HYL1 regulates the balance between adaxial and abaxial identity for leaf flattening via miRNA-mediated pathways

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Abstract

HYPONASTIC LEAVES1 (HYL1) is an important regulator of microRNA (miRNA) biogenesis. Incurvature of rosette leaves in loss-of-function mutants of HYL1 implicates the regulation of leaf flatness by HYL1 via miRNA pathways. Recent studies have identified jba-1D, jaw-1D, and oe-160c, the dominant mutants of MIR166g, MIR319a, and MIR160c genes, respectively, which display three types of leaf curvature. However, it remains unclear whether or how HYL1 controls leaf flatness through the pathways mediated by these miRNAs. To define which miRNAs and target genes are relevant to the hyl1 phenotype in terms of leaf incurvature, the effects of three mutated MIRNA genes and their targets on the direction and extent of leaf curvature in hyl1 mutants were examined. The genetic analysis shows that the hyl1 phenotype is strongly rescued by jba-1D, but not by jaw-1D or oe-160c, whereas the mutant phenotypes of jba-1D, jaw-1D, or oe-160c leaves are compromised by the hyl1 allele. Expression analysis indicates that reduced accumulation of miR166, rather than of miR319a or miR160, causes incurvature of hyl1 leaves, and that miR319a-targeted TCP3 positively regulates the adaxial identity gene PHABULOSA while miR160-targeted ARF16 negatively regulates the abaxial identity gene FILAMENTOUS FLOWER. In these cases, the direction and extent of leaf incurvature are associated with the expression ratio of adaxial to abaxial genes (adaxial to abaxial ratio). HYL1 regulates the balance between adaxial and abaxial identity and modulates leaf flatness by preventing leaf incurvature, wavy margins, and downward curvature. It is concluded that HYL1 monitors the roles of miR165/166, miR319a, and miR160 in leaf flattening through the relative activities of adaxial and abaxial identity genes, thus playing an essential role in leaf development.

Key words: Adaxial–abaxial polarity, Arabidopsis, auxin response, cell division, HYL1, leaf curvature, miR160, miR166, miR319.

Introduction

Formation of a normal leaf is a complicated process that involves the initiation and differentiation of leaf primordia from the shoot apical meristem (SAM), specification of leaf identity, the establishment of leaf polarity, the control of cell division and expansion, and vascular pattern formation. In the genesis of plant form, leaf morphogenesis has long been a focus of study, particularly the roles of polarity, cell division, and auxin response. In contrast to the SAM, which is indeterminate, leaves of higher plants are determinate in development. The leaf primordium arises on the flank of the SAM 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) planes. Polarity along the three dimensions is associated with cell division activities in the respective cell lineages (Poethig, 1987) 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 the success of plant architecture. Any imbalance of these developmental processes results in altered leaf shapes such as curly, crinkly,
twisted, rolled, or shrunken leaves (Serrano-Cartagena et al., 2000; Liu et al., 2010). In recent years, mutants with different curvatures have been isolated and quantitatively described. Meanwhile, different molecular causes for the leaf curvature observed in these mutants have been identified. Interestingly, many of these mutants have defects in microRNA (miRNA) biogenesis and miRNA-mediated gene silencing.

miRNAs are small regulatory RNAs that vary in length from 20 to 24 nucleotides (Bartel and Bartel, 2003). They are thought to play a key role during development by negatively regulating gene expression at the post-transcriptional level. In plants, appropriate miRNA accumulation depends on the activity of the nuclear proteins HYPONASTIC LEAVES1 (HYL1), DICER-LIKE1 (DCL1), SERRATE (SE), and AGONANTE1 (AGO1) (Park et al., 2002; Reinhart et al., 2002; Papp et al., 2003; Han et al., 2004; Vaucheret et al., 2004). DCL1, in combination with HYL1 and SE, is thought to catalyse the processing of pri-miRNAs and pre-miRNAs, subsequently producing a wide variety of miRNAs that control the expression of various important genes (Kurihara et al., 2006; Yang et al., 2006; Fang and Spector, 2007).

AGO1 incorporates miRNAs into the RNA-induced silencing complex (RISC) for cleavage of miRNA target genes. Interestingly, nearly all non-lethal dcl1, hyl1, se, and ago1 mutants are reported to have many morphological, physiological, and biochemical changes (Jones-Rhoades et al., 2006). However, the most characterized leaf phenotypes in hyl1, dcl1-9, and se-1 alleles are different, as the mutant plants show the incurvature, bent tip, and serrated margins of leaves, respectively. The incurvature phenotype was also found in another hypomorphic mutant allele, se-2, with a similar leaf curvature index (CI) to hyll (Grigg et al., 2005). However, the up-curved leaf phenotype was not detected in known dcll hypomorphic mutant alleles. Theoretically, the pleiotropic effects of DCLI, HYL1, and SE mutations are attributable to the differential expression of miRNA-directed targets. Although some progress has been made in characterizing the biochemical processes of miRNA biogenesis, the precise relationship between HYL1 and leaf shape remains elusive.

HYL1 was originally characterized as a nuclear double-stranded RNA (dsRNA)-binding protein required for normal leaf development in Arabidopsis (Lu and Fedoroff, 2000). It is defined by the presence of two conserved dsRNA-binding domains (dsRBDs) in its N-terminal half and two nuclear localization signal (NLS) sequences in its centre. The N-terminal dsRBDs of the protein are sufficient for pre-miRNA processing (Wu et al., 2007). The dsRBD1 is essential for dsRNA binding in vitro, whereas dsRBD2 contributes to its protein–protein interaction activity (Hiraguri et al., 2005). The interaction of HYL1 with DCL1 and SE is important for the efficient and accurate processing of pri-miRNA during plant miRNA biogenesis (Dong et al., 2008). Due to a deficiency in miRNA biogenesis, hyl1 mutants have highly pleiotropic phenotypic abnormalities, and are characterized by incurvature of leaves (Lu and Fedoroff, 2000; Yu et al., 2005). Conversely, the dominant mutants MIR166g and MIR319a are characterized by curling down and wavy margins of leaves, respectively, owing to overexpression of the miRNA genes. Thus, the question arises of how HYL1 directs miRNAs to modulate leaf shape.

miRNAs negatively regulate their target genes by pairing the complementary regions, and guide the polarity, cell division, and auxin response during leaf development (Palatnik et al., 2003). CLASS III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) genes are targets of miR165/166 (Supplementary Table S1 available at JXB online). 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; Mallory et al., 2004). Dominant mutations in the Arabidopsis PHB and PHV transcription factor genes switch the leaf fate from abaxial to adaxial by altering an miRNA complementary site present in processed PHB or PHV (McConnell et al., 2001). The alternation of 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 both antagonistic to those of REV within certain tissues and overlap with those of REV in other tissues (Emery et al., 2003). The TEOSINTE BRANCHED1/CYCLOIDIA/PCF (TCP) family contains five miR319a targets, which represent the Arabidopsis homologues of the Antirrhinum CINCNATA (CIN) gene (Nath et al., 2003). 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 three ARF genes of Arabidopsis that are targets of miR160. Plants expressing an miRNA-resistant version of ARF17 or ARF16 have incurved leaves, corresponding to increased ARF17 or ARF16 miRNA levels (Mallory et al., 2005; Wang et al., 2005).

Recently, it was demonstrated that the YAB family of abaxially expressed genes, which encodes presumptive transcription factors with high-mobility group and zinc-finger domains, is recruited to mould modified shoot systems into flat plant appendages by translating organ polarity into lamina-specific programmes, promoting abaxial cell fates in the lateral organs of Arabidopsis (Eshed et al., 1999; Sawa et al., 1999; Siegfried et al., 1999; Villanueva et al., 1999; Bowman, 2000; Sarojam et al., 2010). One member of the YAB gene family, FILAMENTOUS FLOWER (FIL) (Sawa et al., 1999; Siegfried et al., 1999; Kumaran et al., 2002), is required for normal leaf development. FIL is thought to act redundantly with YAB2 and YAB3 because of their overlapping expression pattern and sequence homology (Siegfried et al., 1999), consistent with the absence of aberrant vegetative phenotypes in fil loss-of-function mutants. Kumaran et al. (2002) reported the analysis of the fil-8 yab3-2 double mutant in which partially radialized leaves were observed, suggesting a partial loss of leaf polarity.

In leaf development, miR165/166, miR319a, and miR160 regulate polarity, cell division, and auxin response, respectively, and should play important roles in the determination of curvature, shape, and size of leaves. In hyl1 mutants, biogenesis of many miRNAs, including miR165/166, miR319a, and miR160, is impaired. However, it remains unknown
whether and how these miRNAs and their target genes contribute to the specification of leaf form. Specifically, it was not known what caused the hyll phenotype, characterized by narrow, sessile, and incurved leaves. To understand the connection between HYL1 and plant phenotypes, the genetic relationship between the hyll allele and the dominant alleles of MIR166g, MIR191a, and MIR160g genes, as well as recessive mutant alleles of corresponding target genes, was analysed. According to analysis of the genetic relationship between the hyll allele and the mutants of the MIR166g, MIR191a, and MIR160c genes, it was found that deficiency in the miR165/166 pathways was the primary cause for leaf incurvature of hyll mutants. On the basis of northern blotting and RT-PCR, it is proposed that HYL1 regulates the balance between adaxial and abaxial identity for leaf flatness by miRNA pathways. The results provide further insight into the understanding of molecular mechanisms involved in the development of leaf forms and plant bodies.

Materials and methods

Plant materials and growth conditions

The hyll, jba-1D, phb-6 phv-5, phb-6 phv-5 rev-9+/-, rev-6, rev-9, CNA::2mCNA oe-160c, ARF16::5mARF16, arf10-3 arf16-2, jaw-1D, and TCP3SRDX mutants and transgenic lines have been previously described (Liu et al., 2010). rev-10d seed samples were from J. Bowman (Monash University). phb-1d (CS3761, Ler) was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). The 35S::mTCP3 seed sample was from Dr Ohme-Takagi. REV::FIL was constructed by amplifying a DNA fragment containing 2.5 kb of sequence upstream of the hyll coding sequence with the coding sequence of FIL into the binary constructs pCAMBIA1301. The REV::FIL transgenic lines were selected after segregation for three generations. Seeds were surface-sterilized in 70% ethanol for 1 min and then in 1% (v/v) NaClO for 10 min, and washed four times in sterile distilled water. Seeds were then placed on top of solid 1% sugar Murashige and Skoog (MS) medium. Plates were sealed with Parafilm, incubated at 24°C in the dark for 3–4 d, and then moved to a growth chamber at 22°C with 16 h of light. Two weeks later, the seedlings were transplanted carefully to peat soil in plastic pots, moved from a growth room to a growth chamber in the phytofoton of SIPPE, and grown at 22°C with 16 h of light per day.

Construction of double, triple, and quadruple mutants

Single, double, or triple mutants hyll, rev-9, arf10-3 arf16-2, phb-6 phv-5, and phb-6 phv-5 rev-9+/- were constructed by transposon or T-DNA tagging, so that PCR-based genotyping was performed to verify their mutations. The single mutants rev-6 and rev-10d result from point mutations and were genotyped using derived cleaved amplified polymorphic sequence (dCAPS) markers. For identification of the double, triple, and quadruple mutants, the plants with novel phenotypes in F2 segregation populations in accordance with Mendelian segregation were selected and further genotyped using molecular markers. For hyll jba-1D, hyll jaw-1D, and hyll oe160c double mutants, the heterozygous jba-1D and homozygous jaw-1D or oe160c mutants were crossed to the homozygous hyll mutants, as the homozygote jba-1D plant is sterile. In F2 progeny, plants with homozygous hyll phenotypes that survived on kanamycin plus Basta or hygromycin MS plates were transferred onto soil in plastic pots for the further step of PCR screening. phb-6phv-5rev-9+/- plants were crossed to hyll plants to generate the hyll phb-6 phv-5 rev-9 quadruple mutant. F2 plants were selected using MS plates with kanamycin plus Basta and then transferred onto soil in plastic pots. Plants exhibiting novel phenotypes were genotyped by PCR to confirm homozygosity for hyll, phb, and phv mutant alleles. These plants were genotyped by PCR to confirm homozygosity for hyll, phb, phv, and rev mutant alleles.

Scanning electron microscopy (SEM)

Leaves were fixed in FAA [50% (v/v) ethanol, 5% (v/v) acetic acid, and 3.7% (v/v) formaldehyde], dried, and then dissected under a stereo microscope and mounted on SEM stubs. Mounted leaves were coated with palladium-gold and then examined using a JSM-6360LV SEM microscope (JEOL, Tokyo, Japan) with an acceleration voltage of 7–15 kV.

Histology

Leaves of 4- to 5-week-old wild-type and mutant plants were fixed in FAA and embedded in paraflin (Sigma), and 7 μm sections were stained with 0.05% (w/v) toluidine blue (Sigma) at 37°C for 15 min and then washed in water. A non-toxic histological clearing agent (Histo-Clear) (National Diagnostics, Atlanta, GA, USA) was used instead of xylene to remove paraflin. For analysis of semi-thin sections, samples fixed in FAA were embedded in epoxy resin. Sections 2 μm thick were cut with glass knives, affixed to glass slides, and stained in 0.05% (w/v) toluidine blue.

Quantitative measurement of leaf curvature and leaf shape

The sixth rosette leaves of 4-week-old wild-type and mutant plants were selected for quantitative measurement of leaf curvature and leaf shape. The CIs of downward and upward curvature were calculated as CI=ab'–ab/ab and CI=(ab–a'b')a'b', where ab is chord length, and the distance between points a and b on two margins of curvature before flattening of leaves, and a'b' is arc length, the distance between a' and b' on two margins after flattening (Liu et al., 2010).

In situ hybridization

Ten-day-old seedling longitudinal sections (7 μm thick) from both wild-type and mutant plants were prepared following pre-treatment and hybridization methods described previously (Jackson, 1991). Hybridization probes corresponding to coding sequences were defined as follows: a REV-specific probe located at 7–2529 bp; and an FIL-specific probe located at 145–567 bp. Digoxigenin (DIG)-labelled probes were prepared by in vitro transcription (Roche) according to the manufacturer’s protocol. Locked nucleic acid (LNA)-modified probes of miR166/319/160 were synthesized and labelled with DIG at the 3’ end by TaKaRa and used for in situ hybridization of miR166/319a/160.

miRNA isolation and northern blot analysis

Total RNA was extracted from 10-day-old seedlings of wild-type and mutant plants. Antisense sequences of miR166, miR160, and miR319 were synthesized and end-labelled as probes with biotin (TaKaRa). A 15 μg aliquot of total RNA was fractionated on a 15% polyacrylamide gel containing 8 M urea and transferred to a Nitran Plus membrane (Schleicher and Schuell). Hybridization was performed at 41°C using hybridization buffer (ULTRAhyb® Ultrasesensitive Hybridization buffer, Ambion). Autoradiography of the membrane was performed using the LightShift Chemiluminescent EMSA Kit (Pierce). A synthesized U6 probe end-labelled with biotin (TaKaRa) was used for the quantification of total RNA content between samples.

Protein gel blot analysis

Rabbit polyclonal antiserum, anti-ARF16, anti-REV, and anti-TCP4, was raised to two synthetic peptides CCGTKEKGLDPQ
and NIDTKNEKGFC corresponding to the Arabidopsis ARF16; CERSSDSMNRHL and AASEENNNNLHC corresponding to the Arabidopsis REV; and CRHRSTSDAADG and CQGQEE-EQHDLGTH corresponding to the Arabidopsis TCP4, respectively. Antibody specificity has been described (Zhao and Last, 1995). Immunoreactions with affinity-purified ARF16/REV/TCP4 antibody were carried out at a final protein concentration of 1.4–7 µg ml⁻¹.

Protein samples were analysed on 12% SDS–polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore Intertech). Membranes were blocked with 5% non-fat dry milk in TRIS-buffered saline plus 0.1% Tween-20 (TBST) and then probed with rabbit polyclonal anti-REV/TCP4/ARF16 antibodies in TBST. Primary antibodies were detected using a horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (Chemicon). Bands were visualized by an enhanced chemiluminescence system according to the manufacturer’s instructions (Chemicon).

**Quantitative real-time RT-PCR**

Ten-day-old seedlings of wild-type and mutant plants were collected from the MS plates and frozen immediately in liquid nitrogen. After being ground in liquid nitrogen, 100 mg of plant sample powder was collected in Eppendorf tubes, and total RNA was isolated and purified according the manufacturer’s protocol (Invitrogen), followed by DNase (Promega) treatment. Then reverse transcription was performed using oligo(dT) primers. Quantitative real-time PCR analysis was performed using the Rotor-Gene 3000 system (Corbett Research, Mortlake, NSW, Australia) using SYBR Premix Ex Taq (TaKaRa). For HD-ZIP III, ARF16, and TCP genes, TUBULIN mRNA was used as an internal control, and relative amounts of mRNA were calculated using the comparative threshold cycle method. Expression ratios of PHB/FIL, REV/FIL, and CNA/FIL were also calculated. The gene-specific primers for RT-PCR are shown in Supplementary Table S2 at JXB online.

**Results**

The mutants deficient in miRNA-mediated pathways display defects in adaxial or abaxial polarity

The rosette leaves of hyll mutants are curved inward along the longitudinal axis due to defects in adaxial/abaxial polarity (Wu et al., 2007). Like hyll leaves, the rosette leaves in mCNA (miR165/166-resistant), mARF16 (miR160-resistant), and mTCP3 (miR319a-resistant) seedlings are incurved transversely while the extent of incurvatures of each mutant are quite different from each other (Fig. 1A–C, I, K, L). To determine whether these miRNA mutants have defects in adaxial/abaxial polarity similar to those in hyll plants, the epidermal cells and mesophyll cells of these mutants were compared with those of hyll plants. For accuracy, the focus was on the lateral areas of rosette leaves in 4-week-old plants where curvature is relatively prominent. Under SEM, the adaxial epidermis of wild-type leaves was characterized by uniform pavement cells, which made the upper surface smooth (Fig. 1E); whereas the abaxial epidermis was mixed with some long and narrow cells, leading to an uneven

![Fig. 1. Phenotypes and leaf polarity of hyll and miRNA-resistant mutants.](image-url)

(A, C, I, K, L) Phenotypes of 4-week-old rosettes of wild-type (A), hyll mutant (C), mCNA (l), phb-1d (J), mARF16 (K), and mTCP3 (P). Bar=100 µm. Adaxial and abaxial epidermal cells of the wild type (E, F) and hyll (G, H). Variable shapes of long and narrow cells are indicated by arrowheads. (B, D, M–P) Transverse sections of leaves in the wild type (B), hyll (D), mCNA (M), phb-1d (N), mARF16 (O), and mTCP3 (P). Bar=200 µm. The substomatic chambers (arrowheads) in the wild type were mostly replaced by adaxialized spongy cells in hyll, mCNA, phb-1d, and mARF16 mutants (asterisks).
surface (Fig. 1F, arrowheads). In cross-sections of wild-type leaves, the palisade parenchyma consisted of thin-walled cells that look like cylindrical spheroids perpendicular to the upper epidermis. These cells were larger than the spongy mesophyll cells, which were usually ball-shaped with large intercellular spaces. The number of chloroplasts in palisade mesophyll cells was much greater than in the spongy mesophyll cells (Fig. 1B). Compared with the wild type, hyl1 leaf cells showed a range of modifications: the adaxial epidermal cells were smaller than abaxial epidermal cells; the abaxial epidermis did not contain long and narrow cells (Fig. 1G, H); both palisade and spongy mesophyll cells looked like oblate spheroids parallel to the upper epidermis; cell size and chloroplast content between palisade and spongy mesophyll cells are a little different; and, most strikingly, the intercellular space in the mesophyll, especially in spongy mesophyll, became small (Fig. 1D). Apparently, the upper side of hyl1 leaves is extremely adaxialized, whereas the lower side has adaxial character. In appearance, the mesophyll cells of hyl1 were similar to those of mCNA and phb-1d, the dominant mutants of CNA and PHB, respectively, in which leaf cells are extremely adaxialized (Fig. 1J, N). Surprisingly, the adaxial/abaxial mesophyll cells in the leaves of mARF16 and mTCP3 plants were the same as those from hyl1 leaves, and both palisade mesophyll and sponge cells were oblate spheroids parallel to the upper epidermis (Fig. 1O, P). These observations reveal that mCNA, mARF16, and mTCP3 leaves have defects in adaxial/abaxial polarity similar to those in hyl1 leaves.

The jba-1D, jaw-1D, and oe-160c (Liu et al., 2010) leaves are characterized with curling down, wavy margins of leaves, and downward curvature of leaves, respectively (Fig. 2A, D, G). In the curved areas of jba-1D leaves, the adaxial epidermis contained a few of the long and narrow cells that normally appear in the abaxial epidermis of the wild type (Fig. 2C), and palisade mesophyll cells were round or irregular with intercellular spaces even larger than those observed in spongy mesophyll of wild-type leaves, suggesting that the abaxial character is on the adaxial side in jba-1D leaves. In the leaf margins of jaw-1D, the adaxial epidermal cells were similar to those of the wild type, and palisade and spongy mesophyll cells were indistinguishable from the wild type except that intercellular spaces between palisade mesophyll cells were larger than in the wild type, showing a little character of abaxialization (Fig. 2E, F). Compared with the wild type, adaxial/abaxial polarity in oe-160c leaves was not changed since palisade and spongy mesophyll cells were the same as those of the wild type (Fig. 2H, I).

Some loss-of-function mutants of miR166-, miR319a- or miR160-targeted genes displayed defects in the adaxial/abaxial polarity of rosette leaves. The rev-6 mutant, with downward leaf curvature, represents the strongest mutant allele of the REV gene (Fig. 2M). It transforms the collateral vascular bundles into nearly amphicribral bundles with the opposite arrangement to that previously reported for rev-10d (Zhong and Ye, 2004). Possibly because of the transformation of vascular bundles in leaves, the adaxial epidermis in rev-6 contained a number of long and narrow cells that normally appear in abaxial epidermis (Fig. 2O). Meanwhile, the shapes of palisade mesophyll cells were indistinguishable from those of spongy mesophyll cells (Fig. 2N). In rev-9 and phb-6 phv-5 leaves, abaxial character on the adaxial side is observable but less obvious (Fig. 2J–L, P–R). Like jaw-1D leaves, TCP3SRDX (Supplementary Fig. S1H, S1K at JXB online) plants, in which TCP3 fused to the EAR-motif repression domain (SRDX) dominantly represses endogenous and functionally redundant transcription factors (Koyama et al., 2007), displayed a weak abaxial character in the adaxial side of leaves (Fig. 2S–U). However, no visual difference in epidermal surfaces and mesophyll cells was observed between the wild type and arf10-3 arf16-2 double mutants (Fig. 2V–X).

The hyl1 allele interacts with dominant mutant alleles of MIR166g, MiR319a, and MiR160c genes

miR165/166, MiR319a, and MiR160 are three miRNAs previously characterized as guiding dorsoventral polarity, cell division, and auxin response, respectively, by post-transcriptional negative regulation of their target genes during leaf development (Palatnik et al., 2003; Wang et al., 2005; Williams et al., 2005) (Supplementary Table S1 at JXB online). Dominant and recessive mutants in these three pathways exhibited different directions and extents of leaf curvature, and showed defects, to various extents, in adaxial/abaxial polarity of leaves. To determine whether and how HYL1 regulates leaf polarity establishment through these three miRNA pathways, hyl1 mutants were crossed with jba-1D, jaw-1D, and oe-160c, and hyl1 jba-1D, hyl1 jaw-1D, and hyl1 oe-160c double mutants were produced after two generations of backcrossing to hyl1 plants. The jba-1D leaves were curling down, in contrast to the incurvature of hyl1 leaves. In the rosette leaves of hyl1 jba-1D double mutants, the extent of incurvature was considerably low compared with that of hyl1 single mutants (Fig. 3G, Supplementary Fig. S2A). Meanwhile, the CI of leaves was calculated to indicate the extent of leaf curvature. The CI of hyl1 plants was much lower than that of hyl1 jba-1D plants (Supplementary Fig. S2), revealing that the hyl1 phenotype, in terms of leaf incurvature, is strongly rescued in hyl1 jba-1D plants, whereas the jba-1D phenotype, in terms of curling down, is compromised. Apparently, the jba-1D allele is epistatic to the hyl1 allele. To examine the contribution of MIR166g to hyl1 leaf morphogenesis further, the epidermal surface and transverse sections of leaves were observed. For hyl1 jba-1D leaves, the abaxial epidermis was mixed with some long and narrow cells like the wild type (Fig. 3H, I), whereas the palisade mesophyll cells were oblate spheroids as in hyl1, and intercellular spaces became larger than those of hyl1.

The hyl1 single mutants had a short petiole and a larger leaf shape index (blade length to width ratio; BLW) compared with the wild type (Supplementary Fig. S2A at JXB online). The petiole to blade length ratio (PBL) of hyl1 jba-1D double mutants was slightly higher than that of hyl1 but lower than that of jba-1D single mutants. The BLWs of the double mutants were lower than that of hyl1 but much higher than that of jba-1D (Supplementary Fig. S2A).
The jaw-1D mutant plants had wavy leaves as a result of excess growth in the marginal area (Nath et al., 2003; Palatnik et al., 2003). In hyl1 jaw-1D seedlings, miR319a accumulated to a low level, in contrast to jaw-1D seedlings where miR319a accumulated to an extremely high level (Fig. 4A). Nevertheless, the CI of hyl1 jaw-1D plants was nearly the same as that of hyl1, indicating that the effect of the jaw-1D allele on the hyl1 phenotype is negligible (Fig. 3M, Supplementary Fig. S2C at JXB online). However, the jaw-1D phenotype was suppressed by the hyl1 allele to some extent. TCP3SRDX plants showed wavy margins of leaves similar to the jaw-1D phenotype, while hyl1 plants with the TCP3SRDX construct had the mutant phenotypes of both hyl1 and jaw-1D: leaf incurvature mixed with wavy margins. Under the microscope, the mesophyll tissues of hyl1 jaw-1D leaves were basically the same as those of hyl1 (Fig. 3N, O). Although hyl1 alleles rescued the jaw-1D phenotype, complementing of the hyl1 phenotype by the jaw-1D allele was limited.

To examine the genetic interaction between HYL1 and MIR160, hyl1 was crossed with oe-160c, a transgenic line overexpressing MIR160c. miR160 accumulated to an extremely high level in oe-160c plants, whose leaves were curved slightly downward (Figs 2G, 4A). In contrast, the accumulation of miR160 in the hyl1 oe-160c seedlings was much lower than in oe-160c, but still higher than in the wild type (Fig. 4A). For hyl1 oe-160c double mutants, the extent of incurvature was a little lower than that of hyl1 seedlings (Fig. 3J, Supplementary Fig. S2B at JXB online). Corresponding to
this, the difference in epidermis and mesophyll tissues between hyl1 oe-160c double mutants and hyl1 single mutants was not distinct (Fig. 3L), and the palisade and spongy mesophyll tissues were normal in the hyl1 oe160c mutant except that intercellular spaces were larger than in hyl1 (Fig. 3K). Clearly, genetic interaction between hyl1 and oe-160c does exist, but it is not obvious in the external layers of leaves.

The hyl1 leaf phenotype is rescued differentially by the mutant alleles of miRNA-targeted genes

To test whether the mutant alleles of miRNA-targeted genes are linked with the hyl1 allele, multiple mutants were constructed on the hyl1 background. hyl1 rev-9 leaves were nearly flat (CI= -0.25) (Fig. 3A, Supplementary Fig. S2A at...
verify further the effects of mutation of adaxial identity genes on polarity defects of hyl1 leaves, a hyl1 phb-6 phv-5 rev-9 quadruple mutant was constructed. In less severely affected phb-6 phv-5 rev-9 triple mutants, two bilaterally symmetrical cotyledons were extremely abaxialized, with radial patterning, and lacked an apical meristem (Emery et al., 2003). Surprisingly, cotyledons of hyl1 phb-6 phv-5 rev-9 quadruple mutants with modest incurvature displayed some lamina expansion (Fig. 3U), whereas the first two leaves showed an abaxialized lotus-like phenotype (Fig. 3W, arrowheads), and the leaves formed afterward displayed a radial needle-like lamina only (Fig. 3V, asterisks). This result indicates that the phb-6 phv-5 rev-9 allele can potentially suppress the hyl1 phenotype while causing defects in shoot meristem and leaf shape.

Along with the partial and complete rescue of the hyl1 leaf incurvature phenotype by jba-1D and rev alleles, investigations were carried out to determine if an miR165/166-resistant allele enhances incurvature of hyl1 leaves. As expected, the leaf phenotype of hyl1 mutants was enhanced by rev-10d in the hyl1 rev-10d double mutant (Fig. 3R), with the mesophyll tissue similar to that of hyl1 (Fig. 3R').

To examine whether mutant alleles of miR160- and miR319-targeted genes affect the hyl1 phenotype, a series of multiple mutants of ARF16 and TCP3 in hyl1 backgrounds were made. In the hyl1 plants containing TCP3SRDX (CI = −0.54) constructs, the rosette leaves were similar either to hyl1 leaves in leaf incurvature or to jaw-1D leaves in wavy margins (Fig. 3Q), indicating that TCP3SRDX does not affect the hyl1 phenotype. However, the transgenic plants expressing 35S::mTCP3 (which is resistant to miR319a) had slightly incurved leaves (CI = −0.37) (Fig. 1L, P). To define whether the hyl1 phenotype is related to TCP genes, the phenotype of hyl1 mTCP3 double mutants was investigated. The plants of hyl1 mTCP3 double mutants were very small, narrow, and their petioles were extremely short, meaning that the effects of hyl1 and mTCP3 are additive (Fig. 3T) and their mesophyll tissue looked like that of the hyl1 single mutant (Fig. 3T'). The hyl1 arf10-3 arf16-2 plants had a phenotype similar to that of hyl1, indicating that the effects of arf10-3 arf16-2 alleles on the leaf incurvature of hyl1 mutants are not apparent (Fig. 3P). Further, hyl1 was crossed with a transgenic plant expressing ARF16:5mARF16 constructs (Fig. 1K). An additive effect was shown in the hyl1 mARF16 double mutants (Fig. 3S), most of which died soon after transfer to pots. The surviving plants showed a phenotype of extremely small leaves and hyl1-like internal mesophyll cells (Fig. 3S'). Thus, the possibility that TCP3 and ARF16 affect the hyl1 phenotype could not be excluded.

The hyl1 allele suppresses expression of miR165/166, miR319a, and miR160, but differentially affects their target genes

To address the relationship between recessive alleles of hyl1 and dominant alleles of miRNAs, the accumulation level of the three miRNAs in hyl1 jba-1D, hyl1 jaw-1D, and hyl1 oe-160c double mutants was analysed. Northern blotting
 showed that the accumulation of miR165/166, miR160, and miR319 was dramatically decreased in hyll seedlings but dramatically increased in seedlings of jba-1D, jaw-1D, and oe-160c, respectively, compared with the wild type (Fig. 4A). In hyll jba-1D and hyll oe-160c seedlings, accumulation of miR165/166 and miR160 was much lower than in jba-1D and oe-160c, respectively, but still higher than in the wild type. In hyll jaw-1D seedlings, however, accumulation of miR319 was much lower than in either jaw-1D or wild-type seedlings. These results reveal that hyll alleles suppress the strength of jba-1D, jaw-1D, and oe-160c alleles, albeit to a different extent.

In hyll leaves, miR166, miR319a, and miR160 accumulate less than in the wild type (Wu et al., 2007). To examine whether the target genes of these miRNAs are up-regulated in hyll seedlings, real-time PCR was performed. As expected, all five miR165/166-targeted genes are up-regulated. Among them, PHB, REV, and CNA were up-regulated with 3.4-, 2.2-, and 2.6-fold changes, respectively. All of the miR160-targeted genes that were tested were up-regulated. In hyll seedlings, expression of HD-ZIP III and ARF genes is increased due to reduced accumulation of miR165/166 and miR160. Surprisingly, none of the miR319a-targeted genes was up-regulated, and TCP2, in contrast, was down-regulated slightly (Supplementary Fig. S1 at JXB online). Possibly, miR319a regulates its targets in the hyll background by translational inhibition rather than cleavage of mRNA. To address this question, levels of REV, ARF16, and TCP4 proteins in hyll seedlings were determined. The results of western blotting indicated that the abundance of REV and ARF16 in hyll seedlings was increased, whereas that of the TCP4 protein was not remarkably changed (Fig. 4B). These results indicate that the reduction of miR319 accumulation in hyll seedlings is not concomitant with up-regulation of TCP genes. In hyll mutants, miR319a accumulated to a lower level, while TCP4 expression was not increased, implying that some elements in hyll mutants inhibit activities of TCP genes, possibly in an indirect way. In other words, HYL1 mutation alters the normal pathway of miR319-mediated gene silencing.

To exclude the possibility that the abnormal activities of miRNA mutants are due to ectopic expression of miRNAs in the single and double mutants, in situ hybridization of miR166, miR319a, and miR160, respectively, was performed. In wild-type seedlings, miR166 accumulated more in SAM, leaf primordia, and developing leaves, and less in stems and developed leaves (Fig. 5A). It was noticed that the amount of miR166 on the abaxial side of leaves was more than on the adaxial side. In hyll mutants, accumulation of miR166 in the SAM, leaf primordia, and developing leaves was reduced in such a way that the expression signal was weak but evenly distributed in all organs of the plant (Fig. 5B). In seedlings of single jba-1D mutants, accumulation of miR166 was increased remarkably but the spatial patterns remained unchanged compared with that of the wild type (Fig. 5C). In wild-type seedlings, miR319a was preferentially accumulated in the SAM, leaf primordia, and vascular tissues of stem, while miR160 accumulated mainly in the SAM and leaf primordia (Fig. 5E, 1). HYL1 mutation caused the reduced accumulation of both miR160 and miR319a in these organs (Fig. 5F, J), while enhanced activation of MIR160c and MIR319a genes led to a remarkable increase in miR160 and miR319a accumulation in these organs (Fig. 5G, K). The observations reveal that miRNA accumulation in plants of single dominant mutants is not ectopic and that HYL1 mutation primarily affects the amount of the miRNAs but not the spatial distribution of miRNAs. It is suggested that the defects in polarity of hyll mutants are predominantly a consequence of PHB, PHV, and REV overexpression (Supplementary Fig. S1 at JXB online).

The mild phenotypes that distinguish double hyll rev-9 and triple hyll phb-6 phv-5 mutants from wild-type plants suggest that HYL1 regulates other genes in addition to PHB, PHV, and REV; including the more distantly related HD-ZIP III gene CNA, which shows an elevated expression in hyll plants (Supplementary Fig. S1 at JXB online).

The extent of leaf curvature is associated with expression levels of adaxial and abaxial identity genes

According to the direction of leaf curvature, hyll and many mutants of miRNAs and their target genes are classified into three types: upward curvature, downward curvature, and flat (Liu et al., 2010). To establish the relationship between the extent of leaf curvature and the expression level of the related genes, expression of adaxial and abaxial genes was detected in seedlings of the mutants mentioned above. Compared with the wild type, the expression of PHB, REV, and CNA was very weak in the jba-1D seedlings with curling down of leaves, high in hyll seedlings with leaf incurvature, and almost the same in hyll jba-1D seedlings with flat leaves (Fig. 6A–C). PHB, CNA, and REV were overexpressed in seedlings of miRNA-resistant mutants such as phb-1d, mCNA, and rev-10d, respectively, with leaf incurvature (Fig. 6A–C). In hyll jaw-1D seedlings with leaf incurvature, expression levels of REV, PHB, and CNA were almost the same as in hyll oe-160c double mutants (Fig. 6A–C). Interestingly, expression of PHB and CNA in mTCP3 seedlings with leaf incurvature was significantly higher than in the wild type, suggesting that miR319a-targeted genes regulate HD-ZIP III genes (Fig. 6A–C). On the other hand, expression of PHB and CNA in mARF16 seedlings with leaf incurvature was not up-regulated, indicating that HD-ZIP III genes are not regulated by miR160-targeted genes (Fig. 6A–C). Apparently, the low expression of the adaxial identity genes is associated with upward curvature of rosette leaves, while the strong expression is associated with downward curvature of leaves.

Enhanced expression of adaxial identity genes can repress expression of abaxial identity genes in leaves (Bowman et al., 2002). Expression of FIL in hyll, phb-1d, mCNA, and rev-10d was lower than in the wild type (Fig. 6D), suggesting that FIL expression is repressed by overexpression of HD-ZIP III genes. Interestingly, FIL expression in mARF16 plants was significantly lower than in the wild type, while expression of HD-ZIP III was not changed; that is, auxin response factor genes regulate the abaxial identity gene.
To verify the contributions of expression of adaxial or abaxial identity genes to the degree and direction of leaf curvature, the adaxial to abaxial (ATA) ratio of relative expression levels was calculated. It was found that ATA ratios of \( \frac{PHB}{FIL} \), \( \frac{REV}{FIL} \), and \( \frac{CNA}{FIL} \) in \( hyl1 \), \( hyl1 oe-160c \), and \( hyl1 jaw-1D \) mutants were remarkably higher than in the wild type (Fig. 7A–C), implying that increased expression of adaxial genes and/or decreased expression of abaxial genes caused the incurvature or upward curvature of leaves in \( hyl1 \), and that only \( jba-1D \) could partially rescue the \( hyl1 \) phenotype. As increased levels of \( PHB \) repressed expression of \( FIL \), extremely high ATA ratios of \( \frac{PHB}{FIL} \) and \( \frac{REV}{FIL} \) were also found in \( phb-1d \) mutants with radial or lotus leaves (Fig. 7A, B). In addition, as a result of decreased expression of \( FIL \) in \( mARF16 \) leaves, high ATA ratios of \( REV/FIL \) and \( CNA/FIL \) were detected in \( mARF16 \) leaves (Fig. 7B, C). In contrast, evidently lower ATA ratios of \( PHB/FIL \), \( REV/FIL \), and \( CNA/FIL \) were found in \( jba-1D \) mutants and \( REV::FIL \) plants ectopically expressing \( FIL \) under control of the \( REV \) promoter, corresponding to downward curvature of the leaves (Fig. 7A–C). All of these results indicated that changes in the expression ratios of adaxial/abaxial genes could generally affect the degree and direction of leaf curvature. The correlation coefficient \( (R^2) \) was calculated between ATA ratios and CIs of mutants. The coefficients of \( \frac{PHB}{FIL} \) CI, \( \frac{REV}{FIL} \) CI, and \( \frac{CNA}{FIL} \) CI were 0.71243 \( (P = 0.0029) \), 0.93854 \( (P < 0.0001) \), and 0.68252 \( (P = 0.005) \), respectively. These results indicate a remarkable positive correlation between ATA ratios and CIs. In this case, polarity defects in \( hyl1 \) leaves were caused by mis-regulation of both adaxial and abaxial genes via the miR166- and miR160-mediated pathways.

**Discussion**

Defects in miR165/166-directed pathways are the primary causes for leaf incurvature of \( hyl1 \) plants

miR165/166, miR319, and miR160 play important roles in establishing polarity, cell division, and auxin response, respectively (McConnell et al., 2001; Mallory et al., 2005; Wang et al., 2005). Gain-of-function mutation of these MIRNA genes leads to curling down, wavy margins, and downward curvature of leaves in \( jba-1D \), \( jaw-1D \), and \( oe-160c \)
mutants, respectively. On the other hand, overexpression of some adaxial identity genes, TCP genes or ARF genes, causes upward curvature of leaves in mCNA, mTCP3, and mARF16 seedlings, respectively. This fact implies that proper accumulation of miR165/166, miR319, and miR160 prevents curling down, wavy margins, and downward curvature, thus maintaining leaves in a flattened state. In the double mutants of hyl1, the hyl1 phenotype in terms of leaf incurvature is partially rescued by jba-1D, but not by the jaw-1D or oe-160c alleles, thus suggesting that miR165/166, rather than miR319a and miR160, is closely related to leaf incurvature of hyl1 mutants. Moreover, loss-of-function mutants of miR165/166-targeted genes such as rev-9 and phb-6 phv-5 are able to suppress the hyl1 phenotype to some extent; miR165/166-resistant plants such as rev-10d aggravate it; and arf10-3 alleles and TCP3-SRDX constructs do not rescue it, meaning that overexpression of adaxial identity genes rather than TCP or ARF genes contributes to the hyl1 phenotype. In this case, HYL1, miR165/166, and some adaxial identity genes, such as PHB, REV, and CNA, act in the same molecular pathways.

Nevertheless, the effect of miR319a and miR160 and their targets on the hyl1 phenotype should not be ignored. Because PHB, REV, and CNA genes are up-regulated in mTCP3 leaves overexpressing TCP3 and the FIL gene is down-regulated in mARF16 leaves overexpressing ARF16, miR319a and miR160 pathways acting on activities of adaxial identity genes should exert some indirect effects on the hyl1 phenotype. Under the present conditions, however, such indirect effects are not so strong.

Fig. 6. Expression levels of miRNA-targeted genes. (A–F) Expression of PHB, REV, CNA, ARF16, TCP3, and FIL in the wild type, mutants, and transgenic plants. Total RNA was extracted from 10-day-old plants and analysed by real-time RT-PCR with two technical replicates and three biological replicates. Expression was normalized relative to that of β-TUBULIN2. Bars indicate the SD.
Direction and extent of leaf curvature are dependent on the ATA ratio of gene expression

Gain-of-function or loss-of-function mutation of several adaxial identity genes causes various types of leaf curvature or rod-shaped leaves (Liu et al., 2010). Under the present conditions, hyll, phb-1d, rev-10d, and mCNA are the mutant or transgenic plants displaying incurvature or upward curvature of rosette leaves. In these lines, expression levels of PHB, REV, and/or CNA are higher than in the wild type. Among them, PHB is strongly overexpressed in phb-1d plants, concomitant with leaf incurvature and some rod-shaped leaves (an extreme type of incurvature); CNA is overexpressed, concurrent with severe incurvature in mCNA plants; and PHB, REV, and CNA are overexpressed in hyll leaves, corresponding to leaf incurvature. Apparently, the appearance of leaf incurvature or rod-shaped leaves in these mutants is dependent on high levels of adaxial identity genes. On the other hand, expression levels of the FIL gene in leaves of these mutants are lower than in the wild type, consistent with the conclusion of Bowman et al. (2002) that enhanced expression of adaxial identity genes could repress expression of abaxial identity genes in leaf. The mutant plants with upward curvature of rosette leaves include mARF16 and mTCP3. In mARF16 leaves, the FIL gene is down-regulated when adaxial identity genes are not up-regulated. This change indicates that low levels of abaxial identity genes are related to leaf incurvature. This deduction is confirmed by REV::FIL plants, in which leaves are curved downward, corresponding to elevated expression of the FIL gene. In mTCP3 leaves, PHB, REV, and CNA are up-regulated when the FIL gene is not changed. It is suggested that leaf incurvature of mTCP3 is due to high expression levels of adaxial identity genes.

CI values of oe-160c, REV::FIL, jaw-1D, jba-1D, and rev-6 are above zero, and they are the mutants with downward curvature of leaves. FIL is up-regulated in oe-160c leaves, whereas certain adaxial identity genes are down-regulated in jaw-1D, jba-1D, and rev-6 leaves. These results reveal that the direction and extent of leaf curvature are dependent not only on expression levels of adaxial identity genes, but also on those of abaxial identity genes.

To clarify the relative expression levels of adaxial/abaxial identity genes in leaf curvature, the ratio of the expression levels of PHB and FIL (A), REV and FIL (B), and CNA and FIL (C) were calculated. The correlation coefficient ($R^2$) was calculated between each leaf polarity gene expression level ratio and CIs. PHB/FIL CI = 0.71243 ($P = 0.0029$), REV/FIL CI = 0.93854 ($P < 0.0001$), CNA/FIL CI = 0.68252 ($P = 0.005$).
level of adaxial identity genes to that of abaxial identity genes was calculated. As indicated in Fig. 7, the ATA of the mutants observed is negatively correlated with CI values. Among the mutants with leaf incurvature, a higher ATA ratio indicates a more severe incurvature; among the mutants with downward curvature of leaves, a lower ATA ratio indicates a more severe downward curvature. Such correlations fit not only for single mutants, but also for double or multiple mutants. It was not possible to determine if the direction and extent of curvature are generally correlated with the ATA ratio beyond the mutants relevant to HYL1. It will be of particular interest to perform general analysis of the relationship between the ATA ratio and the extent of leaf curvature.

**HYL1 regulates the balance between adaxial and abaxial identity for leaf flatness**

Flattening of the leaf is usually the first morphological indication of dorsoventral patterning of tissues. Adaxial/abaxial polarity and asymmetry are most evident at the cellular level, and it has been proposed that the juxtaposition of adaxial and abaxial domains is required for lamina outgrowth (Pozzi et al., 2001; Waite and Hudson, 2001). It was found that the direction and extent of leaf curvature are dependent on the ATA ratio of gene expression, which is affected not only by adaxial or abaxial identity genes, but also by TCP or ARF genes. Importantly, ATA ratios are determined by HYL1. An imbalance between adaxial and abaxial genes causes some types of leaf curvature. Overexpression of the adaxial identity genes REV, PHB, and/or CNA usually leads to upward curvature of leaves, such as in hyl1 and mCNA plants, whereas further overexpression of these genes may result in rod-shaped leaves, the extremely adaxialized structure seen in phb-1d and rev-10d plants. On the other hand, underexpression of adaxial identity gene(s) sometimes causes downward curvature, as in jba-1D and rev-6 plants. The effects of suppressed expression of the FIL gene on leaf curvature, as in mARF16 plants, are equivalent to up-regulated expression of adaxial identity genes, as in mCNA plants. Thus, a model is proposed to illustrate how HYL1 regulates the balance between adaxial and abaxial identity genes for leaf flatness (Fig. 8).

According to the new model, HYL1 controls the type of leaf flattening through mir165/166 and other miRNAs that regulate adaxial and abaxial identity genes. When HYL1 is normally expressed, juxtaposition of adaxial and abaxial domains is perfect because the expression of adaxial and abaxial identity genes is well balanced, and thus the leaves are flat. When HYL1 expression is reduced, activities of adaxial identity genes could be enhanced or that of the FIL gene suppressed, leading to upward curvature of leaves. Sometimes, adaxial identity genes are overexpressed to an extreme extent so that the leaves are extremely abaxialized, resulting in another type of rod-shaped leaves. For this model, the ATA ratio is an internal indicator to explain the molecular mechanism of leaf curvature of various types. Nevertheless, the molecular reasons for leaf curvature are complicated, and a general relationship between the ATA ratio and extent of leaf curvature should be explored after more data about the specific function of individual genes are available. The proposed model provides insight into the complicated process of miRNA-mediated gene silencing under the control of HYL1, and is certainly useful for understanding the mechanisms by which leaves form and function.

Supplementary data are available at *JXB* online.

**Figure S1.** Real-time PCR analyses of HD-ZIPIII genes, TCP genes, ARF genes, and FIL in WT-Nossen and hyl1.

**Figure S2.** Leaf shape index of wild-type and mutant plants in the miR165/166-, miR160-, and miR319-regulating pathways.

**Table S1.** The combinations between MIRNA genes, mutants, miRNA, and the target genes.

**Table S2.** Sequences of probes and primers used in this study.

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