

# PHANTASTICA Regulates Development of the Adaxial Mesophyll in Nicotiana Leaves

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**Initiation and growth of leaf blades is oriented by an adaxial/abaxial axis aligned with the original axis of polarity in the leaf primordium. To investigate mechanisms regulating this process, we cloned the *Nicotiana tabacum* ortholog of PHANTASTICA (NTPHAN) and generated a series of antisense transgenics in *N. sylvestris*. We show that NSPHAN is expressed throughout emerging blade primordia in the wild type and becomes localized to the middle mesophyll in the expanding lamina. Antisense NSPHAN leaves show ectopic expression of NTH20, a class I KNOX gene. Juvenile transgenic leaves have normal adaxial/abaxial polarity and generate leaf blades in the normal position, but the adaxial mesophyll shows disorganized patterns of cell division, delayed maturation of palisade, and ectopic reinitiation of blade primordia along the midrib. Reversal of the phenotype with exogenous gibberellic acid suggests that NSPHAN, acting via KNOX repression, maintains determinacy in the expanding lamina and sustains the patterns of cell proliferation critical to palisade development.**

## INTRODUCTION

Lateral organ primordia are initiated at the periphery of the shoot apical meristem (SAM) as clusters of initials make a fundamental transition from stem cell to organ cell identity. It appears that the foundation for morphogenesis in leaf primordia is linked directly to an asymmetry established at the time of inception by the adjacent meristem (Bowman et al., 2002). The surface of the primordium facing the meristem (adaxial) acquires its identity in a process associated with expression of *PHABULOSA* and *PHAVOLUTA*, two functionally redundant genes in the *HDZ/PIII* family of transcription factors (McConnell and Barton, 1998; McConnell et al., 2001). The abaxial domain facing away from the meristem is marked by the expression of genes in the *KANADI* (Kerstetter et al., 2001) and *YABBY* (Sawa et al., 1999; Siegfried et al., 1999) gene families. On the lateral flanks of the primordium where the adaxial and abaxial domains intersect, lateral outgrowth of blade primordia takes place. The leaf blade is a laminar structure with a fixed set of cell layers, each of which differentiates according to its position along an adaxial/abaxial axis of polarity. The adaxial (upper) mesophyll develops into palisade, the abaxial (lower) mesophyll forms a spongy layer, and the middle mesophyll gives rise to vascular tissue. Because the axis of blade polarity is directly aligned with the original axis of adaxial/abaxial polarity in the primordium, there is reason to suspect that they arise through a common mechanism, but this remains largely unexplored.

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A putative connection between leaf and blade polarity emerged in studies on the *PHANTASTICA* (*PHAN*) gene in *Antirrhinum majus* (Waites and Hudson, 1995). In the adult phase, loss of *PHAN* produces radialized, bladeless leaves thought to result from the loss of adaxial identity in emerging leaf primordia. In juvenile leaves, however, leaf blades are initiated normally, and polarity defects are observed in the expanding lamina itself. This appears in the form of sporadic default to abaxial identity on the upper leaf surface, producing ectopic juxtapositions of adaxial and abaxial identities and formation of ectopic blade primordia. The sporadic phenotype suggested that *PHAN* might be partially redundant with other genes that maintain polarity in the expanding lamina. A candidate gene with an overlapping role in blade polarity is *LAM1*, which was defined in *Nicotiana* by a recessive mutation interfering with lateral outgrowth of blade tissue from the flanks of the primordium (McHale, 1992, 1993). Mutant *lam1* plants show normal adaxial/abaxial polarity in the primordium and produce blade founder cells at the domain boundary, but this region forms only a vestigial strip of blade tissue lacking polarity and fails to grow outward into lamina. Formation of a polarized lamina with normal cell layering is restored in periclinal *lam1* chimeras with wild-type *LAM1* function in L3-derived tissue, indicating that the polarizing function of *LAM1* is nonautonomous (McHale and Marcotrigiano, 1998). In later stages of expansion, however, disorganization and loss of polarity reemerges sporadically in chimeric blades, in a manner roughly analogous to the patches described in *phan* mutants of *Antirrhinum*. Thus, *LAM1* and *PHAN* appear to perform related functions associated with the maintenance of polarity and lateral growth of leaf blades. Another gene involved in blade polarity is *ASYMMETRIC LEAVES2* (*AS2*) (Semiarti et al., 2001; Iwakawa et al., 2002), a member of the *LATERAL ORGAN BOUNDARIES*-domain gene family in *Arabidopsis thaliana*. Loss of *AS2* does not interfere with polarity, but 35S:*AS2* transgenics

show disruptions in adaxial/abaxial symmetry and differentiation of adaxial blade cell types in the abaxial domain (Lin et al., 2003).

Further studies on *PHAN* in Antirrhinum revealed that it coded for a MYB-containing transcription factor involved not only in leaf polarity but also in elaboration of the proximodistal axis in lateral organs (Waites et al., 1998). *PHAN* orthologs were subsequently investigated in maize (*Zea mays*; *ROUGH SHEATH2 [RS2]*) (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999) and Arabidopsis (*ASYMMETRIC LEAVES1 [AS1]*) (Byrne et al., 2000, 2002; Ori et al., 2000; Sun et al., 2002), where loss of the function curiously produced neither bladelessness nor radialization, but a common conclusion nonetheless was a role for *PHAN* orthologs in negative regulation of class I *KNOX* genes. The leaf phenotypes of the *rs2* mutant in maize and the *as1* mutant in Arabidopsis in fact have a striking resemblance to those resulting from *KNOX* overexpression in those species (Lincoln et al., 1994; Schneeberger et al., 1995; Chuck et al., 1996). The Arabidopsis studies show that several *KNOX* genes, including *KNAT1/BREVIPEDICELLUS (BP)*, *KNAT2*, and *KNAT6*, are ectopically expressed in the absence of *AS1* function. This concerted response makes it uncertain as to which *KNOX* gene causes the phenotype, and the functional overlap of *KNOX* genes raises the obvious possibility that they are acting together in *as1* leaves. In fact, analysis of *as1* in combination with single *KNOX* mutations shows that no single *KNOX* gene is solely responsible for the *as1* leaf phenotype (Byrne et al., 2002). An unexpected result emerged from initial studies on a *PHAN* ortholog (*LePHAN*) in tomato (*Lycopersicon esculentum*), where unlike maize, Antirrhinum, and Arabidopsis, the gene is coexpressed in the meristem with the class I *KNOX* gene *LeT6*, apparently at odds with *PHAN*-mediated *KNOX* repression (Koltai and Bird, 2000). Nonetheless, disruption of *LePHAN* in antisense tomato transgenics produced a radialized leaf rachis lacking lateral leaflets, analogous to the bladeless radialized petioles seen in *phan* mutants of Antirrhinum (Kim et al., 2003). Thus, in the context of the leaf primordium, it appears that *PHAN* and *LePHAN* perform the same function, which is required both for the initiation of lateral leaflets in compound leaves and for blade formation in simple leaves. This is consistent with a long standing belief that that these are related developmental events (Hagemann and Gleissberg, 1996) and with the current view that the compound leaves of angiosperms arose through modification of a simple ancestral form (Bharathan and Sinha, 2001).

Whether ectopic *KNOX* actually causes the phenotypes associated with loss of *PHAN* orthologs remains in question, but there is evidence supporting a causal connection from work on *KNOX* regulation of gibberellic acid (GA) metabolism. The tobacco class I *KNOX* gene *NTH15 (STM)* ortholog directly suppresses expression of *Ntc12*, a gene encoding a GA20 oxidase required for biosynthesis of active GA (Sakamoto et al., 2001). Accordingly, GA synthesis is blocked in the meristem where *NTH15* is present but proceeds in the subapical region (where it mediates stem elongation) and in emerging leaf primordia where *KNOX* genes are suppressed. This appears to be the case in Arabidopsis as well because repression of *AtGA20ox1* is observed in 35S:*BP/KNAT1* and 35S:*KN1* transgenics (Hay et al., 2002). This relationship predicts that phenotypes putatively resulting from ectopic *KNOX* (such as

*as1*) would be enhanced by factors antagonistic to GA response and conversely would be suppressed by application of exogenous GA. Hay et al. (2002) report that the *as1* phenotype is in fact enhanced in a *ga insensitive* mutant background and that the phenotype of 35S:*BP/KNAT1* transgenics is suppressed by application of GA. Application of GA likewise suppresses the leaf phenotype in 35S:*NTH15* transgenics of tobacco (Tanaka-Ueguchi et al., 1998). It is unlikely that *KNOX* genes act solely through negative regulation of GA because positive regulation of the cytokinin pathway also appears linked to *KNOX* expression (Ori et al., 1999). Nonetheless, *KNOX* regulation of the GA pathway in the SAM may be a critical component in the acquisition of differentiated cell states and the onset of regulated patterns of cell division in lateral organ primordia.

Our primary interest was the nature of *PHAN* function in the lamina itself and its relationship, if any, to adaxial/abaxial polarity and *KNOX* repression. To investigate this further and examine the relationship between *PHAN* and *LAM1* in the expanding lamina, we isolated the Nicotiana ortholog of *PHAN* and generated a series of antisense transgenics in *N. sylvestris*. In juvenile wild-type leaves, *NSPHAN* is expressed throughout emerging blade primordia and becomes localized to the vascular proximal tissue of the middle mesophyll, where it persists throughout blade expansion. Antisense *NSPHAN* transgenics show that this middle mesophyll function is essential for normal development of the overlying adaxial mesophyll. In the absence of *NSPHAN*, ectopic *KNOX* suspends the upper mesophyll in an indeterminate state, leading to disorganized proliferation, delayed palisade differentiation, and de novo reinitiation of ectopic leaf blades along the flanks of the midrib. Reversal by exogenous GA suggests that the phenotype is associated with a *KNOX*-mediated suppression of GA synthesis in the expanding lamina.

## RESULTS

### *PHAN* Protein Alignment

The MYB region of the Antirrhinum *PHAN* gene was used as a probe for isolation of a full-length clone from an *N. tabacum* leaf cDNA library in  $\lambda$ ZAPII. The gene was sequenced and translated for alignment with the *PHAN*-like proteins of four eudicots, which is shown in Figure 1 with identical residues shaded. All have a highly conserved MYB DNA binding domain at the N terminus, consisting of two imperfect repeats (55 and 51 residues), both of which are essential for sequence-specific DNA binding based on structural analysis of c-MYB (Jin and Martin, 1999). In the Antirrhinum and Nicotiana proteins, 94 of 106 residues are identical in this region. The C-terminal sequences of R2R3 MYB genes are generally quite diverse, falling into 22 different subgroups in Arabidopsis (Kranz et al., 1998), but the C-terminal regions of *PHAN*-like genes in Figure 1 are very similar. This is particularly so for Nicotiana, Lycopersicon, and Antirrhinum, where there is >80% identity in the last 100 residues, suggesting functionality for this domain. In fact, mutations predicted to truncate the C-terminal third of the protein in Arabidopsis *AS1* produce a loss of function (Byrne et al., 2000), and there is evidence that this region mediates homodimerization of *PHAN*

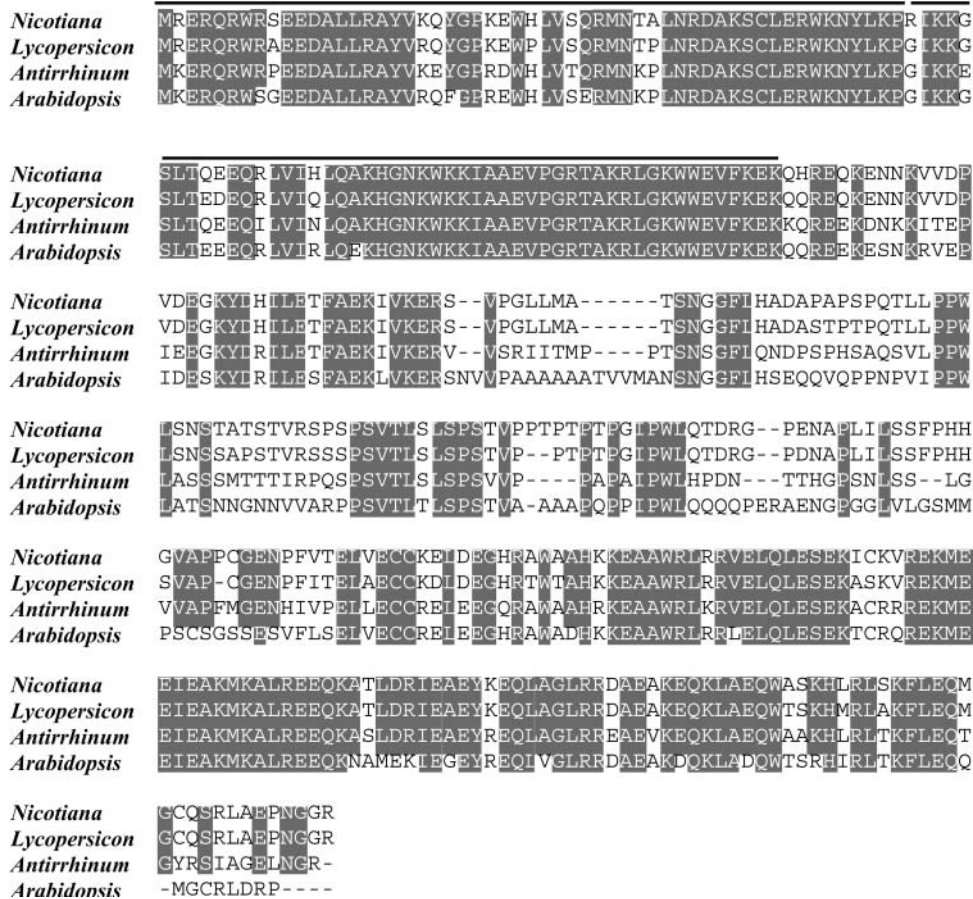


Figure 1. Alignment of PHAN Orthologs.

Amino acid alignment of PHAN proteins. Identical residues are shaded, and dashed lines were added to maximize alignment. Solid lines show the two imperfect N-terminal MYB repeats of 55 and 51 residues.

orthologs in maize (*RS2*) and Arabidopsis (*AS1*) (Theodoris et al., 2003).

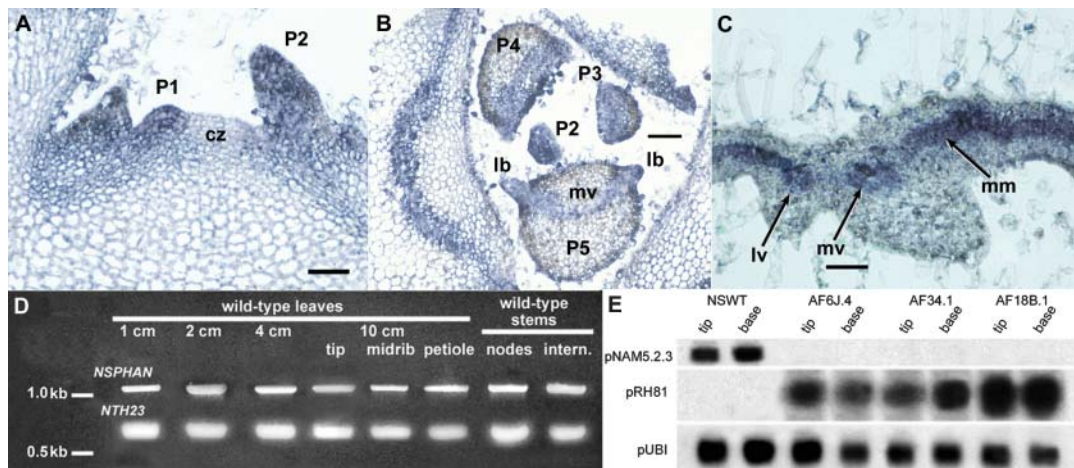
**NSPHAN and LAM1**

Previous evidence that PHAN and LAM1 performed similar functions in the expanding blades of Antirrhinum (Waites and Hudson, 1995) and Nicotiana (McHale and Marcotrigiano, 1998) indicated that they were either orthologs or separate genes with partially redundant functions. The phenotypic distinction between antisense NSPHAN transgenics and lam1 mutants in the juvenile phase of N. sylvestris suggests they are not homologous genes. Mutant lam1 leaves are defective in lateral outgrowth of lamina in all phases of growth (McHale 1992, 1993), as opposed to transgenics, where juvenile leaves produce a polarized lamina and disruptions arise later during lamina expansion. Thus, it appears that LAM1 acts first in the blade pathway, consistent with the observation that antisense NSPHAN lam1 double mutants of N. sylvestris have the bladeless phenotype of lam1 mutants in all phases of growth (data not shown). Beyond this phase, however, it does appear

that NSPHAN and LAM1 have overlapping roles in the expanding lamina, where both genes regulate development of the adaxial mesophyll.

**NSPHAN Expression**

To determine the patterns of expression for the PHAN ortholog in N. sylvestris (NSPHAN), we conducted in situ hybridizations with a digoxigenin-labeled antisense RNA probe specific to the C terminus and 3' untranslated region (UTR) (Figures 2A to 2C). Median longitudinal sections through the juvenile shoot apex revealed NSPHAN mRNA throughout P1 and P2 leaf primordia but not in the central zone of the meristem (Figure 2A). NSPHAN mRNA begins to show an adaxial restriction at P3 and P4. At the P5 stage, NSPHAN is observed throughout the emerging lamina and in regions adjacent to the leaf midvein (Figure 2B). In the expanding leaf blade (Figure 2C), NSPHAN was detected in regions proximal to the midvein and lateral veins and in the middle mesophyll layers of the lamina where vascular tissue differentiates. RT-PCR amplification of NSPHAN from samples of total RNA indicated that the mRNA is present in leaves up



**Figure 2.** *NSPHAN* Expression Analysis.

Expression patterns for *NSPHAN* were examined in situ with digoxigenin-labeled probes (**[A]** to **[C]**), by RT-PCR (**[D]**), and in RNA gel blots (**[E]**). Bars in **(A)**, **(B)**, and **(C)** = 60, 80, and 100  $\mu\text{m}$ , respectively.

**(A)** In situ hybridization with an antisense RNA probe in a juvenile shoot apex shows *NSPHAN* mRNA is undetectable in the central zone of the meristem (cz) but is present throughout P1 and P2 leaf primordia.

**(B)** In stages P4 to P5, *NSPHAN* is expressed throughout emerging leaf blades (lb) and in leaf midveins (mv).

**(C)** In the expanding lamina, *NSPHAN* is localized to middle mesophyll (mm) and lateral veins (lv). mv, midveins.

**(D)** RT-PCR amplification of *NSPHAN* and *NTH23* as control from total RNA samples shows expression in leaves up through the 10-cm stage and in the stems.

**(E)** RNA gel blot analysis with an *NSPHAN*-specific probe (pNAM5.2.3) shows similar mRNA levels at the tip and base of juvenile wild-type leaves (NSWT) and undetectable levels in three antisense transgenics (AF6J.4, AF34.1, and AF18B.1) relative to a ubiquitin control (pUBI). Expression levels of the antisense transgene were analyzed with the pRH81 probe.

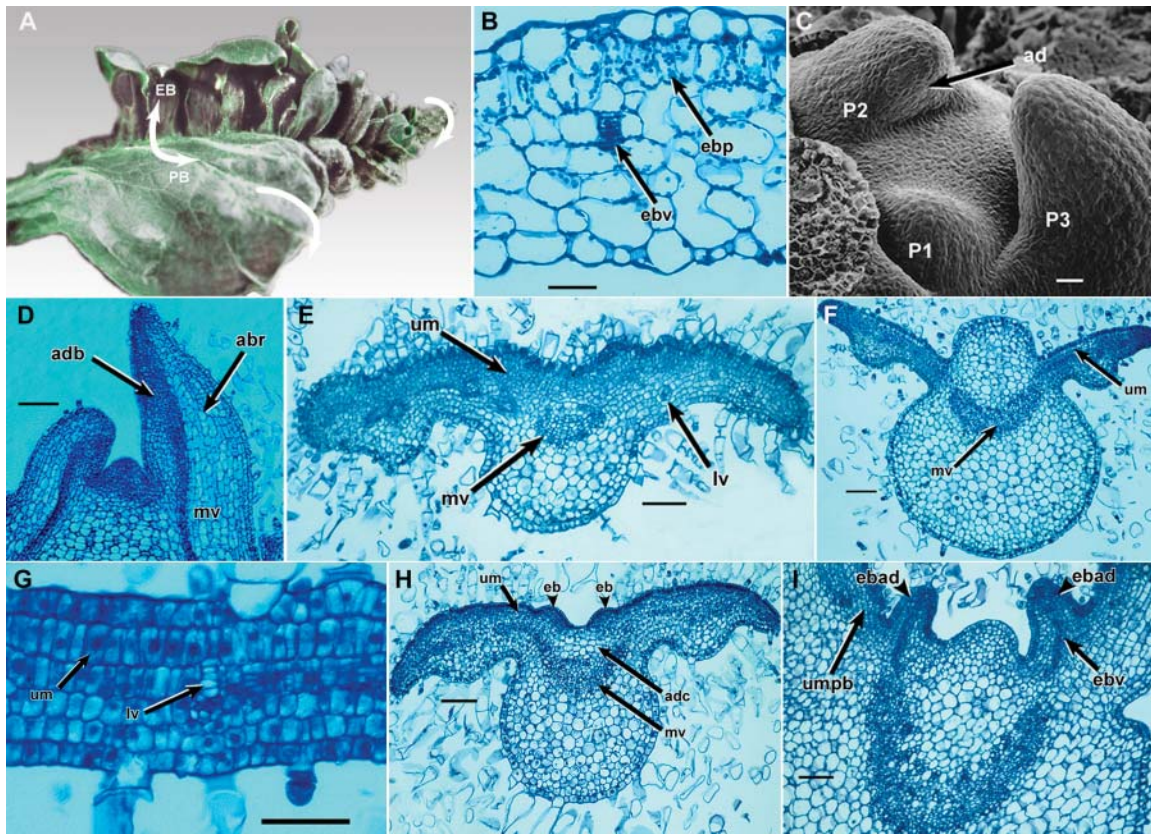
through the 10-cm stage and in the nodal and internodal regions of the stem (Figure 2D).

Disruption of *NSPHAN* function was accomplished by introducing a full-length cDNA in reverse orientation into wild-type *N. sylvestris*. A group of 30 transgenics with a range of phenotypic severity was recovered, and three independent transgenics (AF6J.4, AF434.1, and AF18B.1) with single insertions producing strong leaf phenotypes were chosen for detailed analysis. Expression levels for *NSPHAN* and the antisense transgene were analyzed in RNA gel blots with total RNA samples isolated from the tip and base of wild-type juvenile leaves (5 cm) and matching transgenics and compared with ubiquitin controls (Figure 2E). A gene-specific probe (pNAM5.2.3) revealed that *NSPHAN* mRNA is present at similar levels at the tip and base of the wild type and is not detectable in either location in three independent transgenics with identical leaf phenotypes. The pRH81 RNA probe specific to the antisense transgene revealed differences in transgene expression between these lines but apparently in a range sufficient to bring *NSPHAN* RNA below the limits of detection. All subsequent histological and molecular analyses were performed in homozygous T3 progenies of transgenic AF18B.1.

### Juvenile Antisense *NSPHAN* Leaves

In the juvenile phase, antisense transgenics produce leaves with primary blades in the normal position and then generate an ectopic set of blades along both sides of the midrib, with

a differentiated adaxial surface facing away from the rib (Figure 3A). Transverse sections through ectopic blades confirmed that these are differentiated leaf blades, with a recognizable palisade layer in the upper mesophyll, polarized vascular tissue, and a spongy mesophyll (Figure 3B). A detailed histological analysis of juvenile transgenic leaves with particular attention to the ontogeny of ectopic blades is presented in Figures 3C to 3H. Scanning electron micrographs revealed that transgenic primordia have normal adaxial/abaxial polarity in stages P1 to P3 (Figure 3C). This was confirmed with longitudinal sections through the juvenile apex, showing differentiated adaxial and abaxial leaf domains and a midvein formed at the interface (Figure 3D). The first evidence of a disruption is a highly abnormal pattern of proliferation in the adaxial mesophyll of the leaf blade, which is clearly visible by the P5 stage (Figure 3E). Median transverse sections show numerous layers of undifferentiated upper mesophyll forming a continuous pavement over the developing veins. This is quite pronounced at the tip of the leaf where abnormal proliferation produces mounds of upper mesophyll above the lateral veins, apparently causing the lamina to curl downward at the tip and at the margins (Figure 3A). Wild-type upper mesophyll at this stage is a single cell layer of palisade initials maintained by a strict pattern of anticlinal cell division (Figures 3F and 3G). In transgenic leaves where the adaxial mesophyll abuts the adaxial midrib cortex, periclinal cell divisions generate vertical outgrowths (Figure 3H), which develop into vascularized blade primordia (Figure 3I) and eventually emerge as a fully differentiated ectopic lamina with an adaxial



**Figure 3.** Juvenile Antisense Phenotype.

In the juvenile vegetative phase, antisense *NSPHAN* transgenics produce leaves with normal adaxial/abaxial polarity but with a specific disruption in the adaxial domain, leading to formation of ectopic leaf blades on the lateral flanks of vascular ribs. Bars = 40  $\mu\text{m}$  in (B), 20  $\mu\text{m}$  in (C), 100  $\mu\text{m}$  in (D), and 80  $\mu\text{m}$  in (E) to (I).

(A) Ectopic leaf blades (EB) emerge at the junction between the primary blade (PB) and the midrib and show a fixed polarity, the adaxial surface facing away from the rib.

(B) Transverse sections of ectopic blades show differentiation of palisade (ectopic blade palisade, ebp) and lateral veins (ectopic blade vein, ebv).

(C) and (D) Scanning electron micrograph of transgenic leaf primordia in (C) shows a flattened adaxial surface at the P2 stage, and longitudinal sections in (D) show normal adaxial/abaxial differentiation, indicating that leaf polarity is normal in the absence of *NSPHAN*. ad, adaxial; abr, abaxial rib; adb, adaxial blade; mv, midvein.

(E) The primary disruption in transgenic leaves is abnormal proliferation and lack of palisade differentiation in the upper mesophyll beginning at stage P5, which forms a continuous pavement over the midvein (mv) and lateral veins (lv). um, upper mesophyll.

(F) and (G) Corresponding wild-type leaves have palisade initials at this stage in the upper mesophyll (um), which form a single cell layer over lateral veins (lv). mv, midvein.

(H) Where the abnormal upper mesophyll (um) of transgenics abuts adaxial cortex (adc) over the midvein (mv), periclinal cell divisions produce ectopic blade (eb) primordia.

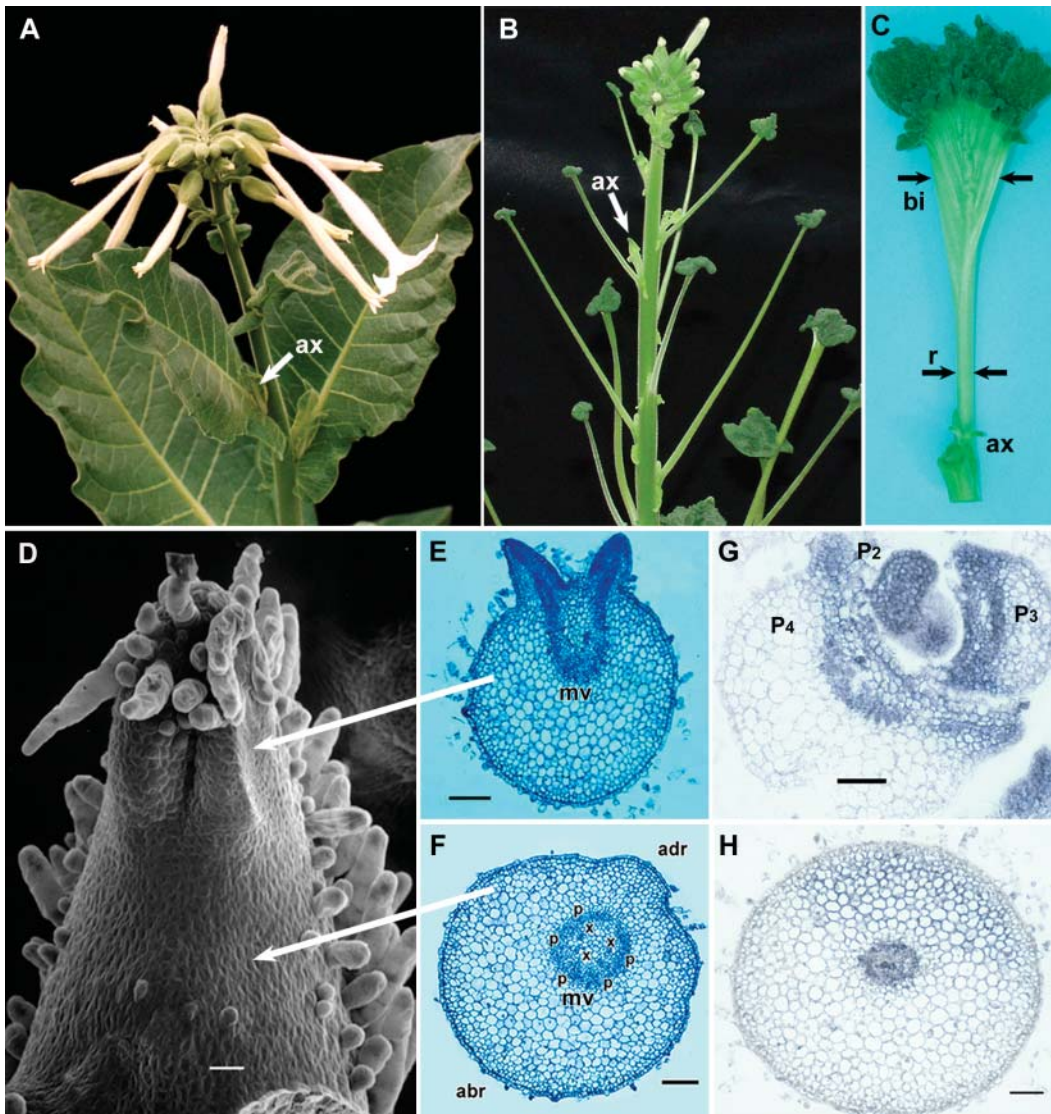
(I) Vascularized blade primordia (ectopic blade vein, ebv) emerge vertically on either side of the midrib, with an adaxial surface (ectopic blade adaxial, ebad) that is contiguous with the upper mesophyll of the primary blade (umpb), thus facing away from the rib.

palisade and abaxial spongy mesophyll (Figures 3A and 3B). Ectopic blades have a fixed polarity, and the adaxial surface is always facing away from the midrib and developing in continuity with the adaxial surface of the primary blade.

### Adult Antisense *NSPHAN* Leaves

Wild-type *N. sylvestris* (Figure 4A) has a simple elliptical leaf 30 to 50 cm at maturity with blade-bearing petioles. In the adult vegetative phase, antisense *NSPHAN* transgenics produce

needle-like leaves with bladeless petioles and a peltate blade at the tip (Figure 4B), directly analogous to the phenotype observed in *phan* mutants of *Antirrhinum* (Waites and Hudson, 1995). In contrast with *Antirrhinum*, however, there is morphological and molecular evidence that bladelessness may not be associated with an outright loss of adaxial/abaxial polarity. Transgenic leaves always produce axillary meristems in the normal position on the adaxial surface of the petiole at the leaf/stem junction (Figure 4B), suggesting that adaxial identity is unaltered at this location. In addition, transgenic leaves show



**Figure 4.** Adult Antisense Phenotypes.

**(A)** and **(B)** Wild-type **(A)** and antisense *NSPHAN* transgenics **(B)** in the adult vegetative phase of growth, with axillary meristems (ax) on the adaxial surface of the petiole at the leaf/stem junction. Adult antisense leaves have radialized petioles lacking leaf blades except at the leaf tips.

**(C)** Antisense leaves formed during the transition from juvenile to adult phase are radialized only at the base of the petiole (r) but are still bladeless in more distal regions where bilateral symmetry is retained (bi). ax, axillary meristem.

**(D)** and **(E)** Adult antisense primordia show blade formation only at the leaf tip, in an abnormal position shifted toward the front of the primordium, in association with abnormal development of the midvein (mv) **(E)** in a closed U shape.

**(F)** The base of the antisense primordium lacks blade formation and has a radialized midvein (mv) closed into a circular structure with phloem (p) surrounding xylem (x).

**(G)** In situ hybridization with an antisense *PHAVOLUTA* probe in wild-type shoot apex shows expression throughout adult leaf primordia in the P2 stage. In P3 to P4 primordia, *PHAV* mRNA is localized to the midvein and adaxial regions of the leaf.

**(H)** *NSHAV* shows a normal pattern of expression in the adaxial domain and in midveins at the base of radialized antisense leaves at the P5 stage.

Bars = 20  $\mu$ m in **(D)** and 80  $\mu$ m in **(E)** to **(H)**.

that bladelessness and radialization are actually separate events. This is particularly evident in leaves formed during the transition from juvenile to adult vegetative growth (Figure 4C). The petiole is bladeless, and the vascular system is radialized at its base, but blade loss is also seen in more distal positions where

the leaf is still bilaterally symmetrical. Here, the leaf is a contiguous span of rib-like cortex associated with lateral veins, and blade tissue appears only at the tip. In adult leaves, scanning electron micrographs show that initiation of lamina occurs only at the leaf tip and in a position shifted dramatically toward the front

of the primordium (Figure 4D). Midvein development is shifted in a similar manner, forming a U-shaped structure extending directly to the adaxial surface (Figure 4E), as opposed to the usual open crescent in wild-type primordia (Figure 4G). In bladeless regions at the base of transgenic primordia, midveins are often fully closed into circular structures with phloem surrounding xylem and do not extend to the surface of the primordium (Figure 4F). This midvein phenotype also occurs in *phan* mutants of *Antirrhinum* (Waites and Hudson, 1995) and was considered to result from a restriction (tip) and eventual elimination (base) of the adaxial leaf domain. Normal formation of axillary meristems in our transgenics was at odds with this interpretation, so we examined *in situ* expression of *PHAVOLUTA*, a molecular marker for the adaxial leaf domain in *Arabidopsis* (McConnell et al., 2001). The *Nicotiana* homolog of *PHAVOLUTA* (*NSPHAV*) shows the expected expression pattern in the wild type, throughout P1 and P2 primordia and becoming restricted to the midvein and adaxial domain by the P3 stage. The bladeless region at the base of transgenic P5 primordia (Figure 4H) revealed a normal pattern of *NSPHAV* expression in the adaxial domain, consistent with the eventual formation of axillary meristems in this location.

### Ectopic Expression of *NTH20*

Because loss of *PHAN* function is associated with ectopic expression of class I *KNOX* genes in other plants, we conducted a preliminary RT-PCR expression analysis in juvenile and adult leaves of antisense transgenics (data not shown), focusing on the class I *KNOX* genes *NTH15* (*STM*-like) and *NTH20* (*KNAT1*-like) (Nishimura et al., 1999). Patterns of *NTH15* expression were indistinguishable in wild-type and transgenic samples, including the SAM, and there was no evidence for ectopic *NTH15* in antisense leaves. By contrast, there was evidence of ectopic *NTH20* expression in juvenile and antisense transgenic leaves, as observed in *as1* mutants of *Arabidopsis*, where *KNAT1/BP* is misexpressed in leaves and *STM* is not (Byrne et al., 2000; Ori et al., 2000). To determine the location of ectopic *NTH20* expression, digoxigenin-labeled antisense probes were applied to sections through the shoot apex and developing leaves of transgenic plants and wild-type controls. Wild-type sections revealed *NTH20* mRNA in the SAM (Figure 5A), primarily in the peripheral zone adjacent to emerging leaf primordia and in the vascular traces of the stem, as previously reported for *N. tabacum* (Nishimura et al., 1999). *NTH20* mRNA was not observed in wild-type P2 to P5 leaf primordia (Figure 5B), except in the basal region of the petiole at the P7 stage, where it is observed in the midvein and in the axillary meristem (Figure 5C). In adult transgenic primordia, sections from the tip (Figure 5D) and bladeless base (Figure 5E) had ectopic *NTH20* mRNA in the vascular tissue and in cortical cells of the midrib. Longitudinal sections through the juvenile apex of transgenics show *NTH20* mRNA in its normal location in vascular traces of the stem and ectopic expression in leaf primordia, particularly on the adaxial surface (Figure 5F). There was no signal above background with the sense probe in wild-type or transgenic tissue (data not shown).

### Suppression of the Juvenile Phenotype with GA

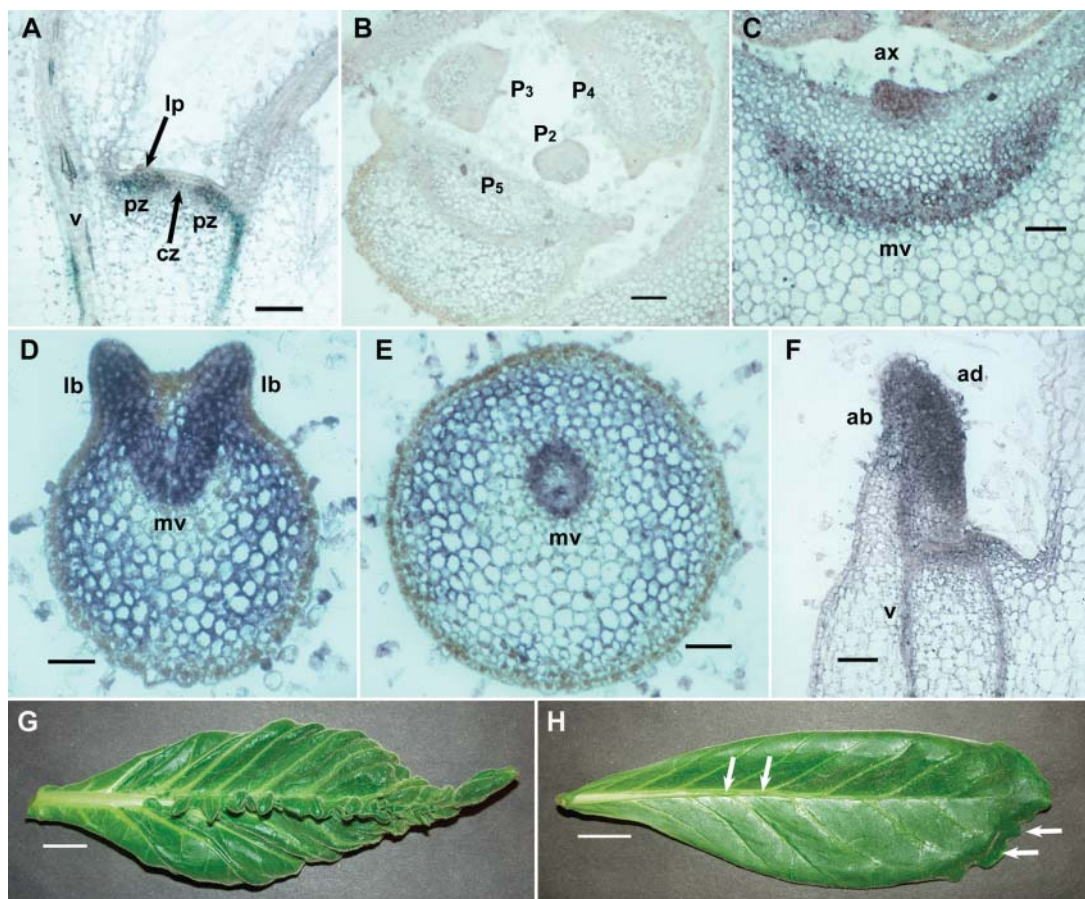
Misexpression of class I *KNOX* genes in the leaves of *Arabidopsis* (Hay et al., 2002) and *Nicotiana* (Tanaka-Ueguchi et al., 1998; Sakamoto et al., 2001) suppresses the expression of GA20 oxidase genes, blocking the final step in synthesis of active GA. In both species, this results in aberrant leaf phenotypes, which are partially or fully reversible by application of GA. To test whether antisense *NSPHAN* phenotypes are caused by ectopic *KNOX*, we applied GA to the leaves of transgenic plants in the juvenile and adult phases of growth. GA was applied daily to the leaves of both heterozygous and homozygous AF18B.1 transgenics to test for reversal of a moderate versus strong antisense phenotype. The treatment had little effect on the strong phenotype of homozygous transgenics but produced nearly a full reversal of the juvenile leaf phenotype in heterozygotes, suppressing the downward curling of the lamina at the tips and margins and blocking formation of ectopic leaf blades (Figures 5G and 5H). By contrast, GA application had no discernible effect on radialization or bladelessness in the petioles of adult heterozygotes or homozygotes (data not shown).

### DISCUSSION

Sequence conservation among *PHAN* orthologs predicts a conserved function in dicots, with all evidence pointing to *KNOX* repression, but disparate loss of function phenotypes in different species left considerable uncertainty as to the role this played in leaf development. Our results show that even within a single species, *PHAN* is regulating different aspects of development in different phases of growth. Analysis of *NSPHAN* function in the juvenile phase was most revealing; the pattern of expression and the loss of function phenotype demonstrated a novel role for *NSPHAN* in development of the blade palisade, with no direct relation to leaf polarity. Results suggest that *NSPHAN*, acting via *KNOX* suppression, regulates GA-dependent patterns of cell proliferation critical to palisade formation. Adult leaves, on the other hand, respond to loss of *NSPHAN* in an entirely different manner. These leaves are bladeless, and their midveins are severely abaxialized, seemingly consistent with loss of adaxial/abaxial polarity, yet other morphological and molecular criteria for polarity remain intact. We discuss this mosaic phenotype and offer an alternative explanation based on a *KNOX*-mediated displacement of stem-like features into leaf petioles.

### Adult Phase: Radial/Bilateral Mosaics

Loss of *PHAN* function in *Antirrhinum* (Waites and Hudson, 1995), tomato (Kim et al., 2003), and, here, in *Nicotiana* produces radialization and loss of blade formation in basal regions of the petiole. One interpretation is that this results from a loss of adaxial identity (abaxialization) in emerging leaf primordia, but certain morphological and molecular observations in antisense *Nicotiana* transgenics are inconsistent with this. Studies in *Arabidopsis* show that the onset of adaxial identity and competence to form axillary meristems are strictly correlated events, each bearing a common connection to expression of the *HD-ZIPIII* genes *PHABULOSA*, *PHAVOLUTA* (McConnell and



**Figure 5.** *NTH20* Expression Analysis.

In situ hybridization of an *NTH20* antisense probe in wild-type and antisense *NSPHAN* transgenics. Bars = 100  $\mu\text{m}$  in (A) and (F), 80  $\mu\text{m}$  in (B) and (C), 50  $\mu\text{m}$  in (D), 60  $\mu\text{m}$  in (E), and 2 cm in (G) and (H).

(A) *NTH20* mRNA is detected in the peripheral zone (pz) of the shoot meristem where leaf primordia (lp) are initiated but absent in the central zone of the meristem (cz). v, vascular.

(B) and (C) *NTH20* is not detectable in leaf primordia (B) in stages P2 to P5 but is present near the leaf/stem junction in P7 primordia (C) where axillary meristems (ax) are formed. mv, midvein.

(D) and (E) Ectopic expression of *NTH20* is observed in adult antisense primordia, both in the midvein and in the surrounding rib cortex. lb, leaf blade; mv, midvein.

(F) Ectopic *NTH20* is also observed in leaf primordia of juvenile antisense transgenics, particularly on the adaxial surface (ad) and in its normal location in vascular traces (v) in the adjoining stem. ab, abaxial.

(G) and (H) The curled lamina and ectopic blade phenotype in juvenile leaves of heterozygous transgenics (G) is strongly suppressed by application of GA as shown in (H). Arrows show vestigial ectopic blades on the midrib and remnants of disorganization at the leaf tip.

Barton, 1998; McConnell et al., 2001), and *REVOLUTA* (Talbert et al., 1995; Otsuga et al., 2001; Emery et al., 2003). Loss of adaxial identity therefore predicts loss of axillary meristems, and this does not occur in antisense *NSPHAN* transgenics. Adult petioles always produce axillary meristems on the adaxial surface and show expression of the adaxial marker gene *PHAVOLUTA* in this domain. But if adaxial identity is intact in these leaves, why are the midveins radialized, particularly toward the base of the petiole, where they roll inward toward the shoot, often into fully circularized structures? Although this is an aberrant pattern of vascular development in the leaf itself, wild-type midveins show this pattern of proliferation at the leaf/stem

junction, turning sharply in an adaxial direction (toward the shoot), facilitating their connection with stem vasculature (Esau, 1977). This points to an alternative explanation for radialized midveins in the absence of *PHAN*, based on displacement of stem-like vascular patterning into leaf petioles. A precedent for this concept is the distal displacement of sheath identity outward into leaf blades, which is observed in maize mutants overexpressing class I *KNOX* genes (Becraft and Freeling, 1994; Schneeberger et al., 1995) and in *rs2* mutants of maize, where loss of *PHAN* produces ectopic *KNOX* (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). This typically extends only a certain distance along the leaf axis in

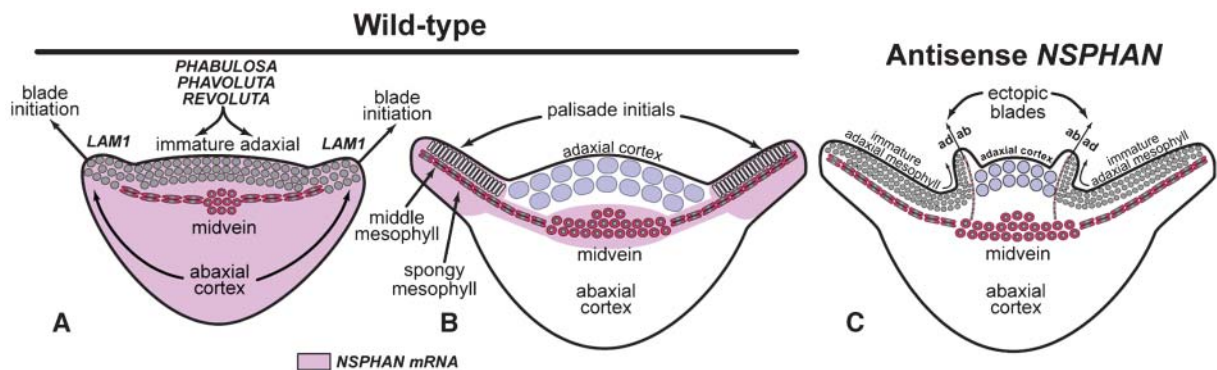


maize, as seen here where there is a proximodistal gradient for radialization in the petiole. Further support for the idea comes from recent studies in Arabidopsis showing that *KNAT1/BP* functions along with *ERECTA* (Douglas et al., 2002) and *PENNYWISE* (Smith and Hake, 2003) to radialize internodes and to promote internode elongation. *NTH20* (*KNAT1/BP* homolog) most likely plays a similar role in tobacco. In the absence of *NSPHAN*, an extension of *NTH20* expression into leaf petioles may promote a radial pattern of vascular growth normally confined to nodal and internodal regions of the stem. Bladelessness itself appears to have no direct link to radialization because leaves formed during the juvenile to adult transition show blade loss in regions with bilateral symmetry (Figure 4C). A revealing observation is that bladeless leaves in the late adult phase are highly elongated, spindly structures (Figure 4B), often reaching twice the length of wild-type leaves (data not shown). This coincides with rapid elongation of stem internodes at this stage, providing further evidence that petioles have adopted a stem-like pattern of development. The phenotype is also consistent with misexpression of *NTH20* in the petiole, given the role of Arabidopsis *KNAT1/BP* in internode elongation (Douglas et al., 2002).

#### Juvenile Phase: Ectopic Blade Formation

Loss of *NSPHAN* function produces a very different result in the juvenile phase, where leaves are neither radialized nor bladeless. Leaf primordia have normal polarity at inception and generate polarized leaf blades in the normal position at the adaxial/abaxial junction. The primary disruption is a highly disorganized upper

mesophyll and vertical outgrowths of ectopic lamina along the flanks of major leaf veins. A similar phenomenon was observed in the first three pairs of juvenile leaves in *phan* mutants of Antirrhinum (Waites and Hudson, 1995). Extra mesophyll cell layers were observed in transverse sections, and ectopic leaf blades grew vertically from the upper surface of the primary blade, though in this case apparently bordering regions where sporadic default to abaxial identity produced an ectopic juxtaposition of adaxial and abaxial identities. Thus, formation of ectopic blades was considered a reiteration of the basic events leading to blade initiation at the adaxial/abaxial junction in young leaf primordia. In *Nicotiana* transgenics, ectopic blades emerge exclusively along the flanks of vascular ribs on the upper leaf surface, where there is no evidence of default to abaxial identity. Juvenile midribs have normal vascular polarity (adaxial xylem and abaxial phloem), suggesting that the upper cortex has adaxial identity. It is possible that ectopic blades in *Nicotiana* do not arise by reinitiation per se, but rather as aberrant extensions of the primary blade. What argues against this, however, is that ectopic blades emerge in the adaxial domain of the leaf, yet reestablish all adaxial and abaxial lamina cell types. By this criterion, ectopic blades appear to arise by de novo reinitiation. Thus, we propose the adaxial mesophyll of transgenic leaves is suspended in a partially indeterminate state by ectopic *KNOX*, where it retains the capacity for lateral outgrowth of blade primordia. The observed delay in differentiation of the palisade is certainly consistent with this idea, but the question remains as to why ectopic outgrowths would occur in the absence of an adaxial/abaxial boundary. Lateral outgrowth of blade primordia occurs here at a juxtaposition of dissimilar cell types, immature



**Figure 6.** Blade Formation.

**(A)** Adaxial leaf identity is a developmental state associated with expression of *HD-ZIPIII* genes (*PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA*) in the meristem and on the adaxial surface of adjoining leaf primordia. Lateral outgrowth of blade primordia occurs where this adaxial tissue encounters the abaxial cortex on the lateral flank of the primordium in a process requiring the function of *LAM1*. *NSPHAN* is expressed throughout the primordium in this early phase.

**(B)** The emerging lamina differentiates along an adaxial/abaxial axis of polarity, producing an adaxial palisade, an abaxial spongy parenchyma, and a vascularized middle mesophyll separating these domains. *NSPHAN* is initially expressed throughout emerging blade primordia and then becomes localized to the middle mesophyll during lamina expansion. *NSPHAN* via *KNOX* suppression maintains determinacy in the expanding lamina and sustains the regulated patterns of cell proliferation critical to palisade development in the upper mesophyll.

**(C)** In the absence of *NSPHAN*, a polarized lamina is generated in the normal position, but the adaxial mesophyll appears suspended in an immature state, showing disorganized patterns of proliferation, delayed differentiation of palisade, and de novo reinitiation of polarized blade primordia along the flanks of the midrib. This is correlated with ectopic expression of the class I *KNOX* gene *NTH20*. GA suppression of the phenotype establishes a causal connection to ectopic *KNOX* and a requirement for GA in palisade development. ab, abaxial; ad, adaxial.

adaxial mesophyll with adaxial rib cortex, without regard to polarity per se. This is actually consistent with current models of blade initiation because these cell types (mesophyll and cortex) are in fact juxtaposed at the adaxial/abaxial boundary in young leaf primordia. A model illustrating this view of blade formation in wild-type and antisense transgenics is presented in Figure 6.

According to current models, early adaxial identity is established by the expression of *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA* in the SAM and on the adaxial surface of adjoining leaf primordia (Figure 6A). Lateral outgrowth of blade primordia occurs on the lateral flanks of the primordium, where this immature adaxial tissue encounters abaxial cortex in a process requiring the function of *LAM1*. *NSPHAN* is initially expressed throughout emerging blade primordia and then localizes to the middle mesophyll, where the function promotes regulated patterns of anticlinal cell division and palisade development in the adaxial mesophyll (Figure 6B). With this process, the adaxial domain loses the capacity for lateral outgrowth where it abuts the adaxial cortex of the midrib. In antisense transgenics, loss of *NSPHAN* leads to ectopic expression of *NTH20* (Figure 6C) and a prolonged period of indeterminacy in the adaxial domain. A polarized lamina is still generated in the normal location (juvenile phase), but the adaxial mesophyll shows highly disorganized patterns of proliferation and retains its capacity for lateral outgrowth where this tissue abuts the adaxial cortex of the midrib and lateral veins. This may not result solely from ectopic *NTH20*, given the functional redundancy of class I *KNOX* genes (Byrne et al., 2002), but the upper mesophyll phenotype is analogous to other *KNOX*-related delays in cell maturation, seen both in the leaves of monocots (Freeling et al., 1992; Becraft and Freeling, 1994; Schneeberger et al., 1995) and dicots (Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996; Jackson 1996). GA suppression of the phenotype likewise points to a causal connection to *KNOX* expression. Thus, it appears that *NSPHAN*, acting via *KNOX* repression, maintains determinacy in the expanding lamina and sustains the regulated patterns of cell proliferation critical to palisade development.

### The GA Connection

Important insights on the leaf/meristem dichotomy have emerged from recent work on *KNOX* regulation of GA metabolism (Sakamoto et al., 2001; Hay et al., 2002). In the shoot apex of tobacco, *Ntc12* (GA20 oxidase) is expressed in the subapical rib meristem of the shoot and in the emerging leaf primordia but not in the corpus of the SAM where its transcription is suppressed by the class I *KNOX* protein *NTH15* (Sakamoto et al., 2001). Downregulation of *NTH15* at leaf initiation sites and the onset of leaf determination is thus associated with induction of GA synthesis. All evidence suggests that *PHAN* orthologs do not condition this initial downregulation of *KNOX* but act immediately afterward to maintain a *KNOX*-off/GA-on status in the primordium. How might GA participate in the onset of determinate cell fates? GA is known to regulate the direction of cell expansion and cell division in plants through the orientation of cortical microtubules (Gunning, 1982; Gunning and Sammut, 1990; Shibaoka, 1994), which serve as templates for the deposition

of cellulose microfibrils. In this manner, cortical microtubules can either enforce a fixed axis of growth or through reorientation impose a new axis, critical components of pattern formation in lateral organ primordia. This would explain the presence of GA in developing organs and conversely its absence in the corpus of the SAM, where indeterminacy is associated with more loosely regulated patterns of proliferation (Sakamoto et al., 2001). When *KNOX* genes are misexpressed in tobacco primordia, whether in *KNOX* transgenics (Sakamoto et al., 2001) or in antisense *NSPHAN* transgenics, regions of the primordium normally characterized by regulated patterns of cell division descend into disorganization. Nowhere is this more evident than in the palisade of the blade mesophyll, which is normally a single layer of cells with a strict pattern of anticlinal division. The disorganized pattern of proliferation in the adaxial mesophyll of transgenic leaves suggests that GA synthesis is a critical component in development of this domain. It is of particular interest that *NSPHAN* mRNA accumulates in the vascularized middle mesophyll layers just below the palisade. Our earlier work with periclinal chimeras had similarly demonstrated that a wild-type middle mesophyll can nonautonomously restore regulated patterns of cell division in a *lam1* mutant palisade (McHale and Marcotrigiano, 1998). This confirms closely related functions for these genes and points to cell layer interaction as a key element in lamina expansion. Further studies on this interplay should advance our understanding of blade development and perhaps tell us more about the overall role of layer communication in plant morphogenesis.

### METHODS

#### Gene Cloning, Transgene Construction, and Molecular Probes

The *MYB* region of the *Antirrhinum majus PHAN* (AJ005586) gene was used as a probe for isolation of a full-length clone (*NTPHAN*; AY559043) from a *Nicotiana tabacum* leaf cDNA library in  $\lambda$ ZAPII. A transgene representing the entire coding region of *NTPHAN* was cloned antisense to the 35S promoter in pFF19 (Timmermans et al., 1990), transferred as a *HindIII/EcoRI* cassette to the pZP 221 binary vector (Hajdukiewicz et al., 1994), and electroporated into *Agrobacterium tumefaciens* strain GV 2260. Cultures were grown overnight at 28°C in 25 mL of liquid YEB medium with spectinomycin (100  $\mu$ g/mL), spun briefly, and resuspended in YEB for inoculation. Leaf discs were taken from wild-type *N. sylvestris* germinated and grown under sterile conditions on MS medium (Sigma, St. Louis, MO) with 2% sucrose. Discs were immersed in the *Agrobacterium* culture for 10 min, blotted and incubated for 48 h on MSR medium (naphthaleneacetic acid at 0.5 mg/L and 6-benzylaminopurine at 1.0 mg/L), and transferred to MSR with timentin (200  $\mu$ g/mL) for suppression of *Agrobacterium*. After 5 d, discs were transferred to MSR with timentin (200  $\mu$ g/mL) and gentamycin (100  $\mu$ g/mL) and incubated for 3 to 4 weeks for selection of transformed tissue. Organized shoots were transferred to an MSO medium (no hormones) with timentin (200  $\mu$ g/mL) and gentamycin (100  $\mu$ g/mL) for growth and rooting of transformed shoots. Plants were transferred to soil, and 25 transgenics were identified by DNA gel blot analysis. Three transgenics (AF6J.4, AF434.1, and AF18B.1) with single insertions producing strong phenotypes were selected for further analysis.

Gene-specific subclones for RNA gel blot and in situ analysis were generated from *N. sylvestris* mRNAs by RT-PCR using primers based on the *N. tabacum* genes. A gene-specific *NSPHAN* fragment (pNAM 5.2.3)

representing the C terminus and 3' UTR was amplified with the following primers: forward, 5'-AGCAGAAGGCAACTCTAGACAGGA-3'; reverse, 5'-CAGATACTAGAAATACAAGTTGCA-3'. Primers based on the *N. tabacum* *NTH20* gene (AB025714) (forward, 5'-TCTTTGATTTAGGGTTGCCGATC-3'; reverse, 5'-GCTTGAGATTGATCATAACAATCGC-3') were likewise used to amplify a gene-specific fragment representing the 5' UTR and N terminus. The *PHAVOLUTA* gene was isolated from *N. sylvestris* (*NSPHAV*; AY560320) by genomic PCR based on homology to the *Arabidopsis thaliana* gene (*ATHB9*; AV10922), and primers amplifying the C-terminal coding region beginning at exon 16 and including the 3' UTR (forward, 5'-CGGAGTTATAGAGTGCACACAGG-3'; reverse, 5'-AAATATGATATAGATTAGGATCCATCCAC-3') were used to isolate a gene-specific cDNA fragment by RT-PCR. All fragments used for synthesis of RNA probes were cloned into pCR TOPO II vectors (Invitrogen, Carlsbad, CA). Digoxigenin-labeled probes were synthesized from SP6 or T7 promoters in linearized TOPO vectors according to the DIG RNA labeling protocol (Roche Molecular Biochemicals, Indianapolis, IN).

### Histology and Probe Synthesis

Plants for histological studies and in situ hybridizations were grown at 27°C under continuous illumination in Metro-Mix 360 (Scotts-Sierra, Marysville, OH) in a Percival growth chamber. Samples were fixed in FAA (50% EtOH, 5% acetic acid, and 3.7% formaldehyde), embedded in Paraplast X-TRA (Oxford Labware, St. Louis, MO), and sectioned at a thickness of 8 µm. For light microscopy, wax sections were stained in 0.1% toluidine blue and photographed with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY). Samples for scanning electron microscopy were fixed in FAA, transferred to 100% ethanol, critical point dried with liquid carbon dioxide in a Polaron pressure chamber (Hertfordshire, UK), sputter coated with gold, and photographed with an ISI-SS40 scanning electron microscope (International Scientific Instruments, Prahran, Australia). Samples for in situ hybridization were fixed in FAA, sectioned at 8 µm, affixed to Probe Plus slides (Fisher Scientific, Loughborough, UK), and processed as described in <http://www.wisc.edu/genetics/CATG/barton/protocols.html> with color substrate incubation overnight.

### RNA Gel Blots

Total RNA samples (15 µg) were fractionated in formaldehyde gels and transferred to nylon membranes as described (McHale et al., 1990) and hybridized with <sup>32</sup>P-UTP-labeled RNA probes synthesized from pCR II TOPO vectors according to the Roche RNA labeling protocol. The transgene probe was pRH81, which represents the 35S poly(A) tail from pFF19. The *NTPHAN* probe pNAM 5.2.3 was as described for in situ hybridization.

### RT-PCR

Total RNA samples were isolated for reverse transcription and PCR amplification by the RNAqueous-4PCR protocol (Ambion, Austin, TX). RNA (2 µg) was reverse transcribed at 42°C for 1 h, and 1-µL aliquots were amplified according to the RETROscript PCR protocol (Ambion) for 30 cycles.

### GA Treatment

GA (G7645; Sigma) was applied daily as a foliar spray (20 µg/mL) to heterozygous and homozygous AF18.B1 antisense *NSPHAN* transgenics (juvenile and adult phase) grown at 27°C under continuous illumination in Metro-Mix 360 (Scotts-Sierra) in a Percival growth chamber.

Sequence data from this article have been deposited with the GenBank data library under the following accession numbers: *NTPHAN*

(AY559043), *PHANTASTICA* (AJ005586.1), *LePHAN* (AF148934), *AS1* (NM129319), *NTH20* (AB025714), *NTH15* (AB004785), *NTH23* (AB004787), and *NSPHAV* (AY560320).

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