Classification and quantification of leaf curvature

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Received 4 September 2009; Revised 25 March 2010; Accepted 30 March 2010

Abstract

Various mutants of Arabidopsis thaliana deficient in polarity, cell division, and auxin response are characterized by certain types of leaf curvature. However, comparison of curvature for clarification of gene function can be difficult without a quantitative measurement of curvature. Here, a novel method for classification and quantification of leaf curvature is reported. Twenty-two mutant alleles from Arabidopsis mutants and transgenic lines deficient in leaf flatness were selected. The mutants were classified according to the direction, axis, position, and extent of leaf curvature. Based on a global measure of whole leaves and a local measure of four regions in the leaves, the curvature index (CI) was proposed to quantify the leaf curvature. The CI values accounted for the direction, axis, position, and extent of leaf curvature in all of the Arabidopsis mutants grown in growth chambers. Comparison of CI values between mutants reveals the spatial and temporal variations of leaf curvature, indicating the strength of the mutant alleles and the activities of the corresponding genes. Using the curvature indices, the extent of curvature in a complicated genetic background becomes quantitative and comparable, thus providing a useful tool for defining the genetic components of leaf development and to breed new varieties with leaf curvature desirable for the efficient capture of sunlight for photosynthesis and high yields.

Key words: Arabidopsis, auxin response, cell division, curvature index, leaf, miRNA, mutants, polarity.

Introduction

Leaf morphogenesis, particularly the roles of polarity, cell division, and auxin response, in the genesis of plant form has long been a focus of study. In contrast to indeterminate shoot apical meristems, leaves of higher plants are determinate in development. The leaf primordium originates from the flank of the shoot apical meristem and is dorsoventrally symmetrical and flattened on its adaxial side. Upon formation of the blades, the three-dimensional leaf form is specified along the proximodistal (base-to-tip), dorsoventral (top-to-bottom), and mediolateral (middle-to-margin) axes. Polarity along the three axes is associated with cell cycle activities in the marginal meristem (Donnelly et al., 1999) and with movements of the arrest front of cell division in the leaves (Nath et al., 2003). Coordination of polarity, cell division, and auxin response is critical for morphogenesis of normal leaves and for plant architecture. An imbalance of these developmental processes results in altered leaf shapes, such as curly, crinkly, twisted, rolled, or shrunken leaves. In recent years, mutants with different curvatures have been isolated and described (Serrano-Cartagena et al., 2000), and various molecular reasons have been identified.

Mutant plants deficient in polarity, cell division, and the auxin response exhibit abnormal leaf curvature. Class III homeodomain leucine zipper (HD-Zip III) genes are targets of miR165/166. Of these, PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) act redundantly to promote the adaxial cell fates of the leaf primordium (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). Dominant mutations in the Arabidopsis PHB and PHV transcription factor genes switch the leaf fate from abaxial to adaxial by altering a microRNA (miRNA) complementary site present in processed PHB or PHV (McConnell et al., 2001). The alternating leaf polarity is related to the interruption of miRNA-mediated regulation of REV (Emery et al., 2003). ATHB8 (HB-8) and CORONA (CNA) encode functions that are antagonistic to...
those of REV within certain tissues and overlap with those of REV in other tissues (Prigge et al., 2004; Green et al., 2005). The TEOSINTE BRANCHED1/CYCLOID1/PCF (TCP) family contains five miR319a targets, which are the Arabidopsis homologues of the Antirrhinum CINNAMATA (CIN) gene (Nath et al., 2003). The TCP transcription factors play a pivotal role in the control of morphogenesis of shoot organs by negatively regulating the expression of boundary-specific genes (Koyama et al., 2007). ARF10, ARF16, and ARF17 are the three targets of miR160. Plants expressing mARF17 or mARF16 have incurved leaves, corresponding to increased ARF17 or ARF16 mRNA levels (Mallory et al., 2005; Wang et al., 2005).

miR165/166, miR319a, and miR160 are implicated in the development of plant forms by regulation of polarity, cell division, or auxin response through target genes (Bartel and Bartel, 2003; Jones-Rhoades et al., 2006). Mutation of these Mirna and target genes causes different types and extents of leaf curvature. Some attempts to uncover the function of the individual genes have been made by analysis of the direction, axis, position, and extent of curvature in mutants (Prigge and Clark, 2006). To date, a number of mutant alleles with curved leaves have been reported. However, comparison of curvature between mutants is difficult in many cases when measurement of leaf curvature is not quantitative. The lack of a quantitative measurement of curvature prevents the accurate assessment of the severity of mutation and the genes involved in the development of plant forms. Several attempts to quantify leaf curvature have been made. For example, Gaussian curvature has been described to account for the excessive growth in leaf margins of cincinnata (cin) mutants (Nath et al., 2003). The ratio of the unflattened width to the width of the flattened leaves has been used to indicate the difference in leaf curvature between poltergeist-like5 (pll5) and pll4 mutants in one direction (Song and Clark, 2005), and the transverse curvature index (TC) and longitudinal curvature index (LC) were defined to measure leaf curvature of hyll mutants according to the distance between the lateral margins of curved leaves (Wu et al., 2007). Despite these advances in quantitative measurement of leaf curvature in a few specific mutants, quantitative comparisons of different types of leaf curvature have not been possible.

Leaves can curve along two axes, the mediolateral axis (middle-to-margin) for transverse curvature and the proximodistal axis (base-to-tip) for longitudinal curvature. In many cases, curvature appears only in some regions of a blade, and simple measurements of leaf length and width cannot account for the extent of curvature. In this study, the formula of the curvature index (CI) is proposed in order to provide a quantitative measure of leaf curvature for all mutants and plant genotypes. Importantly, CI values were able to reveal differences in direction, axis, position, and extent of leaf curvature between the 22 mutants examined, indicating the severity of mutant alleles or transgenic plants. This formula works well for quantitative measurement of leaf curvature of mutants deficient in leaf flatness and is helpful for understanding the roles of the genes functional in plant development.

Materials and methods

Plant materials

The hyll seed sample was obtained from Dr Fedoroff (Han et al., 2004), the dcl1-9, fha-1D, and phb-6 phv-5 rev-9/+ seed samples were from Dr Fletcher (Williams et al., 2005), the rev-6 seed sample was from Dr Clark (Prigge et al., 2006), the oc-160c, ARF16::5mARF16, and arf10-2 arf16-2 (Wang et al., 2005) seed samples were from Dr Chen. The jum-1D seed sample was from Dr Weigel, and the TCP5SRDX seed sample was from Dr Ohme-Takagi (Koyama et al., 2007). The other mutants were purchased from ABRC (Arabidopsis Biological Resources Center). To define gain-of-function phenotypes of the miRNA-targeted genes in the transgenic plants, miRNA-resistant versions of the targets (CNA::2mCNA, REV::2mREV, TCP2::7mTCP2, and TCP7::7mTCP4) were engineered, each of which contains two or seven nucleotide substitutions in the region complementary to miRNAs (Supplementary Fig. S1 available at JXB online). These changed the complementarity to the microRNAs but did not change the amino sequence of the protein product produced by translation. The transgenic lines were selected after segregation for three generations, and the gain-of-function phenotypes due to the disruption of miRNA regulation were observed.

Growth conditions

The seeds of wild-type and mutant Arabidopsis thaliana were surface-sterilized in 70% ethanol for 1 min followed by 0.1% HgCl2 for 10 min. Then, seeds were washed four times in sterile distilled water, mixed in molten 0.1% water agar (BioWest), and plated on top of solid Murashige and Skoog medium with 1% sucrose. The Petri dishes were sealed with Parafilm, incubated at 4 °C in darkness for 2-3 d and then moved to a growth room and incubated at 22 °C under 12 h of light and 8 h of darkness per day. Two weeks later, the seedlings were transplanted to peat soil in plastic pots and moved from a growth room to a growth chamber in the SIPPE phytotron. In this growth chamber, the plants were grown at 22 °C with 16 h of light per day under a light source of warm white fluorescent tubes (colour code 990), an irradiance of 150 μmol m−2 s−1, and a light intensity on the plant canopy of 75 μmol m−2 s−1. The relative humidity was 65–70% and the air velocity was ~0.9 m s−1. All of the seedlings were grouped randomly and grown under identical conditions for 6 weeks. More than 20 individual plants for each mutant were prepared, and samples were taken for various measurements.

Quantitative measurement of leaf curvature

The plants were randomly selected for measurement of leaf curvature, and the leaves were collected at different developmental stages. Before leaf flattening, the type and direction of curvature in the leaves were defined first. To measure the global transverse curvature, two points a and b were found on two lateral margins of the entire leaf at maximum width (Fig. 1E), and a straight distance between a and b was designated as the projected width; after leaf flattening, the distance between a’ and b’ was regarded as the flattened width. Similarly, the projected length and flattened length were defined for the global longitudinal curvature. For some leaves with wavy edges, the straight distance between a and b on two peaks of the longest wave in the leaf was the projected length before flattening and the distance between a’ and b’ was the flattened length after flattening (Fig. 1F). To measure the local curvature, the curved region in the leaf was selected and two points a and b were fixed on two margins of the curved regions at maximum length. In practice, the projected length and flattened length were labelled on a blank sheet of paper and measured using a vernier calliper. The blade width, blade length, and petiole length were measured using a vernier calliper after flattening leaves on a sheet of cardboard with a glass cover. For complete flattening of leaves with wavy
RNA samples were isolated from different tissues at various growth stages. Growth stages were defined according to the methods of Boyes et al. (2001). Stage 1.0 represents seedlings with only cotyledons; stage 1.07 describes seedlings with seven leaves; and stage 6 consists of plants with bolted inflorescences. Total RNA was isolated from different growth stages of cauline and rosette leaves. After grinding in liquid nitrogen, 100 mg of the plant sample powder was collected in an Eppendorf tube, and the total RNA was isolated and purified according to the manufacturer’s protocol (TaKaRa). The first-strand cDNA was synthesized. The primers specific for the cDNA of ACTIN and UBQUITIN (UBQ) were used to normalize the amplification of sample DNA fragments. The gene-specific primers used for RT-PCR are shown in Supplementary Table S1 at JXB online.

miRNA northern blotting

Total RNA was extracted from cauline leaves. A 15 μg aliquot of total RNA was fractionated on a 15% polyacrylamide gel containing 8 M urea and then transferred to a NITRAN PLUS membrane (Schleicher & Schuell). Antisense sequences (20–21 bp) of various miRNAs were synthesized for probes (Supplementary Table S2 at JXB online) and were end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (TaKaRa). Hybridization was performed at 41 °C using hybridization buffer [7% SDS, 1% bovine serum albumin (BSA), 1 mM EDTA, 0.25 M NaH2PO4, pH 7.2]. The synthesized sequences of the corresponding miRNAs were used as positive controls, and tRNA and SS rRNA were used to control the quantity of the total RNA concentrations between samples.

Results

Classification of curvature

Each Arabidopsis leaf was divided into a proximal petiole and a distal blade region. Many of the mutant plants were characterized by upward or downward curvature along the transverse or longitudinal axis of the leaf (Fig. 1A–D). To define the relationship between the mutant alleles and leaf curvature, 22 mutants deficient in flatness with mutant alleles previously identified as affecting leaf flatness were selected. The 22 mutants included miRNA biogenesis mutants (Xie et al., 2003; Vaucheret et al., 2004; Vazquez et al., 2004; Lobbes et al., 2006), loss-of-function mutants of miRNA-targeted genes, transgenic plants, and other mutants related to leaf curvature (Fig. 2). The hyll, hyll-2, and hyll-3 leaves are three hypomorphic alleles of the HYLI gene (Supplementary Fig. S2 at JXB online); se-1, se-2, and se-3 are three hypomorphic alleles of SE; dcll-7 and dcll-9 are two hypomorphic alleles of DCL1; and rev-6 and rev-9 are two hypomorphic alleles of REV. jba-1D, jow-1D, and oc-160c are the semi-dominant mutants of MIR166g, MIR391a, and MIR166c, respectively. CNA::2mCNA, REV::2mREV, TCP2::7mTCP2, TCP4::7mTCP4, ARF10::5mARF10 (AUXIN RESPONSE FACTOR10), and ARF6::5mARF6 are the transgenic plants overexpressing CNA, REV, TCP2, TCP4, ARF10, and ARF16 (Supplementary Fig. S1). Among these mutants, the leaf shape varied in terms of direction, axis, position, and extent of curvature. The curvature direction of the hyll, dcll-9, se-1, se-2, and CNA::2mCNA leaves was upward along the transverse axis, while that of jba-1D, rev-6, and 35S::TCP3SRDX was
downward along the longitudinal axis. The entire leaves of rev-6 mutants curved downward, while those of TCP4::7mTCP4 plants curved upward. The apical regions of the dcll-9 leaves curved upward while the other regions in these leaves were basically flat. In contrast, in jaw-1D and jba-1D leaves, the margins of the curved regions were inside the blades. The mutants were classified as displaying upward (incurvature) or downward curvature according to the direction of curvature, they were grouped into transverse and longitudinal curvatures according to the axis of curvature, divided into apical, lateral, central, and basal curvatures according to the position of curvature, and classified as being high, intermediate, or low curvature (Fig. 1G).

Some leaves curved globally along the transverse or longitudinal axis, resulting in global curvature; in contrast, some regions of the leaves curved locally, giving rise to local curvature. The hyl1, se-1, and se-2 leaves exhibited global curvature as the whole leaves curved upward at the transverse axis. In contrast, the dcll-9, jba-1D, and jaw-1D leaves showed local curvature. The dcll-9 leaves contained an apical upwardly curved region, the jba-1D leaves had a lateral downwardly curved region, and the jaw-1D leaf was characterized by a central upwardly curved region. In many cases, global and local curvature was mixed in one leaf. For example, jaw-1D leaves globally curved downward along the transverse axis. Their apical regions curved downward, whereas their central regions curled upward. In this type of leaf, global and local measures can be used separately to describe the different types of the curved regions.

Global measure of leaf curvature

A curved leaf is in the metric state. In three-dimensional space, leaves may exhibit double and multiple curvatures and may be twisted and ruffled (Fig. 1A–D). To quantify leaf curvature, the origin of the curved lines was described instead of the curved surface. When a curved leaf is transversely cut so that the cross-section is approximately a curved line (arc) (Fig. 1E), the straight distance between the two points a and b on two lateral margins of the leaf is the length of the chord (a straight line joining two points on a arc). The distance between a' and b' on the two lateral margins after leaf flattening is the length of the arc. The arc length a'b' and chord length ab can be determined by measuring the distance between two lateral margins before and after leaf flattening. Some cuts and folds are introduced to allow for the wavy edge to be flattened (Fig. 1F). Based on a previously described formula (Wu et al., 2007), the CI in the downward direction (positive curvature) was defined as $CI_u = (a'b' - ab)/ab$, where ab is the chord length, the distance between points a and b on two margins of curvature before flattening of leaves, and a'b' is the arc length, the distance between a and b on two margins after flattening (Fig. 1D). Accordingly, CI in the upward direction (negative curvature) is $CI_d = (ab - a'b')/ab$. When a leaf curves downward, the $CI_d$ value is positive; when it curves upward, the $CI_u$ value is negative. The CI is almost zero for a flat leaf. Meanwhile, the CI values along the transverse and longitudinal axes were designated as $CI_t$ and $CI_l$, respectively. In contrast to the previously described formula, this method is capable of accounting for the direction, axis, and position of leaf curvature rather than just the extent of curvature. Using this formula, the CI values of all 22 mutants with curved leaves were calculated.

Most of the mutant and transgenic lines were characterized with global curvature. In addition, local curvature was also common. In the hyl1, dcll, and se mutants, miRNA-directed gene silencing was abolished or diminished. To link the function of HYL1, DCL1, and SE to the phenotypes of each mutant, the leaf phenotypes of the hyl1, dcll, and se mutants were compared. The hyl1 leaves were smaller and narrower than the wild-type leaves, curved upward in a transverse direction, and were often helically rotated; the leaves of dcll-9 were round and basically flat in the transverse direction, and bent upwards at the tip; and the leaves of se-1 plants were curved upward with serrated margins. CIs in the transverse axis (transverse CI) of hyl1, dcll-9, and se-1 leaves were $-0.46$, $-0.03$, and $-0.25$, respectively, while the CI values along the longitudinal axis of hyl1, se-1, and dcll-9 were $+0.08$, $+0.02$, and $-0.03$, respectively (Fig. 3). These results indicated that the hyl1 and se-1 leaves curved upward along the transverse axis and downward along the longitudinal axis; in contrast, the dcll-9 leaves curved upward along both the transverse and longitudinal axes. In the dcll-9 leaves, significant upward curvature of apical regions was observed, while curvature in other regions was subtle. The distinct phenotypes of the hyl1 and dcll-9 leaves revealed that the lack of HYL1 function affects the dorsoventral dimension of leaves in the transverse direction, whereas the loss of DCL1 function causes a distinct phenotype in the leaf apical region.

The jba-1D, jaw-1D, and oe-160c plants, the dominant mutants of the MIR166g, MIR319a, and MIR160c genes,
miR319a-targeted TCP genes is repressed, curved downward along the transverse axis (transverse CI = +0.17) and downward in the longitudinal direction (longitudinal CI = +0.06). The TCP3::TCP3SRDX plants display a phenotype similar to jaw-1D, but to a lesser extent. The degree of leaf curvature was compared between the mutants. The transverse CI values of hyll, CNA::2mCNA, and se-2 leaves were lower than –0.4, and thus were classified as having high curvature; 35S::mARF16, TCP4::7mTCP4, and se-1 leaves had transverse CI values of ~0.51 and ~0.10 and therefore had intermediate curvature; and dell-9 and se-3 exhibited low curvature.

**Local measure of leaf curvature**

Among the mutants, local curvature was more prevalent than global curvature. Some mutants were characterized with local curvature of leaves even though the whole leaves curved in a different direction. Because it was helpful to use a definition of local curvature that could be evaluated at all locations along a curve (Silk and Erickson, 1978), the CI of global curvature was applied to local curvature. The chord length ab is the distance between two margins of the curved regions before flattening, while the arc length a’b’ is the distance after flattening. For local curvature, a small region is measured instead of the entire leaf. To calculate the local CI, the curved regions were fixed in the objective leaf. For example, the apical region of a jaw-1D blade (about one-third of the whole blade) curved downward. The apical transverse and longitudinal CI values were calculated to be +0.07 and +0.36, respectively (Fig. 4A, B), indicating that a large ‘global curvature’ assessed by the ratio method could be produced by a large curvature in a small region.

In many cases, local curvature was mixed with global curvature, so both local and global measures were used at the same time. For example, the dell-9 leaves were bent upwards at the tip, while other regions of the blades were curved slightly upward along both the transverse and longitudinal axes. The global longitudinal CI (global CI along the longitudinal axis) for the dell-9 mutants was –0.03, while the apical longitudinal CI (apical CI in the longitudinal axis) was –0.78. The leaf curvature of jaw-1D was more complex because the four regions of the leaf curved in different directions: global transverse CI of ~0.07, global longitudinal CI of +0.15, lateral transverse CI of +0.70, apical longitudinal CI of +0.34, and central transverse CI of –0.21. Accordingly, the apical, central, and lateral regions of the jaw-1D leaf were classified as downwardly curved, upwardly curved, and downwardly curved, respectively. Compared with the global measure, the local measure revealed more significant differences in the direction, axis, position, and extent of curvature, thus allowing for more reliable conclusions to be drawn about the contribution of mutations to the phenotype.

**Spatial and temporal variations of leaf curvature**

Transitions from one vegetative phase to another can be characterized by the leaf shape. As shown in Fig. 5A and B,

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**Fig. 3.** Curvature indices (CIs) of the mutant leaves along the transverse (A) and longitudinal (B) axes. The seventh leaves of plants at the bolting stage (stage 5.1) were globally measured (Boyes et al., 2001). WT-N, Nossen; WT-L, Landsberg; WT-C, Columbia; 2mCNA, CNA::2mCNA plant; 2mREV, REV::2mREV plants; 7mTCP4, TCP4::7mTCP4; 5mARF16, ARF16::5mARF16 plants. Asterisks indicate significant differences between the mutant and the wild type (*P <0.05) by Student’s t-tests. Error bars represent the standard deviation (SD). The number of leaves measured for each genotype was >20. Two asterisks indicate significant differences (t-test, **P <0.01).
the leaves of hyll plants exhibited various degrees of curvature at the seedling, rosette, and flowering stages. Cotyledons and the first, seventh, and 11th leaves had various extents of curvature. The cotyledons were basically flat; the first leaves curved upward at both axes, and the rosette leaves curved upward along the transverse axis and downward at the longitudinal axis. For the jaw-1D mutants, the difference in leaf curvature among the seedling, rosette, and flowering stages was more pronounced (Fig. 5C, D). Not only was the extent of curvature changed, but the direction and position of curved regions were also altered. To assess how much the leaf curvature changed with plant growth, the curvature of cotyledons and first leaves at the seedling stage (stage 1.02) (Boyes et al., 2001) and of the seventh and 11th leaves at the bolting stages (stage 5.1) was measured. In hyll plants, the transverse CI values of cotyledons, first leaves, seventh leaves (seedling stages), and 11th leaves (rosette stages) were −0.02, −0.11, −0.41, and −0.44, respectively, revealing that the extent of transverse upward curvature became higher from the first to the 11th leaves; the longitudinal CI values were −0.04, −0.34, −0.03, and +0.08, respectively, suggesting that the direction and extent of longitudinal curvature changed with plant growth (Fig. 4C, D). Using a global measure for the jaw-1D plants, the transverse CI values of cotyledons and the first, seventh, and 11th leaves were +0.24, −0.06, +0.12, and +0.14, respectively; the longitudinal CI values were +0.71, +0.13, +0.12, and +0.14, respectively. Except for the cotyledons, the differences in extent of curvature between the three types of leaf were relatively small.

**Mutant alleles and the extent of curvature**

Some genes in miRNA pathways have hypomorphic alleles. To assess the statistical difference of curvature between hypomorphic alleles, the CI of mutants derived from the same genes was measured. In contrast to curvatures caused by different mutant genes, the major difference between hypomorphic alleles is due to the extent of curvature. For example, hyll has three hypomorphic mutant alleles: hyl1 (Ds transposon inserted in the second exon), hyl1-2 (T-DNA in the second exon), and hyl1-3 (CS875048) (T-DNA in the third exon). Among these mutants, the hyl1 allele is strongest, as its curvature was the most severe; the hyl1-3 leaves were the same as those of the wild type. The three mutant alleles of SE, se-1, se-2, and se-3, had CI values of −0.22, −0.46, and −0.02, respectively, indicating three different levels of activity. The se-2 allele had a stronger contribution than that of se-1 as its CI was reduced. The rev-6, rev-9, and ifl1 mutants contain mutations in REV. Although these three mutants curved downward, they differed remarkably in their extent of curvature. The CI of
rev-6 leaves was much higher than that of ifli leaves. Taken together, it is concluded that the contribution of mutant alleles to the phenotypes correlates to the leaf curvature to some extent.

A comparison of CI values among wild-type ecotypes of Arabidopsis yielded interesting results. Although the rosette leaves of wild-type Arabidopsis are typically considered to be flat, they actually do curve. The CI values of the Nossen, Columbia, and Landsberg ecotypes were –0.02, +0.08, and +0.09, respectively, revealing that Nossen leaves curve upward slightly, while Columbia and Landsberg leaves barely curve downward. When comparing the curvature among mutants, the ecotype of the mutant alleles should be considered.

**Changed leaf shape associated with curvature**

The blade width, blade length, and petiole length reflect the mediolateral and proximodistal dimensions of leaves, and they therefore are important indices of leaf shape. We noticed that either upward or downward curvature of leaves was concomitant with the changes in the leaf width and length and in the petiole length (Fig. 6A). The BLW ratio of the hyll mutants was 3.01, which is much higher than that of the dcl1-9 mutants but lower than that of the se-1 mutants, indicating that the hyll blades are significantly narrower than the dcl1-9 blades. Meanwhile, the PBL ratios of the hyll and se-1 mutants were also significantly different. The PBL ratio of the dcl1-9 mutants was only 0.02, revealing that the dcl1-9 leaves are almost sessile (Fig. 6B). The PBL ratio of the hyll mutants was much higher than that of dcl1-9 but much lower than that of se-1. Therefore, narrow and sessile leaves are two distinct features of hyll and dcl1 mutants, respectively. It appears that HYL1 regulation has stronger effects on the mediolateral leaf dimension than DCL1. In addition, the effect of DCL1 regulation on the proximodistal dimensions is stronger than that of HYL1. However, a general relationship for correlation between leaf length, leaf width, petiole length, and the extent of leaf curvature was not found.

**Effects of gene expression on leaf curvature**

The jba-1D, jaw-1D, and oe-160c mutant plants display different directions and extents of leaf curvature. To define whether the type of leaf curvature is caused by increased accumulation of the corresponding miRNAs, northern blotting was performed to compare the miRNA accumulation between these mutants at the nine-leaf stage. In jba-1D, jaw-1D, and oe-160c seedlings, the miR165/166, miR319a, and miR160 levels were extremely elevated (Fig. 7A). Accordingly, the expression levels of the target genes REV, TCP4, and ARF16 were remarkably diminished (Fig. 7B). Therefore, it was concluded that the complicated curvature of the leaves of jba-1D and jaw-1D mutants is caused by reduced expression of the miR165/166- and miR319a-targeted genes.

To determine whether the expression level of HYL1 is related to the extent of leaf curvature, HYL1 cDNA was inserted into binary vectors under the control of the cauliflower mosaic virus 35S promoter and then transformed into the hyll mutant plants. The resultant transgenic plants were identified individually by PCR and Southern hybridization. The 35S::HYL1 plants were self-fertilized for five generations and the homozygous lines were selected. As shown in Fig. 7C, most of the 35S::HYL1 plants displayed flat leaves, similar to those of the wild type, indicating that the HYL1 cDNA can completely rescue the hyll mutant phenotypes. However, within the population of the 35S::HYL1 transgenic plants, some plants had rosette leaves that curved less than those of the hyll mutants, indicating that they were partially rescued. The 35S::HYL1 plants were classified into three categories: R1, completely rescued leaves (CI = +0.10 to –0.10); R2, partially rescued leaves (CI = –0.10 to –0.30), and R3, weakly rescued leaves (CI = –0.31 to –0.90) (Fig. 7C). The rosette leaves of the R2 and R3 plants were smaller than the wild-type leaves, but they were larger than the hyll leaves. Of the 35S::HYL1 plants, 58% belonged to R1 (flat leaves), 23% to R2 (weakly curved leaves), and 19% to R3 (strongly curved leaves). To determine whether the three categories of transgenic lines corresponded to the three levels of HYL1 expression and to understand how HYL1 expression is associated with the extent of leaf curvature, real-time PCR was performed to analyse the expression patterns of the exogenous HYL1 gene in the transgenic plants. The expression level of HYL1 in R1 lines was much higher than in the wild-type plants (Fig. 7D) and the rank order of expression from strongest
to weakest was $R_1 > R_2 > R_3$. Consistent with this finding, the rank order of the CI value, indicative of the leaf curvature, from highest to lowest was $R_1 > R_2 > R_3$. Thus, the extent of leaf curvature correlated with the expression levels of $HYL1$ in the leaves. The differences among the $R_1$, $R_2$, and $R_3$ lines and the rank order from the highest to the lowest of $R_1 > R_2 > R_3$ revealed a strong correlation between the expression of $HYL1$ genes and the extent of leaf curvature.

**Discussion**

The CI reflects the direction, axis, position, and extent of leaf curvature.

According to Nath et al. (2003), there are two types of leaf curvature in orthogonal directions. If marginal regions grow more slowly than the centre, the disc will adopt a cupped shape that has a positive Gaussian curvature. Conversely, if the marginal areas grow more rapidly than the centre, the disc will buckle to form a shape with a wavy edge, such as a saddle, with negative Gaussian curvature. Antirrhinum $cin$ mutants have wavy edges owing to more rapid growth of the marginal areas. In the absence of the $PPD$ ($PEAPOD$) genes, lamina tissue of Arabidopsis outgrows the extension capacity of the marginal cells, changing the blade from flat to dome-shaped (White, 2006). In these cases, Gaussian curvature can account for the extent of curvature, as it indicates the excessive growth in the leaf margins. However, unlike the $cin$ and $ppd$ mutants, most of the mutants (such as $hyl1$, $se-1$, $rev-6$, and $TCP4::7mTCP4$ plants) deficient in leaf flatness do not exhibit excessive growth in the leaf margins. An attempt was made to assess the extent and location of curvature in these mutants using equations of Gaussian curvature and geodesic curvature over the leaf surface. Unfortunately, these techniques did not work for the curvature since the values calculated in this way were not consistent with the actual direction and extent of leaf curvature. In curved leaves, the growth rate differed in different parts of the adaxial and abaxial sides, leading to downward and upward curvature and twisted edges. Usually, downward and upward curvature leads to a cylindrical shape: Gaussian curvature is rarely used to quantify this type of curvature. However, the growth rate of the wavy leaves varied greatly in different regions along the edges. The leaf perimeter ($P$) was measured relative to total leaf area ($A$) of the first and sixth blade in the wild type and the $hyl1$, $rev-6$, and $jaw-1D$ mutants. Surprisingly, these leaves have nearly the same perimeter-to-area ratios. Significant changes of the perimeter-to-area ratios were observed only in the $jaw1-1D$ and $TCP3SRDX$ mutants, which both have a wavy leaf edge with negative Gaussian curvature. Therefore, Gaussian curvature is more suitable for describing the curvature of leaves with inhomogeneous growth in different small regions but not for curved leaves with homogeneous growth (e.g. $hyl1$). Nevertheless, the CI is capable of describing the direction, axis, position, and extent of all types of curvature.

The ratio of the unflattened width to the width of the flattened leaves is used for simple comparison of the extent to which different plant genotypes have curved leaves in one direction (Song and Clark, 2005). However, leaf curvature occurs in two directions: upward and downward. In some cases, curvature appears only in local regions of the blade. Simple measurement of the distance between the margins of leaves does not account for the local curvature. In contrast, the CI value can quantify the local curvature in different types of curved leaves.

**Comparison of the CI reveals the roles of the genes that function in plant development**

Leaf morphogenesis has long been investigated for its relationship to polarity, cell division, and auxin response in the genesis of the leaf form. Originating from the flank of the shoot apical meristem, the leaf primordium is dorsoventrally symmetrical and flattened on its adaxial side. Upon the formation of blades, the three-dimensional form of...
leaves is specified along the proximodistal (base-to-tip), dorsoventral (top-to-bottom), and mediolateral (middle-to-margin) planes. Three-dimensional polarity is associated with cell cycle activities in the marginal meristem (Donnelly et al., 1999) and with a movement of the arrest front of cell division in leaves (Nath et al., 2003). Coordination of polarity and cell division is critical for the morphogenesis of normal leaves and successful plant architecture. An imbalance within these developmental processes can result in curly, crinkly, twisted, rolled, or shrunk leaves.

In many cases, leaf curvature is associated with altered activity of the genes controlling leaf adaxial/abaxial polarity. The recessive *phantastica* (*phan*) mutant in snapdragons, which is the first leaf polarity mutant, possesses radially symmetric leaves with abaxial character around their circumference (Waites and Hudson, 1995). *REV, PHB, PHV, and CNA* regulate adaxial/abaxial polarity and asymmetry. Measurement of leaf curvature using the CI indicated that the mutant alleles of these adaxial/abaxial genes cause different types of leaf curvature. The loss-of-function mutants *phv, phb*, and *rev-6* displayed flatness or downward curvature of leaves, and the CI values were *rev-6>**rev-9>**phb-6>*phv-5*, indicating that the contribution of the mutant alleles from strongest to weakest was *rev-6>**rev-9>**phb-6>*phv-5*. Among them, *rev-6* and *rev-9* are hypomorphic alleles, and the higher CI of *rev-6* supports its stronger effect on leaf curvature.

In most cases, leaf curvature is irregular. Several regions in a curved leaf may curve in various directions to different extents. If a global measure is used to quantify the curvature, the resultant CI may be questionable and biased. If a local measure is used to calculate the CI of a local region, the resultant CI should be precise and suitable to assess the function of the mutant genes. For the *jba-1D* mutants, global and local measures generated different CI values: the longitudinal global CI was low (~0.03), whereas the local CI for the apical regions was especially high (~0.78). There is a 26-fold difference between these values. Compared with the global curvature, the local curvature is more accurate and the genetic effect of a mutant allele is more obvious. The curvature of *jaw-1D* is complicated to describe as different types of local curvature occur in one leaf. Under a global measure, the CI value of whole *jaw-1D* leaves was positive along the longitudinal axis. For a local measure, the CI value of the apical regions along the longitudinal axis and lateral regions in the transverse axis were extremely high because of the significant downward curvature and the downwardly rolled leaf margins, highlighting the excessive growth in the leaf margins. To define the identity of these curved regions, a local measure could be used to calculate the CI of each region.

Compared with the *jaw-1D* leaves, 35S::TCP3SRDX leaves curve to a low extent, although the five *TCP* genes targeted by miR319a were down-regulated in both mutants (Palatnik et al., 2003; Koyama et al., 2007). Nevertheless, the relative CI values of the four local regions showed different patterns: the longitudinal CI of the apical region in *jaw-1D* mutants was similar to that of the 35S::TCP3SRDX plants; the transverse CI of lateral regions in the *jaw-1D* mutants was much higher than that of the 35S::TCP3SRDX plants. This difference reveals that the effect of the *jaw-1D* allele on local curvature is distinct from that of the 35S::TCP3SRDX construct. In this example, the extent of digital curvature of the local regions was very useful for identifying differences in the same types of leaf curvature between mutants.

*Arabidopsis asymmetric leaves1* (*asl1*) is a classical mutation that disrupts the development of cotyledons, leaves, and floral organs (Byrne et al., 2000), and is an example of basal leaf curvature. In *asl1* leaves, apical regions curve slightly downward along the transverse and longitudinal axes, whereas the basal regions curve significantly upward, leading to lotus-like leaves. *asymmetric leaves2* (*asl2*) is another mutant deficient in leaf development (Serrano-Cartagena et al., 1999) and is characterized by very similar lotus-like leaves. Double mutations in *CUP-SHAPED COTYLEDON 1 (CUC1)* and *CUC2* cause a lack of an embryonic SAM (shoot apical meristem) and a nearly complete fusion of the cotyledons; notably, each single mutant is basically normal (Aida et al., 1997). Cotyledons of *cuc1 cuc2* mutants are unique, as they curve completely upward along the transverse axis with a transverse CI of ~1.0.

When the difference in direction and extent of leaf curvature between mutants is large enough, visual investigation is possible. When the difference is small or subtle, numerical representation is very useful. In many cases, the mutants of several genes are similar in the phenotypes of curvature, and visual observation relies on the ability and skill of the observer and thus is not certain. Nevertheless, the subtle difference among these mutants could be defined by their CI values. Secondly, leaf curvature of some mutants is complicated as global curvature and different local curvature are mixed, and the regions of alternative curvature must be separated and measured digitally. Thirdly, leaf curvature is affected by environmental conditions; the subtle difference caused by physiological elements should be distinguished from that caused by genetic elements by numerical representation. This study involved the three *hyl1* mutants, eight MIR165/166 mutants, and eight mutants of five HD-ZIP III genes. Most of them displayed leaf incurvature. It was realized that the CI is very useful for defining the strength of the mutant alleles and difference in gene function between the mutants.

**Elastic buckling may explain some types of curvature when the genetic coding is altered**

The leaves of most higher plants are approximately flat, facilitating the efficient capture of sunlight for photosynthesis. Although such flatness is often taken for granted, the probability of this happening by chance is low because there are many ways for a structure to adopt curvature (Nath et al., 2003). In nature, many genes are involved in the initiation and maintenance of leaf flatness. When the genes responsible for leaf flatness act properly, leaves are flat. However, the genes responsible for leaf flatness are often not properly expressed, so some specific types of curvature
appear. Interestingly, elastic buckling may explain some types of curvature. The adaxial identity genes REV, PHY, and PHV, maintain adaxial/abaxial polarity necessary for leaf polarity. When expression is blocked or overexpression occurs, the affected leaves may exhibit a shell structure (Mouli, 2000). In a leaf segment showing a shell structure with double curvature, the leaf bends (curves longitudinally downward) and also rolls (curves transversely) (Hay et al., 2000). In rev-6 mutants with a loss-of-function mutation of REV, the leaves curve downward longitudinally and transversely. In contrast, old leaves of rev-6 mutants are twisted and ruffled along the edges of the blade tip; the rates of longitudinal growth at the edges of a blade exceed the rate of longitudinal growth along the midline of the blade, similar to the ruffles along the edges of the blade produced by elastic buckling (Koehl et al., 2008). In hyl1 mutants with overexpression of REV, the leaves curve downward longitudinally but upward transversely. On the other hand, mutants with wavy leaves may behave like a torn plastic sheet (Sharon et al., 2002), the edge of which forms a complex three-dimensional fractal shape. The equilibrium shape of the sheet consists of a cascade of waves along the newly formed edge. The wavy edge of jaw-1D and TCP3SRDX leaves resembles that of the torn plastic sheet, as the leaves have a wavy edge with a large wave containing many smaller waves. According to elastic theory, the sheet can easily buckle into shapes that remove in-plane compression to reduce its elastic energy. Mutants such as hyl1, rev, jaw-1D, and TCP3SRDX have leaves that follow some rules of elastic theory, but they do this only when the original function of the genes responsible for flatness are impaired. Elastic buckling of each mutant is different in structure and strength, and mutant alleles seem to regulate the time and places of elastic buckling. It will be important to explore the contribution of the genes responsible for leaf flatness and elasticity in formation of leaf shape.

Elasticity theory shows that the same amount of growth inhomogeneity produces greater curvature in a thinner leaf. As the sheet becomes thinner and the compression increases, the possible wavelengths of the buckles are smaller (Sharon et al., 2002). However, this pattern is not observed for some mutant plants. The hyl1 and dellf-9 leaves appear much thicker than the wild type even though they curve transversely and longitudinally (data not shown). The relationship of leaf thickness to the extent of curvature may be conditional on the genes mutated. When several mutant alleles of the same gene are compared, the correlation between leaf thickness and the extent of curvature could be more meaningful.

Curvature is found in many flower petals, lichens, and leaves, and leaf shape is related to curvature. The CI values can be especially useful in describing the severity of mutations and in assays of the genetic contribution to various phenotypes. In some cases, leaf curvature is beneficial for crops, such as Brassica species, which are close relatives of Arabidopsis and include the leafy heads (the edible parts) of Chinese cabbage, cabbage (B. oleracea ssp. capitata), and Brussels sprouts (B. oleracea ssp. gemmifera). The plants of these crops produce flat leaves to capture enough light for photosynthesis at the seedling and rosette stages, and they start to develop curved leaves to store nutrients for head formation at the folding stage. BcpLH, a homologue of the HYLI gene in Chinese cabbage, is related to leaf folding (Yu et al., 2000). Other genes involved in leaf folding are under study. The leaves that curve at the seedling and rosette stages have abnormal leafy heads because the genes responsible for leaf flatness are impaired. Classification and quantification of leaf curvature in Arabidopsis and other important crops will contribute to a better understanding of the molecular mechanism of leaf shape formation.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** The mutated sequences complemented by miRNAs in the target genes.

**Figure S2.** Diagram of the mutant alleles of Arabidopsis used in this study.

**Table S1.** Primers used in this work.

**Table S2.** Probe sequences for northern blot in this work.

**Acknowledgements**

We thank Dr Fedoroff (Pennsylvania State University, USA), Dr Fletcher (Plant Gene Expression Center, USA), Dr Clark (University of Michigan, USA), Dr Weigel (Max Planck Institute for Developmental Biology, Germany), Dr Bowman (University of California, USA), and Dr Chen (Shanghai Institute of Plant Physiology & Ecology, China) for providing the mutant seeds. We also thank Detlef Weigel (Max Planck Institute for Developmental Biology, Germany) for valuable discussions during this study. This work was supported by grants from the Natural Science Foundation of China (Grant nos 39870450 and 90508005) and Major Programs of the Chinese Academy of Sciences (Grant No.30730053).

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