Analysis of the Competence to Respond to KNOTTED1 Activity in Arabidopsis Leaves Using a Steroid Induction System[1]

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Expression of *KNOX* (*KNOTTED1*-like homeobox) genes in the shoot apical meristem of Arabidopsis is required for maintenance of a functional meristem, whereas exclusion of *KNOX* gene expression from leaf primordia is required for the elaboration of normal leaf morphology. We have constructed a steroid-inducible system to regulate both the amount and timing of *KN1* (*KNOTTED1*) misexpression in Arabidopsis leaves. We demonstrate that lobed leaf morphology is produced in a dose-dependent manner, indicating that the amount of KN1 quantitatively affects the severity of lobing. The KN1-glucocorticoid receptor fusion protein is not detected in leaves in the absence of steroid induction, suggesting that it is only stable when associated with steroid in an active state. By using a second inducible fusion protein to mark exposure of leaf primordia to the steroid, we determined the stage of leaf development that produces lobed leaves in response to KN1. Primordia as old as plastochron 7 and as young as plastochron 2 were competent to respond to KN1.

Leaves arise from the shoot apical meristem (SAM) at regular intervals of time termed plastochrons. An Arabidopsis leaf consists of a distal lamina and proximal petiole with a pair of stipules flanking the leaf base. Like most plants, Arabidopsis exhibits heteroblasty such that juvenile rosette leaves have small, round lamina with smooth margins, and adult leaves have larger, elongate lamina with serrated margins. Cauline leaves differ from rosette leaves by lacking a petiole, and the lamina tends to be narrower.

Molecular evidence of the transition from meristem to leaf identity comes from a change in the expression of *KNOX* (*KNOTTED1*-like homeobox) genes. In Arabidopsis, the class I *KNOX* genes *STM* (*SHOOT-MERISTEMLESS*), *KNAT1* (*KNOTTED1*-like in Arabidopsis1), and *KNAT2* are expressed in the SAM but excluded from cells destined to become leaf primordia (Lincoln et al., 1994; Long et al., 1996; Pautot et al., 2001). This expression pattern suggests that KNOX gene products are required for meristem function. Genetic evidence supports such a hypothesis: Loss-of-function mutations in *STM* result in a failure

to initiate or maintain a SAM (Long et al., 1996). In addition, double-mutant analysis between weak *stm* alleles and the *KNAT1* loss-of-function mutation *bp* (*brevipedicellus*) demonstrate that *KNAT1* assumes a redundant role with *STM* in the SAM (Byrne et al., 2002).

Conversely, ectopic expression of *KNOX* genes alters normal leaf morphology by conferring less determinate characters to the leaf. Expression of *KNAT1* driven by the constitutive *CaMV 35S* promoter transforms simple Arabidopsis leaves into lobed leaves with ectopic meristems and stipules (Lincoln et al., 1994; Chuck et al., 1996). Lobed leaf margins with ectopic stipules are produced in both *35S:KNAT2* and DEX-induced *35S:KNAT2-GR* plants (G. Chuck and S. Hake, unpublished data; Pautot et al., 2001). Ectopic expression of *STM* also results in alterations of leaf shape, meristem formation on the adaxial surface of the cotyledons, and growth arrest (K. Barton, personal communication; Williams, 1998; Gallois et al., 2002).

Mutations at two independent loci, *AS1* (*ASYMMETRIC LEAVES1*) and *AS2*, condition misexpression of *KNAT1*, *KNAT2*,and *KNAT6* in Arabidopsis leaves, correlating with lobed leaf morphology (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). *AS1* encodes a myeloblastosis domain protein and is likely to act as a transcriptional regulator of *KNOX* expression (Byrne et al., 2000). *AS2* is a member of the *LATERAL ORGAN BOUNDARIES* gene family and contains a Leu zipper motif and Cys repeats, suggesting that AS2 may associate with DNA or protein partners to negatively regulate *KNOX* gene expression (Iwakawa et al., 2002; Shuai et al., 2002). Less is known regarding the components that act downstream of KNOX function. In Arabidopsis, KNOX

transcription factors repress the GA biosynthetic gene *AtGA20ox1*, thus promoting low GA conditions favorable for meristematic activity (Hay et al., 2002).

In contrast to Arabidopsis, the dissected leaves of tomato (*Lycopersicon esculentum*) plants express *KNOX* genes. Overexpression of maize (*Zea mays*) *KN1* or tomato *KNOX* genes leads to a dramatic increase in leaf dissection (Hareven et al., 1996; Janssen et al., 1998). These results suggest that dissected leaves have a greater capacity for indeterminate growth than do simple leaves and that differential regulation of *KNOX* genes confers this indeterminacy to the development of dissected leaves. A recent study further supports this idea by demonstrating that *KNOX* expression early in leaf development correlates with formation of complex leaf primordia across a broad spectrum of vascular plants (Bharathan et al., 2002).

To regulate *KN1* misexpression in vivo, a steroid-inducible fusion between the maize *KN1* open reading frame (Vollbrecht et al., 1991) and the steroid-binding domain of the glucocorticoid receptor (GR), driven by the *CaMV 35S* promoter (Lloyd et al., 1995), was constructed and transformed into Arabidopsis. The GR steroid-binding domain maintains the constitutively expressed transcription factor in an inactive state by tethering it in the cytoplasm. This inactivation is reversed by application of the steroid dexamethasone (DEX), which triggers translocation of the fusion protein into the nucleus. As a homeodomain-containing protein, KN1 is expected to function as a transcriptional regulator in the nucleus. The subcellular localization of KN1 in the nucleus (Smith et al., 1992) and its ability to specifically bind DNA support this expectation (Smith et al., 2002).

Here, we demonstrate a dose-dependent response of Arabidopsis leaves to KN1 induction in the production of lobed margins, broad petioles, and arrested growth. We show that the KN1-GR fusion protein is only detected in leaves after DEX treatment. We also define a window of competence in which primordia as old as plastochron 7 (P_7) and as young as P_2 are competent to respond to KN1 by producing lobed margins.

RESULTS

35S:KN1-GR Confers a Lobed Morphology to Arabidopsis Leaves upon DEX Induction

35S:KN1-GR seeds germinated on media containing DEX have perturbed leaf development (Fig. 1, B and D) similar to severe *35S:KNAT1* transformants and DEX-induced *35S:KNAT2-GR* and *35S:STM-GR* plants (Chuck et al., 1996; Pautot et al., 2001; Gallois et al., 2002). In the absence of DEX, *35S:KN1-GR* plants show no phenotypic effects (Fig. 2A). Leaf growth is reduced in DEX-treated *35S:KN1-GR* plants, resulting in a tight rosette of small, lobed
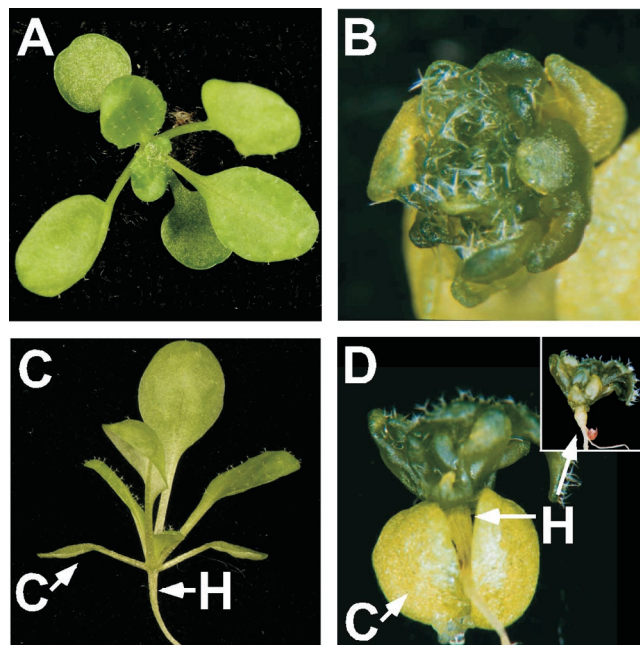

Figure 1. 35S:KN1-GR plants show severe perturbations in leaf development when germinated on DEX-containing media. A, Wild-type top view; B, *35S:KN1-GR* top view; C, wild-type side view, arrows indicate hypocotyl (H) and cotyledon (C); D, *35S:KN1-GR* side view, arrows point to short, thick hypocotyl and epinastic cotyledon. Insert, Hypocotyl with cotyledons removed.

leaves lacking petioles (Fig. 1B). Leaf primordia show very delayed lateral expansion of the lamina, such that primordia appear radial for some time before expansion occurs to produce a lobed morphology. Cotyledons are normal in size and shape but they demonstrate epinastic growth in response to KN1-GR activity. Elongation of the hypocotyl is inhibited, resulting in a short, thick hypocotyl (Fig. 1D). No ectopic shoot meristems were seen at the concentration of DEX used. Wild-type seeds germinated on DEX showed no phenotypic effects (Fig. 1, A and C).

To further investigate the effects of KN1 misexpression on leaf development, we analyzed the response of *35S:KN1-GR* plants to increasing doses of DEX hormone. The effect of the hormone application overlays a heteroblastic leaf series that is identical in *35S:KN1-GR* and wild-type plants grown under long-day conditions. We applied a single dose of increasing concentration of DEX at the time when the first true leaves were visible. This allowed us to assess the effects of KN1 misexpression on the development of juvenile leaves, which have a more entire margin than adult leaves. Lobed leaf morphology was produced in a hormone-dependent manner with a dose-dependent increase in both the number and depth of lobes and the number of leaves affected (Fig. 2, A–F).

To describe the severity of the leaf margin phenotypes, we focused on the depth of the sinuses. We defined serrations as having sinuses less than one-fourth of the distance to the midvein, whereas lobes
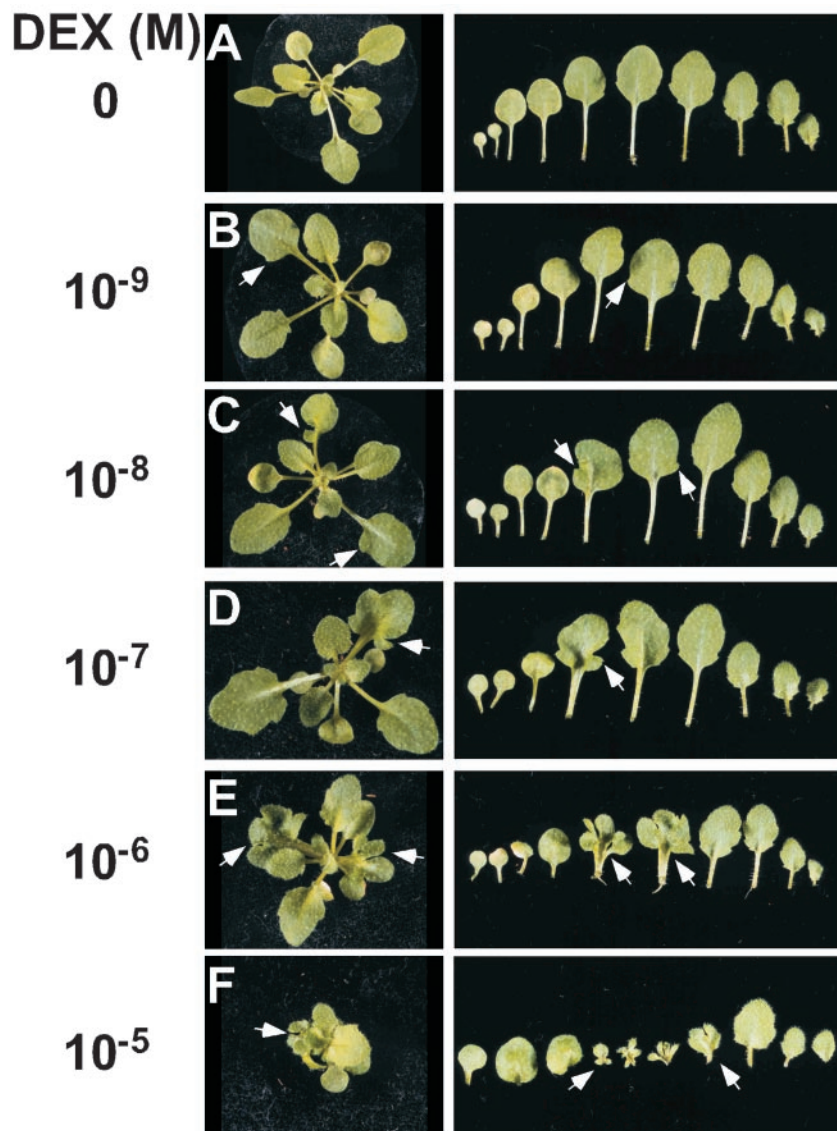
Figure 2. Dose response of *35S:KN1-GR* phenotype. Whole-plant and heteroblastic leaf series from cotyledons to leaf number 8 of *35S: KN1-GR* plants in response to a single application of: A, no DEX; B, 10^{-9} M DEX, arrows point to serration on leaf 4; C, 10^{-8} M DEX, arrows point to serrations on leaves 3 and 4; D, 10^{-7} M DEX, arrows point to lobe on leaf 2; E, 10^{-6} M DEX, arrows point to deep lobes on leaves 3 and 4; and F, 10^{-5} M DEX, arrows point to small, deeply lobed leaves 3 to 6. Only one cotyledon is shown in F. Each panel is representative of 36 plants per treatment.

were defined as having sinuses extending one-fourth or more of the distance to the midvein (Groot and Meicenheimer, 2000). Application of low concentrations of DEX (10^{-9} and 10^{-8} M) results in single, conspicuous serrations on juvenile *35S:KN1-GR* leaves (arrows, Fig. 2, B and C). At 10^{-7} M DEX, a number of more deeply indented lobes and serrations form on the juvenile leaves and a broader petiole forms in lobed leaves (arrow, Fig. 2D). At 10^{-6} M DEX, deep lobes form resembling leaflets with round lamina and narrow petiolules (arrow, Fig. 2E). The petiole is broader and shorter in these lobed leaves. At 10^{-5} M DEX, reduced growth results in small, highly lobed leaves that lack petioles (arrows, Fig. 2F), similar to those produced when seeds are germinated on 10^{-6} M DEX (Fig. 1, B and D). Four consecutive leaves are fully affected by the induction of KN1 activity with 10^{-5} M DEX, compared with one to two leaves with lower concentrations. These DEX-dependent changes in leaf morphology seen in *35S:*

KN1-GR plants suggest that KN1 is indeed regulating target gene expression upon steroid induction.

Variation in vascular patterning is associated with different leaf morphologies (Nelson and Dengler, 1997). Lobed *35S:KN1-GR* leaves, induced using a single application of 10^{-6} M DEX, show prominent, widely spaced veins on either side of the midvein, correlated with a broad petiole (Fig. 3, D–F). No significant alterations in vascular pattern are apparent in the leaves immediately preceeding or after those that are lobed (Fig. 3, C, G, and H; data not shown), suggesting that changes in vasculature are tightly correlated with the lobed morphology.

KN1-GR Is Stably Expressed Only in the Leaves of DEX-Treated Plants

The induction of activity of a transcription factor, such as KN1, is predicted to alter expression levels of
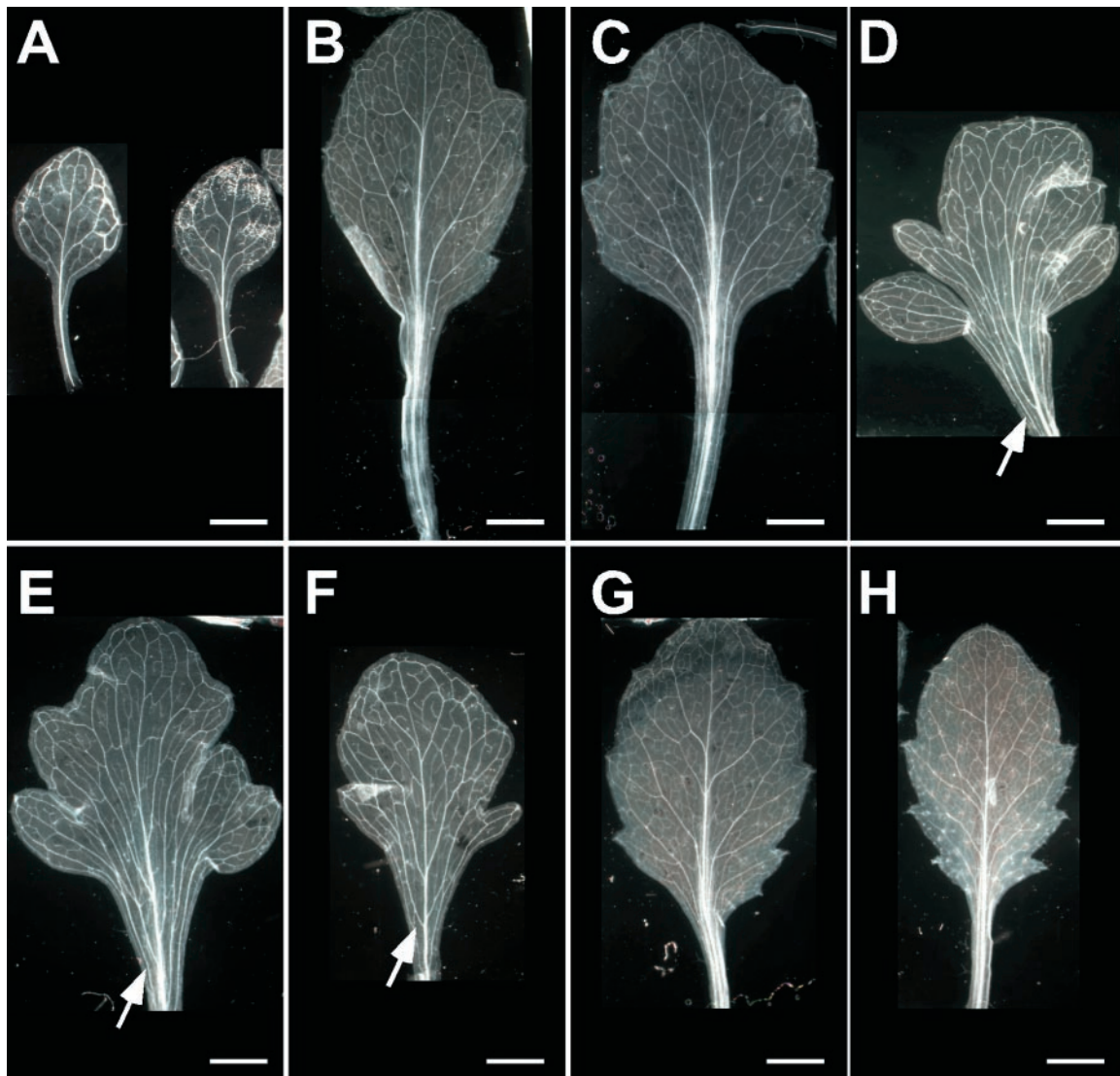
Figure 3. Vascular pattern of leaves 1 to 9 of a mature *35S:KN1-GR* plant after DEX induction. A to C, Leaves 1 to 4 showing a normal vascular pattern in the petiole in which the veins are appressed to the midvein; D to F, leaves 5 to 7 with short, broad petioles; arrows indicate widely spaced veins, not appressed to the midvein; G and H, leaves 8 and 9 with normal vascular pattern in the petiole. Scale bars = 5 mm.

downstream genes. We previously demonstrated that the GA biosynthetic pathway is a downstream component of *KNOX* function in Arabidopsis (Hay et al., 2002). Transcript levels for the GA biosynthetic gene *AtGA20ox1* are reduced in response to *KNOX* misexpression. By inducing KN1 activity for different time intervals in *35S:KN1-GR* seedlings, we established that *AtGA20ox1* expression is reduced within 30 min of DEX application (Fig. 4, lanes 4 and 8; Hay et al., 2002). This rapid repression of *AtGA20ox1* expression suggests that it may be directly regulated by KN1, similar to the direct interaction between the tobacco (*Nicotiana tabacum*) KNOX protein, NTH15, and *Ntc12* (Sakamoto et al., 2001). We tested this hypothesis by analyzing *AtGA20ox1* expression after DEX induction in the presence of the protein synthe-

sis inhibitor CYC. The effect of CYC was monitored by expression of the CYC-inducible gene, *IAA1* (Fig. 4; Abel et al., 1995). The presence of CYC inhibits the repression of *AtGA20ox1* transcription by KN1-GR (Fig. 4, lanes 3 and 7).

The inability of KN1-GR to repress *AtGA20ox1* transcription in the absence of protein synthesis suggests that the regulation of *AtGA20ox1* is indirect, or that the KN1-GR fusion protein is rapidly degraded in the absence of steroid binding. The stability of the KN1-GR fusion protein was investigated by western-blot analysis. Protein was extracted from leaves of wild-type plants and *35S:KN1-GR* plants that had been treated with either 10^{-6} M DEX or a control treatment. Forty micrograms of each protein sample was blotted and immunoreacted with antibodies
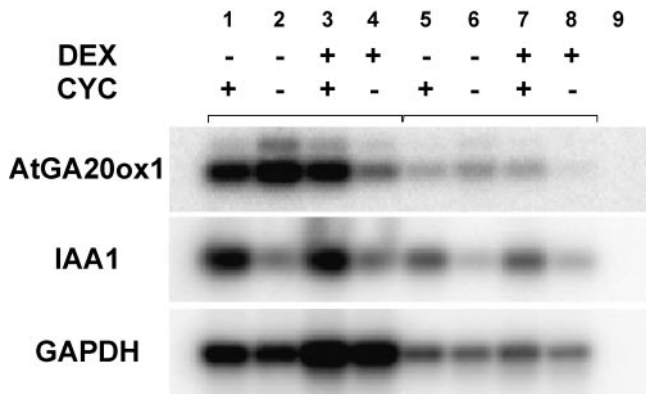
Figure 4. Reverse transcriptase-PCR gel-blot analysis of *AtGA20ox1* expression in *35S:KN1-GR* seedlings 30 min after DEX and/or cyclohexamide (CYC) induction. Lane 1, CYC application only; lane 2, surfactant application only; lane 3, both DEX and CYC application; lane 4, DEX application only. Lanes 5 to 8, as for lanes1 to 4 with PCR performed using one-tenth dilution of cDNA. Lane 9, PCR performed with no cDNA. *IAA1* expression is induced in response to CYC, and *GAPDH* expression indicates a similar abundance of cDNA in each sample.

raised against either KN1 or GR (Fig. 5, lanes 1–10). The KN1 antibody recognizes a 44-kD protein in an extract from bacteria that express a recombinant KN1 protein (Fig. 5, lane 5). A 76-kD protein that corresponds to the full-length KN1-GR fusion protein is detected by both anti-KN1 and anti-GR antibodies in the DEX-treated *35S:KN1-GR* sample (Fig. 5, lanes 2 and 7). No protein is detected in either wild-type or untreated *35S:KN1-GR* leaves (Fig. 5, lanes 1, 3, 6, and 8).

The absence of KN1-GR protein in untreated *35S: KN1-GR* leaves suggests that the fusion protein is only stable in association with DEX. Perhaps KN1-GR is only stable upon translocation to the nucleus after DEX treatment and is unstable in the cytoplasm. Instability in the cytoplasm, however, is not true of all KN1 fusion proteins. The biologically active fusion between KN1 and green fluorescent protein is stably expressed in both the cytoplasm and nucleus (Kim et al., 2002). The GR steroid-binding domain maintains a transcription factor in an inactive state in the absence of ligand via interactions with a protein complex involving the heat shock protein hsp90 (Picard et al., 1988, 1990; Scherrer et al., 1993). It is possible that KN1-GR is unstable in association with this complex. If KN1-GR is degraded in the cytoplasm, then inhibition of protein synthesis by CYC would limit the amount of fusion protein available for DEX-induced nuclear translocation. It is still possible, therefore, that KN1 directly regulates *AtGA20ox1*, but in the presence of CYC, too little fusion protein is present to have an effect after DEX induction.

Developmental Competence of Leaf Primordia to Form Lobes in Response to KN1

To analyze the competence of primordia to respond to KN1 activity, we marked primordia that had been exposed to DEX using a second DEX-inducible transgene, R-GR. R, a maize basic helix loop helix transcription factor, produces trichomes in a hormone-dependent background when the transgene is expressed in the trichomeless background of

Figure 5. KN1-GR fusion protein is detected only in the presence of DEX. Western-blot analysis detects a 76-kD KN1-GR fusion protein using antibodies raised to both KN1 and GR in *35S:KN1-GR* leaves after DEX application (lanes 2 and 7) but not in the absence of DEX (lanes 1 and 6). No fusion protein is detected in wild-type leaves (lanes 3 and 8). Forty micrograms of total protein of each sample was loaded. A 44-kD recombinant KN1 protein (pKN1) was detected only by the KN1 antibody (lanes 5 and 10). Prestained marker was used to determine protein size (lanes 4 and 9).

ttg (*transparent testa glabrous*; Lloyd et al., 1995). The range of plastochrons that respond to DEX treatment by producing trichomes in *35S:R-GR* plants is wider than that which produces lobes in *35S:KN1-GR* plants, indicating that R-GR is a suitable marker to assess the competence of primordia to respond to KN1. Plants were constructed that contained both *35S:KN1-GR* and *35S:R-GR* transgenes in a *ttg* background. These plants produced both lobed margins and trichomes in response to DEX induction (Fig. 6, A–J). Hence, each leaf of these plants could be scored for exposure to DEX by trichome production and response to DEX-induced KN1 activity by a lobed morphology.

To correlate each mature leaf with its position at the apex at the time of DEX induction, a sample of plants was fixed at the time of induction ($n = 10$). Transverse sections were stained with Toludine Blue-O and the number of primordia initiated was determined. An average of 10.2 ± 0.3 leaf primordia had been initiated at the time of induction, with leaf 10 corresponding to P_0 (Fig. 6K). The remainder of plants was allowed to grow until bolting, and each mature leaf was scored for both trichomes and lobed margins ($n = 13$). A heteroblastic leaf series for a representative plant is shown in Figure 6. Arrows point to trichomes on leaves 3 to 9 (Fig. 6, D–J), indicating that these leaves were exposed to DEX and
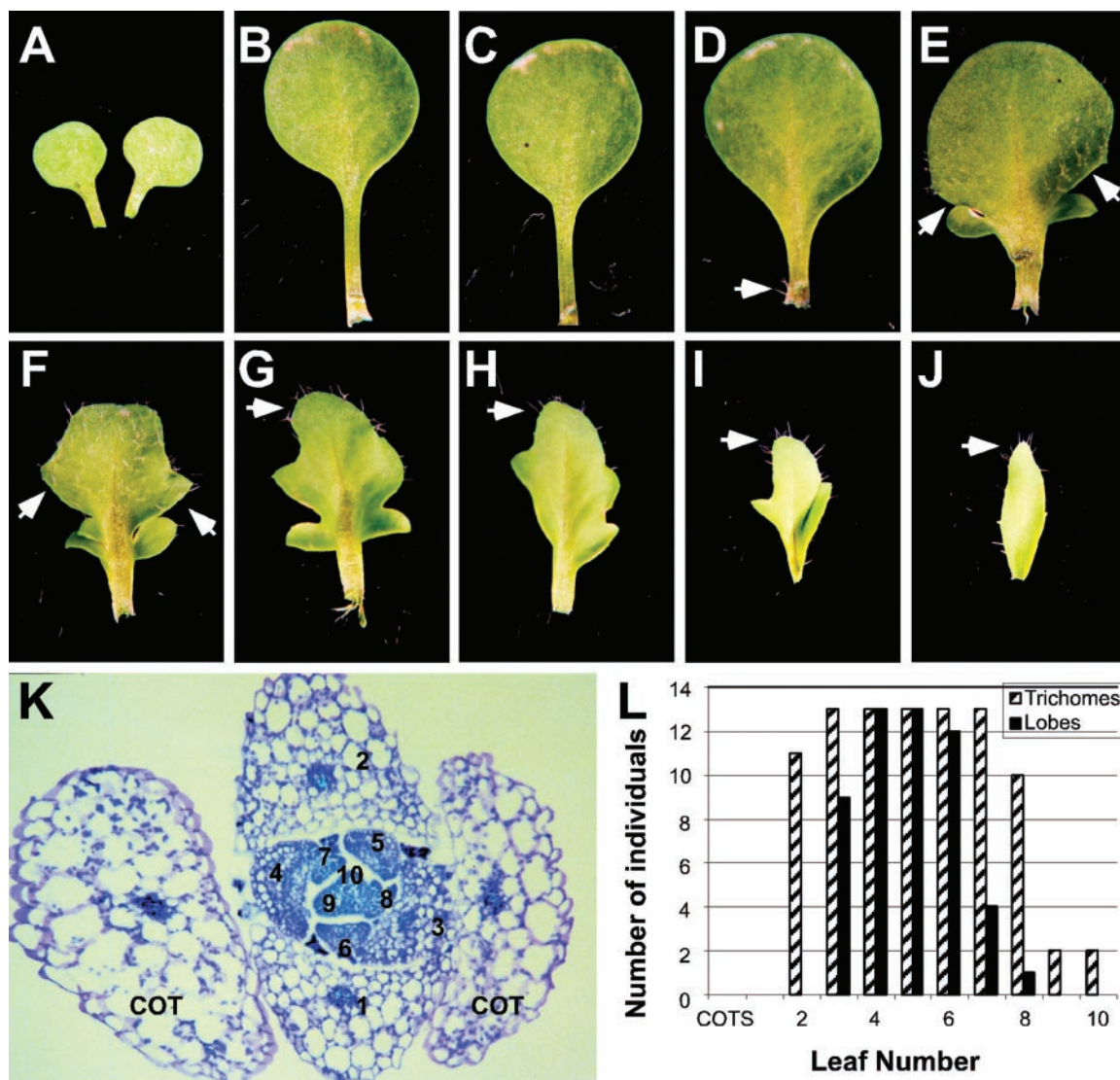
Figure 6. Developmental competence of leaf primordia to respond to KN1 activity. Heteroblastic leaf series of a representative *35S:R-GR;35S:KN1-GR;ttg* plant after DEX induction. A to C, Cotyledons and leaves 1 and 2; D, leaf 3 with entire margin, arrow indicates trichomes at the petiole base; E to I, leaves 4 to 8 with lobed margins, arrows indicate trichomes; J, leaf 9 with entire margin, arrow indicates trichomes. K, Transverse section through the apex of a *35S:R-GR;35S:KN1-GR;ttg* plant at the time of DEX induction, stained with Toluidine Blue O. Mature leaf number is indicated on the corresponding primordia where leaf 10 is at the P_0 stage. L, Graphical representation of the number of *35S:R-GR;35S:KN1-GR;ttg* individuals scored for the presence of trichomes (striped bar) and lobes (black bar) on each leaf number 1 to 10.

competent to produce trichomes. A subset of these leaves (leaves 4–8) produced lobed margins (Fig. 6, E–I), indicating that a window of competence exists for leaf primordia to respond to KN1.

The number of lobes per leaf increases from two lobes in leaf 4 (Fig. 6E) to at least three lobes in leaves 5 and 6 (Fig. 6, F and G). The position of the lobes moves from the base of the lamina in leaf 4 to more distal positions in the lamina in leaves 5 and 6. This may reflect the age of each leaf in terms of the heteroblastic changes in margin shape, or it may reflect a temporal pattern in the competence to respond to KN1, such that the tip of the lamina differentiates and loses competence to respond to KN1 before the base.

Correlating each mature leaf number with its plastochron number at the time of induction reveals the developmental stages of leaf primordia that are competent to respond to KN1 activity. Leaf 3 is the oldest mature leaf that produces a lobed margin in the majority of individuals in this experiment (Fig. 6L). This leaf corresponds to P_7 at the time of induction (Fig. 6K). P_7 is a late stage of leaf development when vascular pattern has been established and basipetal tissue differentiation has occurred (Telfer and Poethig, 1994). Leaf 8 is the youngest leaf that produced lobes in one individual in this experiment (Fig. 6L) and corresponds to P_2 at the time of induction (Fig. 6K). Although P_0 and P_1 are exposed to DEX, indicated by the presence of trichomes on leaves 9 and 10 of some individuals (Fig. 6L), these incipient primordia are not competent to respond to the presence of KN1.

DISCUSSION

We have constructed and characterized a steroid-inducible system to regulate KN1 misexpression in vivo. We show that lobed leaf morphology is produced in a hormone-dependent manner. The KN1-GR fusion protein appears to be unstable in the absence of hormone induction, suggesting that protein degradation may be one mechanism by which KNOX proteins are regulated. We also show that a window of competence exists during which leaf primordia can respond to KN1 activity by producing a lobed margin.

Hormone-Dependent Production of Lobed Leaf Morphology

Increasing concentrations of DEX produce a dose-dependent increase in the severity of the *35S:KN1-GR* phenotype. The number of leaves affected also increases with DEX concentration, perhaps due to higher levels of residual hormone. The increased severity is manifest in the number and depth of lobes, length and width of the petiole, and overall growth of the leaf.

Serrations are produced at the margin in response to low concentrations of DEX and replaced by lobes as the concentration of DEX increases. These results suggest that serrations and lobes are both a consequence of *KN1* misexpression and that the difference between them is due to the relative level of *KN1* activity. The same range of phenotypes, mild serrations to deep lobes, was also seen in independent *35S:KNAT1* transformants, although no correlation with RNA levels could be detected (Chuck et al., 1996). The leaf lamina remains a constant size despite increases in the circumference of the leaf margin with increasing DEX concentrations. This finding supports the hypothesis that lobes result from a lack of growth in the sinus rather than an increase in growth of the lobe (Chuck et al., 1996).

35S:KN1-GR petioles decrease in length and increase in width in response to increasing DEX concentrations. At high concentrations of DEX, leaves do not develop a petiole; instead, they assume a palmate leaf shape with lobes extending directly from the leaf base. A similar leaf shape is seen in *35S:KN1-GR* plants germinated on DEX. Dominant *KNOX* mutants in maize also show a decrease in length and increase in width of the basal half of the leaf (Foster et al., 1999). Analysis of the changes in vascular pattern produced in these deeply lobed *35S:KN1-GR* leaves suggests that the lack of petiole is correlated with precocious radiation of secondary veins out from the midrib. Rather than remaining appressed close to the midvein, secondary veins radiate out toward the lobes almost immediately at the base of the leaf. This change in vascular patterning may indicate that lobes have features of leaves, such that the vascular pattern of the leaf reiterates in each lobe.

A reduction in expansive leaf growth also correlates with increasing concentrations of DEX. The small mature leaves produced in response to high DEX concentrations undergo a period of growth arrest early in development from which they recover and expand to a highly dissected form. This stage of primordia arrest also occurs in *35S:KN1-GR* plants germinated on media containing DEX. A reduction in localized growth, therefore, correlates with the level of *KN1* activity.

A Window of Competence Exists in Which Primordia Respond to KN1

The steroid-inducible system enables regulation of not only the amount of KN1 misexpression but also the timing of misexpression. The ability to mark leaf primordia that have been exposed to DEX by expressing a second DEX-inducible fusion protein in *35S:KN1-GR* plants allowed us to determine which leaf primordia were competent to respond to KN1 activity. Although it is technically possible that different leaf primordia received variable levels of DEX, and that R-GR was able to respond to lower levels of

DEX than KN1-GR, we made the assumption that all leaf primordia could respond to DEX, given that the application was 10^{-6} M and we observed a leaf phenotype even at 10^{-9} M. By determining the number of leaf primordia that had been initiated at the time of DEX-induction, we could correlate mature leaves with their position from the apex at the time of induction.

These experiments revealed that a window of competence exists during which leaf primordia can respond to KN1. Primordia outside of this window, at both earlier and later plastochron stages, are not competent to respond to KN1 by producing a lobed morphology. The window defined in these transgenic lines is quite broad. Primordia as late as P_7 and as early as P_2 are competent to respond to KN1 activity. Thus, KN1 has a similar effect when misexpressed over a continuum of stages of leaf development.

Similarities in lobed morphology between *35S: KNAT1* leaves and the recessive mutants *as1* and *as2* led to the identification of the AS1 and AS2 gene products as regulators of *KNAT1, 2,* and *6* expression in the Arabidopsis leaf (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002). Analysis of the pattern of *KNOX* expression in *as1* and *as2* by in situ hybridization demonstrates that *KNAT1* is negatively regulated in P_0 and P_1 leaf primordia, as seen in normal plants, but is misexpressed in later primordia (Ori et al., 2000). This temporal pattern of *KNOX* misexpression fits within the window of competence defined by *KN1-GR* induction, suggesting that misexpression of any one *KNOX* gene within this window can result in lobed morphology. Loss of all three genes, *KNAT1, 2,* and *6*, is, therefore, likely to be required to suppress the lobed leaf phenotype of *as1* or *as2*.

The inability of P_0 and P_1 leaf primordia to respond to KN1 misexpression is reminiscent of earlier findings. Mutations in both Arabidopsis and maize that result in misexpression of *KNOX* genes all show correct down-regulation of KNOX proteins in P_0, even when KNOX proteins accumulate as early as P_1 (Schneeberger et al., 1998; Foster et al., 1999). Further, in a broad study of KNOX expression in various vascular plants, all species showed down-regulation of KNOX protein at P_0, but differed in whether KNOX was expressed later in leaf development (Bharathan et al., 2002). This conservation in expression patterns suggests that mechanisms exist to exclude KNOX from the cells of incipient primordia that may be different from mechanisms that negatively regulate KNOX expression in older leaf primordia.

The KN1-GR fusion protein is only stable when associated with DEX in an active state in the nucleus. Therefore, one mechanism to exclude KNOX activity from incipient primordia may be to export KNOX proteins from the nucleus or block their import.

KNOX proteins also contain PEST motifs (Vollbrecht et al., 1991; Nagasaki et al., 2001), which target proteins for degradation via proteolytic pathways. The context in which KNOX proteins are expressed is likely to influence whether these PEST domains are exposed or not. Interactions between KNOX proteins and/or other protein partners (Smith et al., 2002) may regulate exposure of protein degradation motifs.

It is also possible that incipient primordia fail to respond to KN1 for reasons unrelated to the stability or subcellular localization of the protein. The environment of these leaf founder cells is different from that of established leaf primordia. Lobed leaf morphology is thought to involve the juxtaposition of novel boundaries in the Arabidopsis leaf (Ori et al., 2000). Gene products required early in leaf development to specify the initiation of dorsiventral polarity could be involved in specifying such boundaries. Members of the *YABBY* gene family and both *KANADI1* (*KAN1*) and *KAN2* promote abaxial cell fate in Arabidopsis leaves, whereas dominant mutations in both *PHABULOSA* and *PHAVOLUTA* transform abaxial leaf fates to adaxial (Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001). The expression patterns of these genes do not resolve into clear abaxial or adaxial domains until primordia become distinct from the SAM at the P_2 stage (McConnell et al., 2001). Therefore, the domains of gene expression required to establish boundaries with ectopic *KNOX* expression may not exist in P_0 and P_1 primordia. This would suggest that young primordia are not competent to respond to the ectopic *KNOX* expression.

CONCLUSION

Lobed leaf morphology is produced in *35S:KN1-GR* plants in a dose-dependent manner, indicating that the amount of KN1 quantitatively affects the severity of lobing. KN1-GR fusion protein is not detected in leaves in the absence of steroid induction, suggesting that it is only stable in an active state as a transcriptional regulator. P_0 and P_1 leaf primordia are not developmentally competent to produce lobed morphology in response to KN1. This lack of effect at early leaf stages could be due to the stability or subcellular localization of KN1-GR protein in these young primordia, or to a difference in the expression patterns of genes that are required for developmental competence.

MATERIALS AND METHODS

Plasmid construction: *35S:KN1-GR* is a C-terminal fusion of the *KN1* cDNA with the steroid-binding domain of GR from pBI-ΔGR (Lloyd et al., 1995). The cloning was performed by amplifying a PCR fragment using a 5′ primer inside the *KN1* cDNA with the 3′ primer DJKBS2 (tcacggatccccgagc-cggtac), which replaces the *KN1* stop codon with a *Bam*HI site. This fragment was sequenced to confirm fidelity, then cut with *Kpn*I and *Bam*HI and cloned along with an *Xba*I/*Kpn*I *KN1* cDNA fragment cut from pKOC10 into *Xba*I/*Bam*HI sites in the pBI-ΔGR plasmid.

Plant Transformation

The Columbia (Col) ecotype was transformed by the floral dip method using the *Agrobacterium tumefaciens* strain GV3101. A large number of transformants were selected on Murashige and Skoog media containing 50 μg mL^{-1} kanamycin, and at least 20 independent lines were screened on media containing 10^{-6} M dexamethazone. Three lines were pursued, which gave consistent inducible phenotypes. *35S:KN1-GR* transgenic line 46-10 is used as a heterozygote in the Col background for all analyses presented here.

Plant Growth Conditions

Plants were grown to maturity in a greenhouse, and seedlings were grown on DEX plates in a growth chamber. Both greenhouse and growth chamber were set at long day conditions of 16 h of light and 8 h of dark, day temperature was 20°C, and night temperature was 18°.

Chemical Treatments

DEX (Sigma, St. Louis) was dissolved in water to 1 mM stock and applied by spraying at 10^{-6} M concentration, unless otherwise specified, with 0.2% (w/v) silwet surfactant. Control plants were treated with the same concentration of surfactant in water. Plants were treated when the first two leaves were visible and analyzed just before bolting unless otherwise specified. *35S:KN1-GR* and Col plants were germinated on Murashige and Skoog media containing B5 vitamins and 10^{-6} M DEX.

Reverse Transcriptase-PCR Gel-Blot Analysis

One microgram of total RNA extracted using RNeasy (Qiagen USA, Valencia, CA) was used for cDNA synthesis with an oligo(dT) primer and SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA). PCR primers specific for *AtGA20ox1*, *IAA1*, and *GAPDH* amplified products of 359, 508, and 542 bp, respectively, that were detected by Southern hybridization with gene-specific probes. Twenty-one PCR cycles were performed. Primers are as follows: AtGA20ox1-F, gccgtaagtttcgtaacaacatctcc; AtGA20ox1-R, gagagaggcatatcaaagaagcgg; IAA1-F, atggaagtcaccaatgggc; IAA1-R, tcataaggcagtaggagcttcggatcc; GAPDH-F, cacttgaagggtggtgccaag; and GAPDH-R, cctgttgtcgccaacgaagtc.

Histology

Tissue was fixed in 4% (w/v) paraformaldehyde and paraffin embedded as described (Jackson, 1992). Sections were stained with 0.1% (w/v) Toluidine Blue O. Tissue clearing was carried out as described (Aida et al., 1997) and viewed under dark-field microscopy.

Protein Extraction and Western-Blot Analysis

Total protein was extracted from leaves of five 16-d-old plants using the EZ extraction procedure (Martinez-Garcia et al., 1999), which allows protein quantification in the extract using the *DC* protein assay (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of each protein sample was loaded in duplicate, separated by SDS-PAGE on a 10% (w/v) acrylamide gel, and transferred to a nitrocellulose membrane using standard techniques. Western-blot analysis was performed on the duplicate membranes using an antibody raised in rabbit to KN1 at 1:500 (w/v) dilution (Smith et al., 2002) and an antibody raised in rabbit to GR (Amersham, Buckinghamshire, UK) at 1:1,000 (w/v) dilution. Goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was used at 1:5,000 (w/v) dilution, and signal was detected using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color detection.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining permissions will be the responsibility of the requestor.

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