

2001). Therefore, we examined the possible dimerization of *AN* in a yeast two-hybrid system. We found that *AN* was able to form dimers in yeast cells (Figure 10), as is the case for animal CtBPs. The proposed dimerization of *AN* was also supported by quantitation of the interaction in terms of the activity of β -galactosidase (host cells, $170 \pm 8 \mu\text{M}/\text{min}/\text{mg}$ protein; cell lines with pAS2-1/*AN*1911 and pACT2 plasmids, $159 \pm 10 \mu\text{M}/\text{min}/\text{mg}$ protein; and cell lines with pAS2-1/*AN*1911 and pACT2/*AN*1911 plasmids, $1484 \pm 61 \mu\text{M}/\text{min}/\text{mg}$ protein; three independent lines were examined in each case and results are means \pm SD for each set of three lines).

The *an* mutation specifically affects the expression of the gene for a meristem-specific enzyme that is involved in cell-wall formation, *MER15*

To examine the possible role of the *AN* protein as a regulator of the transcription of other genes, we performed microarray analysis under the auspices of the Monsanto *Arabidopsis* Microarray Program. Microarray analysis showed that the *an* mutant expressed some genes at higher levels than the wild type, suggesting that *AN* might function as a repressor of such genes. Possible candidates for target genes of the *AN* protein include a cell-wall modulating enzyme, a Zn-finger protein and a MAP kinase (Table II). To confirm the results of microarray analysis, we performed RT-PCR analysis of the expression of candidate genes. We confirmed that some genes were up-regulated in the *an* mutant but down-regulated in wild-type plants and transgenic *an* plants that expressed the wild-type *AN* gene (data not shown). In the present study, we focused on the expression of genes in the xyloglucan endotransglucosylase/hydrolase (XTH) family. These enzymes are thought to regulate loosening of cell walls, and one member of this family, *MER15* (Medford *et al.*, 1991), was among the genes that were specifically up-regulated in the *an* mutant.

We analyzed the levels of expression of genes in the XTH family in rosette leaves by quantitative, real-time RT-PCR. We found that *MER15* was expressed at a 3-fold higher level in the *an-1* mutant than in the wild type, as indicated by the microarray analysis (3.3-fold higher by microarray analysis; 3.27-fold higher by RT-PCR analysis). In contrast, levels of expression of two other genes for members of the XTH family, namely *EXGT-A1* (*EXT*; Okazawa *et al.*, 1993) and *EXGT-A2* (Akamatsu *et al.*,

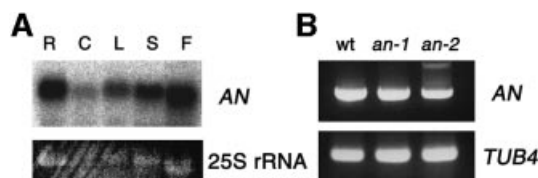


Fig. 6. Northern blotting and RT-PCR analysis of the *AN* transcript. (A) Expression of *AN* mRNA in various tissues of *Arabidopsis*. Aliquots of 20 μg of total RNA were loaded in each lane. Total RNA was isolated from roots (R), cotyledons (C), rosette leaves (L), stems (S) and floral buds (F). The intensity of the band of 25S rRNA served as an internal control (lower panel). (B) Levels of expression of *AN* mRNA in wild-type (wt, Columbia), *an-1* and *an-2* mutant plants, as determined by RT-PCR. Total RNA was isolated from aerial parts of 3-week-old seedlings and 0.5 μg of total RNA was used in each case. Results of the amplification by RT-PCR of the mRNA for β -tubulin 4 (*TUB4*) are shown as an internal control (lower panel).

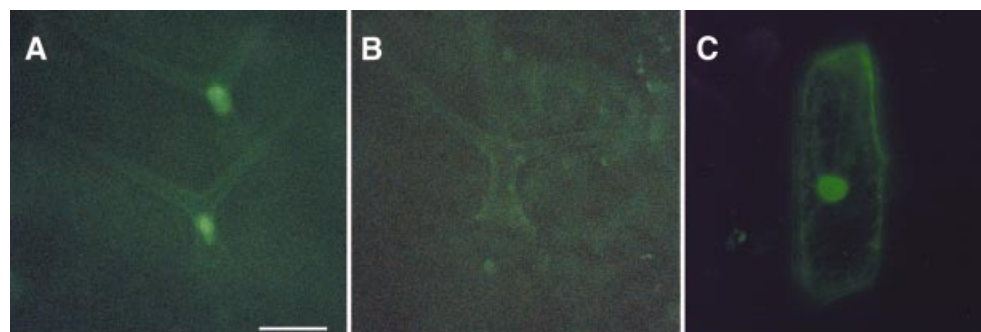


Fig. 7. Intracellular localization of *AN::GFP* in plant cells. (A) Intracellular localization of *AN::GFP* in trichomes of transgenic *Arabidopsis* leaves. (B) Non-transgenic wild-type trichomes that failed to generate a signal under the same conditions for fluorescence microscopy as those that yielded (A). (C) Transient expression of *AN::GFP* in onion epidermis. Bar, 100 μm .

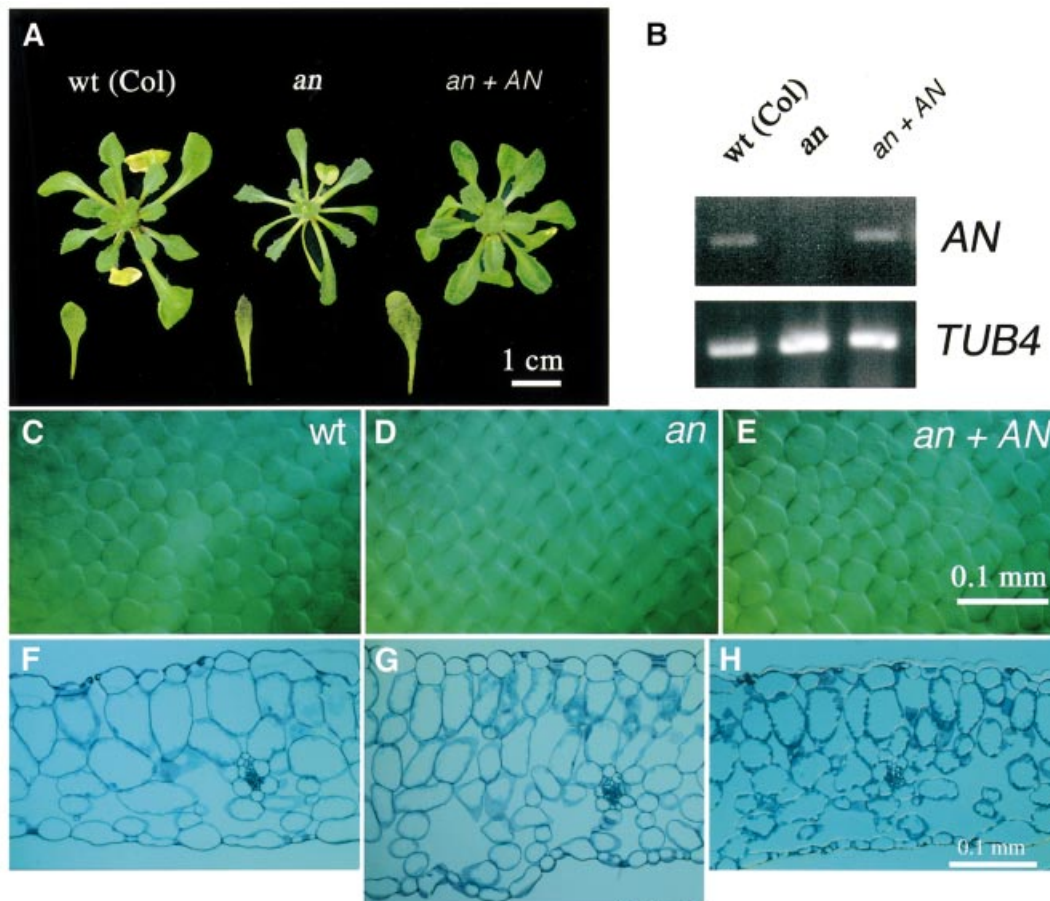


Fig. 8. Anatomical and molecular characterization of transgenic plants that expressed the *AN::GFP* transgene under control of the CaMV 35S promoter. (A) From left, rosette leaves of wt, *an-1* mutant, and transgenic plants with the *an-1* mutant background that expressed the *AN::GFP* fusion protein, respectively. (B) Analysis by RT-PCR of the levels of expression of *AN* mRNA in the transgenic plants shown in panel A. (C-E) Paradermal view of the cleared palisade layers of the fifth leaves of the wt (C), *an-1* mutant (D) and transgenic *an-1* plant that expressed *AN::GFP* (E). Bar, 0.1 mm. (F-H) Cross sections of the fifth leaves of the wt (F), of *an-1* mutant plant (G) and of a transgenic *an-1* that expressed *AN::GFP* plant (H). Bar, 0.1 mm.

1999; Yokoyama and Nishitani, 2001) did not show such differences (not significantly different from the wild type; level of significance, 5%) when we compared *an-1* and wild-type plants (Figure 11). This result suggested that the level of expression of *MER15* was specifically affected by the *an* mutation, as compared to other members of the *XTH* family.

Discussion

In the present study, we showed that the *AN* gene, the first member of the family of *CtBP* genes to be identified in a plant genome, regulates the polarized growth of leaf cells via control of the arrangement of cortical MTs in epidermal cells (Figure 2) and in mesophyll cells (Figure 3). In the *an* mutant, the orientation of the cortical MTs was dramatically disrupted in leaves at an early stage of their development (Figure 2). The stage at which we examined palisade cells corresponded to the stage immediately after stage I, when the polar elongation of cells is initiated (Tsuge *et al.*, 1996; Kim *et al.*, 1998b). We proposed previously that the *AN* gene governs the polar elongation of leaf mesophyll cells after stage I (Tsuge *et al.*, 1996). The mean value of the angle between cortical

MTs and the paradermal plane (plane of the epidermis), was significantly smaller (61%; level of significance, 1%) in transverse sections of the *an* mutant than in those of the wild type. The smaller this angle, the greater is the extent to which rod-shaped cells elongate in a perpendicular direction relative to the paradermal plane. When cortical MTs (and the associated cellulose microfibrils) are arranged parallel to the paradermal plane and the volume of cells increases, the cells are forced to expand exclusively in the leaf-thickness direction and not in the leaf-width direction. We postulated previously (Tsuge *et al.*, 1996) that the increase in cell length in the leaf-thickness direction in the *an* mutant might be a result of restricted elongation of cells in the leaf-width direction. The results in the present study now allow us to explain the unusually narrow and unusually thick leaves of *an* mutant plants in terms of a single phenomenon: the altered arrangement of cortical MTs in *an* mutant cells. Similarly, the reduced number of protrusions of epidermal cells that is specific to the leaf-width direction in the *an* mutant (Tsuge *et al.*, 1996) can also be explained by the orientation of cortical MTs that we observed in the present study (Figure 3). Hogetsu (1989) reported that the pattern of the cortical MTs is the critical factor in the directional expansion of

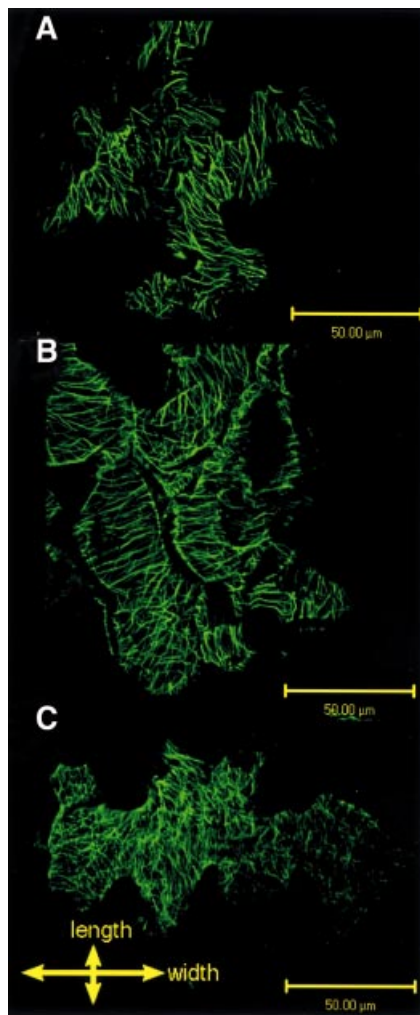


Fig. 9. Restoration of a normal arrangement of cortical MTs in the leaf epidermis by introduction of the *AN* gene into the *an* mutant. Confocal images of the adaxial epidermal cells of leaves are shown for the wt (A), the *an-1* mutant (B) and a transgenic *an-1* plant that expressed the wild-type *AN* gene (C). The direction perpendicular to the images in the figure corresponds to the longitudinal axis of the leaves (leaf-length direction), as shown in (C). Bars, 50 μ m.

the leaf epidermis. Thus, the *AN* gene appears to play a role in the regulation of the orientation of cortical MTs in leaf morphogenesis.

Our analysis also revealed that the cortical MTs in the palisade cells in the subepidermal layer of the *an* mutant were abnormally oriented in different planes (in transverse sections and in longitudinal sections; Figure 3). The fact that the orientation was affected in a specific plane is, itself, of interest with respect to the regulation of cell polarity. The concept of the regulation of cell polarity in a specific plane is further supported by observations that changes in the orientation of cortical MTs, e.g. caused by treatment with a phytohormone, are also associated with changes in a specific plane (Yuan *et al.*, 1995; Lloyd *et al.*, 1996; Wymer and Lloyd, 1996).

The abnormal patterns of branching of the trichomes on *an* leaves (Hülkamp *et al.*, 1994; Oppenheimer, 1998) can now also be readily explained. The branching of trichomes is controlled by cortical MTs in *Arabidopsis* (Mathur and Chua, 2000). Thus, as we speculated earlier (Tsuge *et al.*,

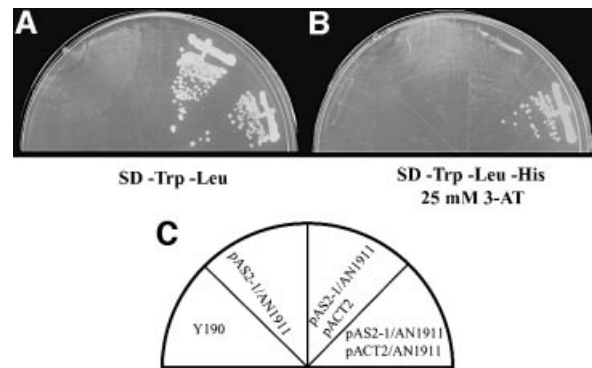


Fig. 10. Self-association of *AN* in a yeast two-hybrid system. (A and B) Growth of yeast strain Y190 that harbored constructs as indicated in (C). (A) Growth on SD medium (-Trp -Leu) to confirm insertion of pAS2-1/*AN* and pACT2/*AN* into strain Y190. (B) Growth on selective SD medium (-Trp -Leu -His) that contained 25 mM 3-amino-1,2,4-triazole (3-AT) for the self-association test (see text for details).

1996), less extensive branching (two branches in *an* leaves and three or four in wt leaves) might be a result of the defect in the arrangement of cortical MTs in *an* plants that suppresses elongation of cell protrusions in the leaf-width direction.

***AN* as a regulator of the polar expansion of leaf cells**

As discussed above, the *AN* gene appears to control the arrangement of cortical MTs and, in this way, *AN* is thought to influence the shape of leaf cells in a polarity-dependent manner. The leaf specificity of the mutant phenotype, with respect to gross morphology (Tsuge *et al.*, 1996), was reflected by the leaf specificity of the abnormality in the arrangement of cortical MTs (Table I). In other words, the leaf-specific abnormality in the orientation of cortical MTs was consistent with the leaf-specific phenotype. Moreover, since the *AN* gene functions independently of the *ROT3* gene (Tsuge *et al.*, 1996), if the *AN* gene is involved in the control of the arrangement of cortical MTs, it would seem unlikely that the *ROT3* gene would be involved in regulation of the same process in leaf cells. In the present study, *rot3* mutant plants were found to have normally arranged cortical MTs in their leaf cells. In addition *AN* cDNA reversed the abnormality in the arrangement of cortical MTs in *an* leaf cells. Thus, all the available data indicate a parallelism between the arrangement of cortical MTs in leaf cells and the activity of the *AN* gene.

Previous studies have shown that disruption of the arrangement of cortical MTs can be responsible for a disturbance in the appropriate enlargement of cells (Aeschbacher *et al.*, 1995; Hauser *et al.*, 1995; Traas *et al.*, 1995; McClinton and Sung, 1997). However, genetic correlations between the regulation of the orientation of cortical MTs and the direction of cell elongation have not been fully clarified, except in the case of the *SPR* genes of *Arabidopsis* (Furutani *et al.*, 2000). As shown in the present study, the close relationship between the change in gross morphology and the abnormal arrangement of cortical MTs strongly suggests that the *AN* gene plays an important role in regulation of the orientation of cortical MTs in leaf cells. This is the first reported proof, to

Table II. Genes up-regulated in the *angustifolia* leaves, as compared with wild-type leaves and identified by microarray analysis^a

Accession ^b	Encoded protein	Up-regulation (-fold; <i>an</i> /wt) ^c
AF08279	Cys-3-His zinc finger protein	11.3
Monsanto	unknown	8.3
D21839	MAP kinase 3	3.5
Monsanto	putative mitochondrial uncoupling protein	3.5
Monsanto	putative protein	3.4
U40217	homolog of eukaryotic release factor 1	3.4
D63508	MER15 (xyloglucan endo-transglycosylase)	3.3
AF0(2109)	Hs1pro-1-related protein	3.1

^aGenes for which levels of transcripts were more than three times higher in *angustifolia* leaves than in wild-type leaves are shown.

^bDesignations and accession numbers of registered genes that are identical to the identified cDNAs.

^cRatio of the level of transcripts in the *angustifolia* mutant to that in wild type.

our knowledge, of the genetic regulation of the arrangement of cortical MTs in leaf cells.

***AN* is a member of the CtBP family and might act as a repressor of transcription**

The *AN* gene is the first homolog of *CtBP* gene to be identified in a plant genome. The CtBP family has been identified as a new family of co-repressors from animal genomes and its members have been shown to play important roles in cell differentiation (for review see Turner and Crossley, 2001). Human *CtBP* (now *hCtBP*) was first isolated by yeast two-hybrid cloning, as the gene for a cellular phosphoprotein that interacts with a C-terminal domain of the E1A protein of adenovirus (Schaeper *et al.*, 1995, 1998). It has been proposed that binding of *hCtBP* to E1A modulates E1A-mediated transformation (tumorigenesis). It has also been proposed that *hCtBP* might be a negative regulator of transcription (Schaeper *et al.*, 1995; Sollerbrant *et al.*, 1996; Molley *et al.*, 1998). Schaeper *et al.* (1995) noted the sequence homology between *hCtBP* and D-2-hydroxy acid dehydrogenase but they did not detect any such enzymatic activity associated with *hCtBP*. Since several amino acids that are important for the enzymatic activity of D-lactate-dehydrogenase (Bernard *et al.*, 1997) were not found in the amino acid sequence of *AN*, it is unlikely that *AN* has such dehydrogenase activity.

After the identification of *hCtBP*, many homologs of *hCtBP* were isolated from animal genomes. A homolog of *hCtBP* in mouse, *mCtBP*, was also isolated as a co-repressor that associates with regulators of transcription (Turner and Crossley, 1998) and a CtBP from *Xenopus* (*xCtBP1*) was identified as a transcriptional repressor that interacts with Polycomb which, itself, is involved in the repression of certain genes during the course of vertebrate development (Sewalt *et al.*, 1999). Several extensive studies have revealed recently that a homolog of *hCtBP* from *Drosophila*, *dCtBP*, plays an important role in the repression of genes which is required for normal development of the embryo (Nibu *et al.*, 1998, 2001; Poortinga *et al.*, 1998; Mannervik and Levine, 1999; Rosée-Borggreve *et al.*, 1999; Zhang and Levine, 1999; Phippen *et al.*, 2000; Wen *et al.*, 2000; Morel *et al.*, 2001;

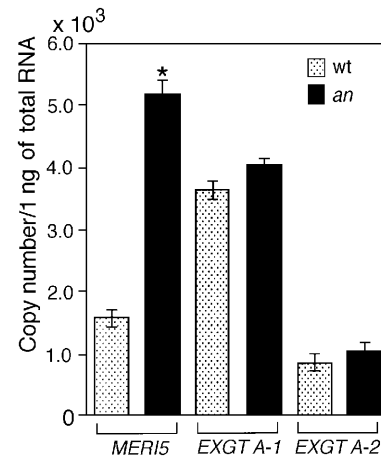


Fig. 11. Levels of expression of genes for three members of the XTH family in wild-type and *angustifolia* leaves, as detected by real-time RT-PCR. Average results from triplicate assays are shown with SD. An asterisk indicates a significant difference from the wild-type level (level of significance, 5%).

Nibu and Levine, 2001). *dCtBP* is a key protein that regulates aspects of developmental processes via repression of various developmental genes, acting as a context-dependent transcriptional cofactor that interacts with several kinds of co-repressor. Thus, CtBPs in animals appear likely to be engaged in an evolutionarily conserved mechanism of transcriptional repression. It is also true, however, that brefeldin A ADP-ribosylated substrates (BARS; Spanfö *et al.*, 1999; Weigert *et al.*, 1999), which appear to play an important role in maintenance of the Golgi apparatus are also strongly homologous to products of *CtBP* genes (Figure 5) and the CtBPs are also known as CtBP/BARS.

Animal and plant CtBPs might share evolutionarily conserved functions as transcriptional co-repressors. The intracellular localization of the product of the *AN::GFP* gene (Figure 8) supports this hypothesis since it indicates that the *AN* protein is present in the nucleus. However, appreciable amounts of *AN* protein were also found in the cytoplasm. In animal cells, CtBPs are also abundant in nuclei but they are not exclusively nuclear (for review see Turner and Crossley, 2001).

Northern analysis of the *AN* transcript showed that the gene is expressed in all organs of *Arabidopsis*, even though the *an* phenotype is restricted to leaves and floral organs. The target gene(s) of the *AN* protein that is responsible for the regulation of the polar expansion of cells might be specifically expressed in leaf cells but the target gene(s) remains to be identified. The target genes of animal CtBPs are not conserved among species. In addition, as shown in Figure 5, the molecular phylogeny of the *AN* gene suggests that *AN* might have a unique function that is not shared by animal CtBPs. Our preliminary microarray analysis of cDNAs (performed in collaboration with Monsanto as part of their *Arabidopsis* Microarray Program) suggests that, in the *an* mutant plant, levels of transcripts of some genes (~10 genes among 10 000 clones) might be more than 3-fold higher than in the wild type (Table II). We found no gene that was expressed at a lower level in the *an* mutant than in the wild type.

While we postulate that the shape of leaf cells might be regulated by the *AN* gene via control of the arrangement of MTs in leaf cells, some researchers have noted recently that, on occasion, a simple correlation between the orientation of MTs and cell shape cannot be observed (Wasteneys, 2000). Thus, it is also possible that the construction or composition of cell walls might be regulated by *AN*. It is of interest that one of the candidates for target genes identified by microarray analysis was *MER15*, which encodes a member of the XTH family and is considered to play a role in the molecular grafting of components of cell walls (Nishitani, 1997). While levels of expression of genes for two other members of the XTH family, *EXGT-A1* and *EXGT-A2*, were unaffected by the *an* mutation, the level of transcription of *MER15* in rosette leaves of the *an* mutant was three times higher than in the wild type (Figure 11). The *MER15* gene is preferentially expressed in apical meristems and leaf primordia in *Arabidopsis* (Medford *et al.*, 1991), an observation that suggests that *MER15* might play a role at the early stages of leaf morphogenesis. Overexpression of *MER15* cDNA under control of constitutive CaMV 35S promoter caused the precocious elongation and/or abnormal expansion of leaf cells (Verica and Medford, 1997). Although the gross morphology of leaves of transgenic plants that carried CaMV 35S::*MER15* (Verica and Medford, 1997) was different from those of the *an* mutant, it is possible that the difference might have been due to a lack of tissue specificity of the overexpression in the transgenic plants. Since both *MER15*-overexpressing transgenic plants and *an* mutant plants show alterations in the process of expansion of leaf cells, it is possible that the *AN* gene might control cell shape in leaves by regulating the expression of *MER15* directly or indirectly. In this case, the effect of *AN* gene on arrangement of MTs in leaf cells might be a secondary result of alteration of cell walls by activity of *MER15*.

Our results support the hypothesis that the product of the *AN* gene might act as a repressor of transcription. The relationship between regulation of the arrangement of cortical MTs and such negative regulation of transcription is unclear. However, such a relationship is not implausible. Detailed analysis of the target(s) of the *AN* protein should reveal new aspects of gene regulation in plants and, in particular, of the regulation of the polarity-specific expansion of cells. As Folkers *et al.* (2002) strongly suggest, *AN* might interact with *ZWICHEL* (*ZWI*), a kinesin-like protein, in trichome cells. Since the morphological phenotype of *zwi* mutants is restricted to the shape of trichomes and since *zwi* leaves are normal in shape (Oppenheimer *et al.*, 1997), *AN* should interact with some other component(s) in other leaf cells in the regulation of leaf width. It is noteworthy that *AN* self-associates in yeast cells (Figure 10). Homodimerization is thought to be necessary for linkage by animal CtBPs of two different proteins (for review see Turner and Crossley, 2001). The proteins interacting with animal CtBPs depends on circumstance. It is very possible that *AN* might interact with *ZWI* during the morphogenesis of trichomes and with other components in other types of cell or at other stages of leaf development.

Details of the functions of animal CtBPs remain to be determined. It is possible that CtBPs regulate gene

expression directly via their enzymatic activities and/or by simply linking other proteins (Turner and Crossley, 2001). Our phylogenetic analysis suggests that plant CtBPs, including the product of the *AN* gene, might have evolved to play a role that is specific to plants since plant CtBPs appear to be monophyletic and to form a unique subfamily among homologs of hCtBP. *AN* is the first member of the CtBP family to be identified in plants and future studies of *AN* should help us to understand how the functions of members of the CtBP family have been conserved in the control of the development of multicellular organisms.

Materials and methods

Plant culture

Seeds were sown on rockwool and/or vermiculite, moistened with MGRL medium, as described elsewhere (Tsukaya *et al.*, 1991; Tsuge *et al.*, 1996). Plants were grown at 23°C under continuous white fluorescent light ($67.4 \pm 14.0 \mu\text{M/s/m}^2$). For observations of root cells, plants were cultivated on agar plates, as described by Okada and Shimura (1992). The *an-1* single-mutant lines were established as described by Tsukaya *et al.* (1994). Another allele, *an-2*, was isolated by Dr T.Hattori of Mie University, Tsu, Japan, from mutagenized seeds of the Columbia ecotype and kindly donated to the authors. The directions relating to the analysis of leaf blades are defined in an earlier report (Tsuge *et al.*, 1996).

Anatomical and immunohistochemical analysis

The procedures for preparing sections of samples embedded in Technovit 7100 resin (Kulzer & Co. GmbH, Wehrheim, Germany) were described previously (Tsukaya *et al.*, 1993; Tsuge *et al.*, 1996). Immunohistochemical observations of cortical MTs in cells of internodal stems and primary roots were made as described earlier (Tsukaya *et al.*, 1995). Epidermal cells from leaf petioles were collected from the first set of foliage leaves at the fully mature stage. Stem samples were collected from the first internodal stem of type II metamers when they had reached 5–7 mm in length. Root samples were collected from primary roots of seedlings 3–7 days after sowing. Cells were examined at the growth stage prior to elongation.

The MTs in leaf cells were visualized by immunohistochemical staining, which was performed by a modified version of previously described methods (Ishida and Katsumi, 1991; Tsukaya *et al.*, 1995) or by a freeze-shattering method (Wasteneys *et al.*, 1997). Samples of mesophyll cells were prepared immediately after stage I (Tsuge *et al.*, 1996), when the polar elongation of cells had just begun. We examined the arrangement of cortical MTs in cells in the subepidermal layer on the adaxial side of the leaf. We used monoclonal mouse antibodies against chicken α - and β -tubulin (Amersham Japan Co., Tokyo, Japan) diluted 500-fold in phosphate-buffered saline (PBS) plus 0.1% NaN₃ and fluorescein isothiocyanate-labelled (FITC-labelled) sheep antibodies against mouse IgG (Amersham Japan Co.). Alternatively, we used an Alexa488-labelled Signal Amplification Kit for mouse antibodies (Molecular Probes, Leiden, The Netherlands). For observations of cellulose microfibrils, we treated samples with 0.01% Fluostain-I (Wako, Osaka, Japan) in 50 mM sodium phosphate buffer pH 7.0 for 5 min, with three subsequent washes with 50 mM sodium phosphate buffer. Samples on glass slides were set in mounting solution [1 mg/ml *n*-propyl gallate (Wako), dissolved in a 50% solution of glycerol in water plus 0.1% NaN₃]. Samples were observed with a fluorescence microscope (DAS Mikroskop DMR; Leica, Wetzlar, Germany) or a confocal laser microscope (LEITZ DMIRB, with a True Confocal Scanner, LEICA TCS 4D; Leica).

For observations of cortical MTs in living cells, we also used transgenic *Arabidopsis* (ecotype Columbia) that harbored the *TUB6::GFP* fusion gene (a kind gift of Dr T.Hashimoto, NAIST, Nara, Japan; Ueda *et al.*, 1999). This gene encodes GFP fused to a gene for tubulin, *TUB6*, under the control of the CaMV 35S promoter. We crossed this transgenic line with the *an* mutant to generate *an* mutant plants that expressed the fusion gene, in which we were able to examine the arrangement of cortical MTs with a confocal laser microscope (Olympus FLUoview/BX50; Olympus, Tokyo, Japan).

Molecular biological analysis

Routine procedures, including the isolation of DNA, Southern and northern blotting analysis, and nucleotide sequencing, were performed

essentially as described elsewhere (Sambrook *et al.*, 1989). The genomic DNA clone including the *PVV4* fragment was a kind gift of Dr T. Golden (University of Rochester, Rochester, NY). TAC clones that gave positive signals in dot-blot analysis with the *PVV4* fragment as a probe were identified and used to transform *an-1* mutant plants. The *Arabidopsis* EST clone YAY141 was obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH) and it was sequenced in its entirety using primers derived from the previously determined DNA sequence. The deduced amino acid sequence encoded by the *AN* gene in the Columbia ecotype was identical to that in the *Ler* ecotype. The cDNA sequence of the *AN* gene was identical to that of the coding region of the genomic DNA sequence with the exception of a missense alteration at position 299 (Met to Ile) in the cDNA sequence. The cDNA sequence of the *AN* gene has been deposited in the DDBJ/EMBL/GenBank database (accession No. AB032060).

To identify the *an-1* mutation, we constructed a genomic DNA library from an *an-1* mutant plant by ligation into *Hind*III-digested lambda DASHII of fragments from a partial *Sau*3AI digest of the mutant genome using an *In vitro* Packaging Kit (Stratagene, La Jolla, CA). Southern blotting and plaque-hybridization analysis were performed with a Gene Images Random-Prime Labelling and Detection System and an AlkPhos Direct System (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. We chose three independent genomic clones that gave positive signals for sequencing. To identify the mutation in *an-2*, we amplified the region that corresponded to the open reading frame (ORF) of the *AN* gene in the genome of the *an-2* mutant using the total RNA of an *an-2* mutant plant and a Superscript One-Step RT-PCR Kit (Gibco BRL, Rockville, MD) and subcloned the fragment into pBluescript SK (Stratagene). Three independent clones were chosen for sequencing.

Northern blotting analysis was performed with [³²P]ATP-labelled *AN* cDNA as the probe. Total RNA was purified from various tissues as described previously (Kim *et al.*, 1998a) and 20 µg aliquots of total RNA were used for northern analysis. Aliquots of 0.2–0.5 µg of total RNA were treated with DNase I at 37°C for 30 min before RT-PCR and each set of primers for PCR was designed such that a different exon sequence was included in each set of primers, to avoid amplification of any contaminating genomic DNA. The conditions for direct amplification by RT-PCR with a Superscript One-Step RT-PCR Kit (Gibco BRL) were one cycle at 50°C for 30 min and 94°C for 2 min, then 20, 25 or 30 cycles at 94°C for 15 s, 55°C for 15 s and 72°C for 90 s. The following oligonucleotides were used as primers for RT-PCR: AN-start, 5'-ATGAGCAAGATCCGTTTC-3'; AN-stop, 5'-TTAATCGATCCAACGTGTA-3'; AN-RT-for1, 5'-TGAGACGGTGCCGTGGTATGG-3'; AN-RT-rev1, 5'-GTTGCTACTGGTGGATTCC-3' and AT-RT-rev2, 5'-CTTACCGGATCTGCTACGTC-3'. Amplification of β -*TUB4* noncoding sequence was performed with two primers, as described previously (Kim *et al.*, 1998a) as a positive control in the analysis by RT-PCR.

For microarray analysis, total RNA was extracted from 2-week-old rosette leaves (including shoot apical meristems) of plants that had been cultivated aseptically on MS0 plate, as described earlier (Tsukaya *et al.*, 1991). The tissue was frozen and ground in liquid nitrogen and RNA was extracted with a Trizol™ reagent kit (Gibco BRL, Grand Island, NY). Microarray analysis of the RNA was carried out under the auspices of the Monsanto *Arabidopsis* Microarray Program (Monsanto, St Louis, MI).

Analysis of levels of expression of transcripts of *EXGT-A1*, *EXGT-A2* and *MER15* in rosette leaves of 20-day-old seedlings of wild-type and *an* mutant plants was carried out by real-time RT-PCR (ABI Prism 5700 Sequence-Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA) using specific primers as described earlier (Yokoyama and Nishitani, 2001).

Yeast two-hybrid assay

For two-hybrid analysis, we used the yeast Y190 [*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4-*, *gal80-*, *cyh2*, *LYS2::GAL1_{UAS}-HIS3_{TATA}-HIS3*, *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*] as host strain. Two full-length *AN* cDNAs that differed in terms of a 5' restriction site were constructed by PCR. *AN* cDNA with an *Nde*I–*Bam*HI site inserted into pAS2-1 (Clontech Japan, Tokyo, Japan) that generated GAL4 DNA-binding domain (GAL4BD) was used as bait, and *AN* cDNA with an *Nco*I–*Bam*HI site inserted into pACT2 (Clontech Japan, Tokyo, Japan) generating GAL4 activation domain (GAL4AD) was used as prey. Both clones were sequenced to confirm the nature of the products. SD medium (–Trp –Leu –His; prepared according to the manufacturer's instructions) that contained 25 mM 3-amino-1,2,4-triazole (3-AT) was used for test of two hybrids.

Phylogenetic analysis

We generated phylogenetic trees using PROTPARS, a maximum-parsimony algorithm that is included in the PHYLIP version 3.5 software package (distributed by Dr J. Felsenstein, University of Washington, Seattle, WA). Topological robustness was assessed by bootstrap analysis with 1000 replicates using simple taxon addition (Felsenstein, 1985).

Sequences used for alignments were identified by BLAST searches of DDBJ/EMBL/GenBank. Sequences conserved in *AN*, homologs of *AN*, animal CtBPs and human BARS were aligned with the CLUSTAL_W program (Thompson *et al.*, 1994) and alignments were refined manually. Several short sequences in the N-terminal region that could not be aligned unambiguously were excluded from the analysis.

Construction of transgenic *Arabidopsis*

For complementation tests, genomic TAC clones were introduced into *an-1* mutant plants by a modified version of a previously published method for the *Agrobacterium*-mediated transformation of roots (Ueda *et al.*, 1996). For construction of the *AN::GFP* fusion gene, we amplified a 1.9 kbp coding fragment of the *AN* gene by PCR, using oligonucleotides that introduced a *Sa*I and an *Nco*I restriction site at the N- and C-terminal ends, respectively. Amplified fragments were sequenced and ligated into the *Sa*I and *Nco*I sites of the sGFP vector pTH-2 (a kind gift of Dr Y. Niwa, Shizuoka Prefectural University, Japan; Chiu *et al.*, 1996). The *AN::GFP* fusion gene was introduced into the pBI vector, which included a CaMV 35S promoter and a NOS terminator cassette. Lines of transgenic plants derived from the wild type (ecotype Columbia) and the *an-1* mutant and harboring this construct were established by *Agrobacterium*-mediated transformation, which was performed by a simplified *in planta* infiltration method, as described by Kim *et al.* (1999). An experiment to examine the transient expression of the fusion gene in onion epidermal cells was performed using a particle delivery system (PDS-1000/He; Bio-Rad Japan, Tokyo, Japan) according to the manufacturer's protocol.

Accession number

The DDBJ/EMBL/GenBank accession No. of the ORF in the *AN* gene is AB032060.

Acknowledgements

The authors thank Mr N. Inoue (Japan University, Tokyo, Japan) for his help with experiments. Drs I. Yabe (University of Tokyo) and S. Mano (NIBB, Okazaki, Japan) kindly taught H.T. to manipulate the confocal microscopes. The authors also thank Dr Y. Niwa (Shizuoka Prefectural University, Shizuoka, Japan) for the sGFP vector, Dr G. Wasteneys (Australia National University, Canberra, Australia) for kindly introducing them to his freeze-shattering method for staining MTs, Dr T. Hashimoto (NAIST, Nara, Japan) for the kind gift of transgenic *Arabidopsis* that expressed the *TUB6::GFP* fusion gene, Dr T. Golden (University of Rochester, Rochester, NY) and Dr D. Shibata (formerly of the Mitsui Plant Biotechnology Research Institute, Tsukuba, Japan and now at the Kazusa DNA Research Institute, Kazusa, Japan) for kind gifts of genomic clones and TAC clones of *Arabidopsis*. Dr T. Hattori (Mie University, Tsu, Japan) isolated the *an-2* allele and kindly supplied the authors with seeds. Dr E. Nitasata (Kyushu University, Fukuoka, Japan) and Dr H. Takano (Kumamoto University, Kumamoto, Japan) kindly permitted the authors to use their unpublished sequence data for construction of the phylogenetic tree of *AN* homologs. The authors thank Dr C. W. Lloyd (John Innes Institute, Norwich, UK) for his critical reading of the MT-related parts of an early version of the manuscript. The authors also thank Dr H. Shibaoka (Osaka University, Osaka, Japan) for valuable discussions. Ms A. Shimoto no of the University of Tokyo (Tokyo, Japan) helped with the sequencing of the ORF of the *AN* gene. Microarray analysis was carried out by Monsanto (St Louis, MI) under a Monsanto *Arabidopsis* Microarray Program Award. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (to G.-T.K. and H.T.); by a grant from the Bio-Design Program of the Ministry of Agriculture, Forestry and Fisheries of Japan (to H.T.) and by a grant from PRESTO, Japan Science and Technology Corporation, Japan (to H.T.).

References

- Aeschbacher, R.A., Hauser, M.-T., Feldmann, K.A. and Benfey, P.N. (1995) The *SABRE* gene is required for normal cell expansion in *Arabidopsis*. *Genes Dev.*, **9**, 330–340.

- Akamatsu, T., Hanzawa, Y., Ohtake, Y., Takahashi, T., Nishitani, K. and Komeda, Y. (1999) Expression of endoxyloglucan transferase genes in *acaulis* mutants of *Arabidopsis thaliana*. *Plant Physiol.*, **121**, 715–721.
- Arthur, M., Molinas, C., Dutka-Malen, S. and Courvalin, P. (1991) Structural relationship between the vancomycin-resistance protein VanH and 2-hydroxycarboxylic acid dehydrogenases. *Gene*, **103**, 133–134.
- Bernard, N. *et al.* (1997) D-2-Hydroxy-4-methylvalerate dehydrogenase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. 2. Mutagenic analysis of catalytically important residues. *Eur. J. Biochem.*, **244**, 213–219.
- Boyd, J.M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S.T. and Chinnadurai, G. (1993) A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *EMBO J.*, **12**, 469–478.
- Chiu, W.-L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr. Biol.*, **6**, 325–330.
- Cyr, R.J. (1994) Microtubules in plant morphogenesis: role of the cortical array. *Annu. Rev. Cell Biol.*, **10**, 153–180.
- Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.
- Folkers, U. *et al.* (2002) The cell morphogenesis gene *ANGUSTIFOLIA* encodes a CtBP/BARS-like protein and is involved in the control of the microtubule cytoskeleton. *EMBO J.*, **21**, 1280–1288.
- Furutani, I., Watanabe, Y., Prieto, R., Masukawa, M., Suzuki, K., Naoi, K., Thitamabee, S., Shikanai, T. and Hashimoto, T. (2000) The *SPIRAL* genes are required for directional control of cell elongation in *Arabidopsis thaliana*. *Development*, **127**, 4443–4453.
- Hauser, M.-T., Morikami, A. and Benfey, P.N. (1995) Conditional root expansion mutants of *Arabidopsis*. *Development*, **121**, 1237–1252.
- Hogetsu, T. (1989) The arrangement of microtubules in leaves of monocotyledonous and dicotyledonous plants. *Can. J. Bot.*, **67**, 3506–3512.
- Hülkamp, M., Misera, S. and Jürgens, G. (1994) Genetic dissection of trichome cell development in *Arabidopsis*. *Cell*, **76**, 555–566.
- Ishida, K. and Katsumi, M. (1991) Immunofluorescence microscopical observation of cortical microtubule arrangement as affected by gibberellin in d5 mutants of *Zea mays* L. *Plant Cell Physiol.*, **32**, 409–417.
- Kim, G.-T., Tsukaya, H. and Uchimiya, H. (1998a) The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev.*, **12**, 2181–2191.
- Kim, G.-T., Tsukaya, H. and Uchimiya, H. (1998b) The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana*. *Planta*, **206**, 175–183.
- Kim, G.-T., Tsukaya, H., Saito, Y. and Uchimiya, H. (1999) Changes in the shapes of leaves and flowers upon overexpression of the novel cytochrome P450 in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **96**, 9433–9437.
- Koornneef, M., van Eden, J., Hanhart, C.J., Stam, P., Braaksma, F.J. and Feenstra, W.J. (1983) Linkage map of *Arabidopsis thaliana*. *J. Hered.*, **74**, 265–272.
- Lee-Chen, S. and Steinitz-Sears, L.M. (1967) The location of linkage groups in *Arabidopsis thaliana*. *Can. J. Genet. Cytol.*, **9**, 381–384.
- Lee, J.-O., Alicia, A., Russo, A.A. and Pavletich, N.P. (1998) Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature*, **391**, 859–865.
- Liu, Y.-G., Shirano, Y., Fukaki, H., Yanai, Y., Tasaka, M., Tabata, S. and Shibata, D. (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. *Proc. Natl Acad. Sci. USA*, **96**, 6535–6540.
- Lloyd, C.W., Shaw, P.J., Warn, R.M. and Yuan, M. (1996) Gibberellic-acid-induced reorientation of cortical microtubules in living plant cells. *J. Microscopy*, **181**, 140–144.
- Mannervik, M. and Levine, M. (1999) The Rpd3 histone deacetylase is required for segmentation of the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA*, **96**, 6797–6801.
- Mathur, J. and Chua, N.-H. (2000) Microtubule stabilization leads to growth reorientation in *Arabidopsis* trichomes. *Plant Cell*, **12**, 465–477.
- McClinton, R.S. and Sung, Z.R. (1997) Organization of cortical microtubules at the plasma membrane in *Arabidopsis*. *Planta*, **201**, 252–260.
- Medford, J., Elmer, J.S., Klee, H.J. (1991) Molecular cloning and characterization of genes expressed in shoot apical meristem. *Plant Cell*, **3**, 359–370.
- Molloy, D.P., Milner, A.E., Yakub, I.K., Chinnadurai, G., Gallimore, P.H. and Grant, R.J.A. (1998) Structural determinants present in the C-terminal binding protein binding site of adenovirus early region 1A proteins. *J. Biol. Chem.*, **273**, 20867–20876.
- Morel, V., Lecourtois, M., Massiani, O., Maier, D., Preiss, A. and Schweisguth, F. (2001) Transcriptional repression by Suppressor of Hairless involves the binding of a Hairless–dCtBP complex in *Drosophila*. *Curr. Biol.*, **11**, 789–792.
- Nibu, Y. and Levine, M.S. (2001) CtBP-dependent activities of the short-range Giant repressor in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA*, **98**, 6204–6208.
- Nibu, Y., Zhang, H. and Levine, M. (1998) Interaction of short-range repressors with *Drosophila* CtBP in the embryo. *Science*, **280**, 101–104.
- Nibu, Y., Zhang, H. and Levine, M. (2001) Local action of long-range repressors in the *Drosophila* embryo. *EMBO J.*, **20**, 2246–2253.
- Nishitani, K. (1997) The role of endoxyloglucan transferase in the organization of plant cell walls. *Int. Rev. Cytol.*, **173**, 157–206.
- Okada, K. and Shimura, Y. (1992) Mutational analysis of root gravitropism and phototropism of *Arabidopsis thaliana* seedlings. *Aust. J. Plant Physiol.*, **19**, 439–448.
- Okazawa, K., Sato, Y., Nakagawa, T., Asada, K., Kato, I., Tomita, E. and Nishitani, K. (1993) Molecular cloning and cDNA sequencing of endoxyloglucan transferase, a novel class of glycosyltransferase that mediates molecular grafting between matrix polysaccharides in plant cell walls. *J. Biol. Chem.*, **268**, 25364–25368.
- Oppenheimer, D.G. (1998) Genetics of plant cell shape. *Curr. Opin. Plant Biol.*, **130**, 630–650.
- Oppenheimer, D.G., Pollock, M.A., Vacik, J., Szymanski, D.B., Ericson, B., Feldmann, K. and Marks, M.D. (1997) Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis. *Proc. Natl Acad. Sci. USA*, **94**, 6261–6266.
- Phippen, T.M., Sweigart, A.L., Moniwa, M., Krumm, A., Davie, J.R. and Parkhurst, S.M. (2000) *Drosophila* C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both Mad and Groucho transcriptional repression. *J. Biol. Chem.*, **275**, 37628–37637.
- Poortinga, G., Watanabe, M. and Parkhurst, S.M. (1998) *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and Hairy-mediated transcriptional repression. *EMBO J.*, **17**, 2067–2078.
- Potikha, T. and Delmer, D.P. (1995) A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. *Plant J.*, **2**, 453–460.
- Rédei, G.P. (1962) Single locus heterosis. *Z. Vererbungs.*, **93**, 164–170.
- Rosée-Borggreve, A.L., Häder, T., Wainwright, D., Sauer, F. and Jäckle, H. (1999) hairy stripe 7 element mediates activation and repression in response to different domains and levels of Krüppel in the *Drosophila* embryo. *Mech. Dev.*, **89**, 133–140.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schaeper, U., Boyd, J.M., Verma, S., Uhlmann, E., Subramanian, T. and Chinnadurai, G. (1995) Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl Acad. Sci. USA*, **92**, 10467–10471.
- Schaeper, U., Subramanian, T., Lim, L., Boyd, J.M. and Chinnadurai, G. (1998) Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif. *J. Biol. Chem.*, **273**, 8549–8552.
- Sewalt, R.G.A.B., Gunster, M.J., van der Vlag, J., Satijn, D.P.E. and Otte, A.P. (1999) C-terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. *Mol. Cell Biol.*, **19**, 777–787.
- Shibaoka, H. (1994) Plant hormone-induced changes in the orientation of cortical microtubules: alterations in the cross-linking between microtubules and the plasma membrane. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 527–544.
- Singh, R. and Green, M.R. (1993) Sequence-specific binding of transfer

- RNA by glyceraldehyde-3-phosphate dehydrogenase. *Science*, **259**, 365–368.
- Sollerbrant, K., Chinnadurai, G. and Svensson, C. (1996) The CtBP binding domain in the adenovirus E1A protein controls CR1-dependent transactivation. *Nucleic Acids Res.*, **24**, 2578–2584.
- Spanfö, S. *et al.* (1999) Molecular cloning and functional characterization of Brefeldin A-ADP-ribosylated substrate. *J. Biol. Chem.*, **274**, 17705–17710.
- Terryn, N. *et al.* (1998) Sequence analysis of a 40-kb *Arabidopsis thaliana* genomic region located at the top of chromosome 1. *Gene*, **215**, 11–17.
- Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL_W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
- Traas, J., Bellini, C., Nacry, P., Kronenberger, J., Bouchez, D. and Caboche, M. (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. *Nature*, **375**, 676–677.
- Tsuge, T., Tsukaya, H. and Uchimiya, H. (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L.) Heynh. *Development*, **122**, 1589–1600.
- Tsukaya, H. (1995) Developmental genetics of leaf morphogenesis in dicotyledonous plants. *J. Plant Res.*, **108**, 407–416.
- Tsukaya, H. (1998) Genetic evidence for polarities that regulate leaf morphogenesis. *J. Plant Res.*, **111**, 113–119.
- Tsukaya, H. (2002) Interpretation of mutants in leaf morphology: genetic evidence for a compensatory system in leaf morphogenesis that provides a new link between Cell and Organismal theory. *Int. Rev. Cytol.*, **217**, in press.
- Tsukaya, H., Ohshima, T., Naito, S., Chino, M. and Komeda, Y. (1991) Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis thaliana*. *Plant Physiol.*, **97**, 1414–1421.
- Tsukaya, H., Naito, S., Rédei, G.P. and Komeda, Y. (1993) A new class of mutations in *Arabidopsis thaliana*, *acaulis1*, affecting the development of both inflorescences and leaves. *Development*, **118**, 751–764.
- Tsukaya, H., Tsuge, T. and Uchimiya, H. (1994) The cotyledon: a superior system for studies of leaf development. *Planta*, **195**, 309–312.
- Tsukaya, H., Inaba-Higano, K. and Komeda, Y. (1995) Phenotypic characterization and molecular mapping of *acaulis2* mutant with flower stalks of much reduced length in *Arabidopsis thaliana*. *Plant Cell Physiol.*, **36**, 239–246.
- Turner, J. and Crossley, M. (1998) Cloning and characterization of mCtBP2, a co-repressor that associates with basic Krüppel-like factor and other mammalian transcriptional regulators. *EMBO J.*, **17**, 5129–5140.
- Turner, J. and Crossley, M. (2001) The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *BioEssays*, **23**, 683–690.
- Ueda, T., Anai, T., Tsukaya, H., Hirata, A. and Uchimiya, H. (1996) Characterization and subcellular localization of a small GTP-binding protein (Ara-4) from *Arabidopsis*: conditional expression under control of the promoter of the gene for heatshock protein HSP81-1. *Mol. Gen. Genet.*, **250**, 533–539.
- Ueda, K., Matsuyama, T. and Hashimoto, T. (1999) Visualization of microtubules in living cells of transgenic *Arabidopsis thaliana*. *Protoplasma*, **206**, 201–206.
- Verica, J.A. and Medford, J. (1997) Modified *MER15* expression alters cell expansion in transgenic *Arabidopsis* plants. *Plant Sci.*, **125**, 201–210.
- Wasteneys, G.O. (2000) The cytoskeleton and growth polarity. *Curr. Opin. Plant Biol.*, **3**, 503–511.
- Wasteneys, G.O., Willingale-Theun, J. and Menzel, D. (1997) Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. *J. Microscopy*, **188**, 51–61.
- Weigert, R. *et al.* (1999) CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature*, **402**, 429–433.
- Wen, Y., Nguyen, D., Li, Y. and Lai, Z.-C. (2000) The N-terminal BTB/POZ domain and C-terminal sequences are essential for Tramtrack69 to specify cell fate in the developing *Drosophila* eye. *Genetics*, **156**, 195–203.
- Wymer, C. and Lloyd, C. (1996) Dynamic microtubules: implications for cell wall patterns. *Trends Plant Sci.*, **1**, 222–228.
- Yokoyama, R. and Nishitani, K. (2001) A comprehensive expression-analysis of all members of a gene family encoding cell wall enzymes allows us to predict *cis*-regulatory regions involved in the cell wall construction in specific organs of *Arabidopsis*. *Plant Cell Physiol.*, **42**, 1025–1033.
- Yuan, M., Warn, R.M., Shaw, P.J. and Lloyd, C.W. (1995) Dynamic microtubules under the radial and outer tangential walls of microinjected pea epidermal cells observed by computer reconstruction. *Plant J.*, **7**, 17–23.
- Zhang, H. and Levine, M. (1999) Groucho and dCtBP mediate separate pathways of transcriptional repression in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA*, **96**, 535–540.

Received August 30, 2001; revised December 3, 2001;
accepted December 20, 2001