Blind Homologous R2R3 Myb Genes Control the Pattern of Lateral Meristem Initiation in Arabidopsis

Dörte Müller, Gregor Schmitz, and Klaus Theres
Max Planck Institute for Plant Breeding Research, D-50829 Cologne, Germany

In seed plants, shoot branching is initiated during postembryonic development by the formation of secondary meristems. These new meristems, which are established between the stem and leaf primordia, develop into vegetative branches or flowers. Thus, the number of axillary meristems has a major impact on plant architecture and reproductive success. This study describes the genetic control of axillary meristem formation in Arabidopsis thaliana by a group of three R2R3 Myb genes, which are homologous to the tomato (Solanum lycopersicum) Blind gene and were designated REGULATORS OF AXILLARY MERISTEMS (RAX). rax mutants show new phenotypes that are characterized by defects in lateral bud formation in overlapping zones along the shoot axis. RAX genes are partially redundant in function and allow a fine-tuning of secondary axis formation. As revealed by monitoring of SHOOT MERISTEMLESS transcript accumulation, the RAX genes control a very early step of axillary meristem initiation. The RAX1 and RAX3 expression domains specifically mark a cell group in the center of the leaf axil from which the axillary meristem develops. Double mutant combinations of lateral suppressor and rax1-3 as well as expression studies suggest that at least two pathways control the initiation of axillary meristems in Arabidopsis.

INTRODUCTION

The diversity of plant forms observed in nature is the result of the activities of different meristems that are established during postembryonic development. In higher plants, the primary axis of growth as well as the primary shoot and root apical meristems are laid down during embryonic development. During the phase of postembryonic development, secondary axes of growth originate from meristems that are initiated in the axils of leaf primordia. The timing of axillary meristem initiation and the pace of its further development is dependent on the genetic constitution of a plant, its stage of development, and the environmental conditions. Axillary meristems develop into axillary buds, which means that they form several leaf primordia. These buds can either become inactive for periods of varying length or they continue to grow. One cause of suppression of bud outgrowth is due to an inhibitory effect by the primary shoot apex and is termed apical dominance.

In Arabidopsis thaliana, different observations suggest that there is considerable variation in the process of axial meristem formation in different phases of development. During prolonged vegetative development, axillary meristems are initiated in an acropetal sequence at a distance from the shoot apical meristem (SAM) (Hempel and Feldman, 1994; Stirnberg et al., 1999, 2002; Grbic and Bleecker, 2000). In the reproductive phase, however, axillary meristems are formed evenly (Stirmberg et al., 1999, 2002) or in a basipetal sequence (Hempel and Feldman, 1994; Grbic and Bleecker, 2000) along the bolting stem at close proximity to the SAM. Depending on the phase of development, newly formed axillary meristems differ also with respect to their size (Leyser, 2003). Whereas axillary meristems established during vegetative development are very small in relation to the supporting leaf primordium and the primary SAM, those meristems initiated during the reproductive phase are relatively large at their inception. The above observations are also relevant with respect to the question of how axillary meristems originate. One hypothesis, which was termed the detached meristem concept, proposes that axillary meristems are initiated from cell groups detached from the primary apical meristem (Stieves and Sussex, 1989). The alternative concept suggests that axillary meristems originate de novo from partially or fully differentiated cells of the leaf axil (Snow and Snow, 1942; McConnell and Barton, 1998). Analyses of the expression patterns of the shoot meristem marker gene SHOOT MERISTEMLESS (STM) and the axil identity gene LATERAL SUPPRESSOR (LAS) revealed that both genes are coexpressed in the axils of young leaf primordia from the P1 primordium until the formation of the axillary meristem (Greb et al., 2003). These results are in favor of the idea that axillary meristems are formed from cells that are directly derived from the primary SAM and retain their meristematic potential.

In several plant species, including tomato (Solanum lycopersicum), Arabidopsis, rice (Oryza sativa), and maize (Zea mays), mutants have been described that are characterized by a reduction in the number of axillary shoots. The mutant phenotypes result either from a defect in axillary meristem formation (e.g., in Arabidopsis, las [Greb et al., 2003], revoluta [Talbert et al., 1995], and pinhead [McConnell and Barton, 1995]; in tomato, Is [Schumacher et al., 1999] and blind [Schmitz et al., 2002]; in maize, barren stalk1 [Gallavotti et al., 2004]; in rice, monoculum1
[Li et al., 2003] and lax panicle [Komatsu et al., 2003]) or a decrease in the outgrowth potential of axillary buds (in Arabidopsis, auxin resistant3 [Leyser et al., 1996]; in maize, teosinte branched1 [Doebly et al., 1997]). In Arabidopsis, loss of LAS function leads to a suppression of axillary meristem formation during vegetative development. Similar phenotypes have been observed in mutants that harbor mutations in the LAS homologous genes in tomato and rice. Introduction of the Arabidopsis LAS gene into the tomato is mutant led to complete restoration of the wild-type phenotype, indicating that the mechanism of axillary meristem initiation is conserved between these two species (Greb et al., 2003). In the tomato blind (bl) mutant, axillary meristem formation is strongly suppressed during shoot and inflorescence development, leading to a severe reduction in the number of lateral axes. Molecular characterization revealed that the bl phenotype is caused by a loss of function in an R2R3 class Myb gene (Schmitz et al., 2002).

In this study, we have characterized Bl homologous genes in Arabidopsis. Characterization of knockout mutants revealed that different R2R3 Myb genes control the formation of axillary meristems at different developmental times. Double mutant analysis demonstrates that different members of the Bl-related subgroup of R2R3 Myb genes act in a partially redundant fashion to regulate axillary meristem formation. Furthermore, double mutant combinations, including las and one of the myb loss-of-function alleles, suggest that LAS and the Bl homologous genes act in independent control pathways.

RESULTS

Identification of Bl-Related Gene Functions in Arabidopsis

The tomato Bl gene encodes an R2R3 Myb transcription factor that plays a central role in the regulation of axillary meristem initiation during vegetative and reproductive development (Schmitz et al., 2002). To compare the control of axillary meristem formation in sympodial versus monopodial plants, we have searched for a Bl homologous gene function in Arabidopsis. BLAST analysis demonstrated that in Arabidopsis, a subgroup of R2R3 Myb genes encompassing MYB36, MYB37, MYB38, MYB68, MYB84, and MYB87 shows the highest similarity to the tomato Bl gene. Proteins of this subgroup show 76 to 86% sequence identity to Bl within the Myb domain (Schmitz et al., 2002) and are characterized by an additional amino acid between the first and the second conserved Trp residues of the R2 repeat (Stracke et al., 2001). Outside the Myb domain sequence, conservation can only be detected between MYB68 and MYB84. Phylogenetic analysis revealed that the tomato Bl gene is most closely related to the Bl-like sequences Blind-like2 and Blind-like1 from tomato (Figure 1; see Supplemental Figure 1 online).

To identify candidates for Bl-related gene functions within this subgroup, we have analyzed the patterns of transcript accumulation. RT-PCR analysis was performed with total RNA from different plant organs. Transcripts of the genes MYB37, MYB38, MYB84, and MYB87 could be detected in different plant tissues, including the shoot tip (Figure 2). By contrast, MYB36 and MYB68 mRNA was almost exclusively found in the root. Because of its influence on axillary meristem formation and inflorescence development, we expected a gene functionally related to Bl to be expressed in the shoot apex. Based on this rationale, we have selected MYB37, MYB38, MYB84, and MYB87 for further analysis.

Different collections of Arabidopsis insertion lines were screened for knockouts in the genes MYB37, MYB38, MYB84, and MYB87 (see Methods). For MYB37 and MYB38, mutants were identified in different T-DNA insertion collections (Krysan et al., 1999; Alonso et al., 2003). A MYB84 mutant was found in a collection of Arabidopsis plants mutagenized by the maize transposable element Enhancer-1 (En-1) (Baumann et al., 1998; Wisman et al., 1998). Because these genes regulate the formation of axillary meristems (see below), we refer to them as regulators of axillary meristems (RAX) [RAX1 (MYB37), RAX2 (MYB38), and RAX3 (MYB84)]. In coordination with the work described in the accompanying article, the mutant alleles used have been designated rax1-3, rax2-1, and rax3-1, respectively. Insertion points were determined by sequencing diagnostic PCR products obtained from plants homozygous for the respective insertion. The T-DNA insertions in RAX1 and RAX2 are positioned in the third exon and the second intron, respectively, whereas the En-1 insertion in RAX3 is localized in the first exon (Figure 3). As described by Feng et al. (2004), a Ds insertion mutant of MYB68 did not show a visible mutant phenotype. A MYB87 knockout allele is not yet available.

Transcript accumulation of the rax1-3, rax2-1, and rax3-1 insertion alleles was analyzed by RT-PCR using RNA obtained...
from plants homozygous for the T-DNA or En-1 insertions. In each case, primers localized 5’ and 3’ of the insertion did not yield amplification products, whereas two primers localized 5’ of the insertion point led to the amplification of products of the expected sizes (see Supplemental Figure 2 online). These findings demonstrated that in all three cases no functional transcript can be detected. \( rax2-1 \) and \( rax3-1 \) are very likely null alleles, whereas we cannot exclude that the protein encoded by the \( rax1-3 \) allele, which does not contain the last 43 amino acids of RAX1, has a residual activity.

**A Mutation in the RAX1 Gene Affects the Ability to Form Axillary Meristems in the Early Phase of Vegetative Development**

The phenotype of homozygous mutant plants was characterized under long- and short-day conditions. In \( rax1-3 \), phenotypic abnormalities could be observed when plants were grown to maturity under short-day conditions. Different from the wild type, \( rax1-3 \) plants showed a reduction in the number of axillary buds originating from the axils of rosette leaves (Figure 4). Very few of the rosette leaves formed early in vegetative development supported the formation of axillary buds (Figures 4B to 4D). Toward the top of the rosette, the number of axillary buds increased, and a very high proportion of the late rosette leaves produced axillary buds resulting in an acropetal gradient of axillary bud formation. Closer inspection of empty leaf axils with a stereo-microscope and by scanning electron microscopy did not uncover any morphological structures within the empty leaf axils (Figure 4B; see Supplemental Figure 3 online). In addition, removal of the primary bolt did not stimulate the outgrowth of any axillary shoots from these bare leaf axils as it did in wild-type plants. From these experiments, we concluded that the barren leaf axils of \( rax1-3 \) plants were due to a defect in axillary meristem formation rather than a defect in axillary bud outgrowth. During the reproductive phase, the formation of side shoots from the axils of cauline leaves did not deviate from the wild type. The primary bolts of \( rax1-3 \) mutants grew significantly taller than those of wild-type plants (\( rax1-3 \): 34.9 cm ± 1.53, \( n = 15 \); Col: 22.0 cm ± 1.03, \( n = 18 \); errors are standard errors of the mean). In addition, these mutants flowered significantly earlier than the Columbia (Col) wild type (Figure 4A, Table 1). The described phenotypic alterations were strongly diminished or not found when \( rax1-3 \) plants were grown in long photoperiods. The pattern of lateral root development in this mutant did not deviate from the wild type (see Supplemental Figure 4 online).

**RAX2 Affects Accessory Side Shoot Formation during Inflorescence Development**

In comparison with the corresponding wild types, \( rax2-1 \) and \( rax3-1 \) mutants grown either in short or long photoperiods did not show alterations in the pattern of axillary bud formation or any other obvious phenotypic alteration (see Supplemental Figure 5 online). As an alternative test for a possible function of RAX2, we have expressed the RAX2 open reading frame under the control of the cauliflower mosaic virus 35S promoter. The chimeric gene was introduced by \( Agrobacterium tumefaciens \)–mediated transformation into the Col ecotype, and 63 independent transgenic plants containing a single-copy T-DNA insertion each were analyzed for phenotypic alterations. With respect to most aspects of shoot development, 35S:RAX2 plants did not differ from Col wild-type plants. However, they were characterized by a reduction in plant stature (Figure 5A), and the branching pattern
Figure 4. Different Members of the RAX Gene Family Redundantly Regulate Axillary Bud Formation.
Table 1. Flowering Time of rax Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Days to Flowering</th>
<th>Total Leaf Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>rax1-3</td>
<td>80.1 ± 0.7**</td>
<td>60.1 ± 0.8*</td>
</tr>
<tr>
<td>rax2-1</td>
<td>71.8 ± 0.6</td>
<td>37.9 ± 0.6**</td>
</tr>
<tr>
<td>rax3-1</td>
<td>84.1 ± 0.4</td>
<td>61.8 ± 0.7</td>
</tr>
<tr>
<td>rax1-3 rax2-1</td>
<td>75.8 ± 0.9**</td>
<td>46.7 ± 1.1**</td>
</tr>
<tr>
<td>rax1-3 rax2-1 rax3-1</td>
<td>69.8 ± 1.3**</td>
<td>43.6 ± 1.8**</td>
</tr>
<tr>
<td>Col-0</td>
<td>84.3 ± 0.6</td>
<td>62.7 ± 0.6</td>
</tr>
<tr>
<td>Ws</td>
<td>71.6 ± 0.7</td>
<td>40.5 ± 0.5</td>
</tr>
<tr>
<td>Col-0 Ws</td>
<td>78.7 ± 0.6</td>
<td>60.2 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE, and errors are standard error of the mean (n = 20 to 33). Differences between the mutants and the corresponding wild-type plants are significant at the 0.05 > P > 0.01 level (*) or the P < 0.01 level (**). rax1-3 and rax3-1 are in the Col-0 background, whereas rax2-1 is in the Ws background. For the double and triple mutants, a mixed Col-0 Ws background was used as a control.

of the flowering stem clearly deviated from the Col reference. Whereas only a single axillary shoot was found in nearly all cauline leaf axils of control plants, 35S:RAX2 lines developed accessory paralades in almost all leaf axils of the flowering stem (Figure 5B). Furthermore, 35S:RAX2 transgenic plants showed a reduction in sepal length (Figures 5C and 5D), a curling of cauline leaves (Figure 5B). Furthermore, accessory paraclades in almost all leaf axils of the flowering stem (Figure 5B). Additionally, a specific position along the shoot axis.

During vegetative development, wild-type plants (Col, Ws) have developed axillary buds in most of their leaf axils (Figure 4L), indicating a very early loss of function of the respective axillary meristem. Furthermore, we frequently observed deviations from the 137° angle between subsequent leaves along the bolting stem and a delay in petal abscission. To monitor the influence of the mixed genetic background on shoot branching in rax1-3 rax2-1, we have analyzed the progenies of sister plants of the double mutants, which were homozygous for RAX1 and RAX2 wild-type alleles, together with the parental accessions Col and Wassilewskija (Ws). Whereas both parental lines developed lateral buds in every leaf axil, this potential was slightly reduced in the progenies of wild-type segregants of the double mutants (Figure 4G). The reduction in axillary bud formation observed in rax1-3 rax2-1 double mutants was however much stronger than the inhibition seen in segregating wild-type plants.

Comparable to rax1-3 rax2-1, rax1-3 rax3-1 double mutants also displayed an enhancement of the rax1-3 branching defect during vegetative development (Figure 4J). The acropetal gradient of lateral bud formation observed in the rax1-3 mutant was modified in the rax1-3 rax3-1 plants, and branching during the reproductive phase was not affected. These observations indicate that RAX1 and RAX2 have a greater impact on axillary meristem formation than RAX3.

In rax2-1 rax3-1 mutants, many axillary buds developed from the basal nodes of the rosette, but lateral bud formation was reduced in the middle and at the top of the rosette (Figures 4M and 4N). Along the flowering stem, axillary shoots were found in all cauline leaf axils. These findings suggest that RAX2 and RAX3 act redundantly, controlling the formation of axillary meristems predominantly at late stages of vegetative development.

Figure 4. (continued).

(A) rax1-3 plants (right) flower earlier than Col wild-type plants (left).
(B) Close-up of rosette leaf axils of a rax1-3 plant.
(C) Close-up of rosette leaf axils of a Col wild-type plant.
(D), (G), (J), (M), and (P) Graphic representations of axillary bud formation of different rax mutants during vegetative development in comparison with corresponding wild-type plants. Genotypes are indicated next to the graphs. Position -1 corresponds to the uppermost rosette leaf, and position -43 corresponds to the oldest rosette leaf analyzed. The percentage values indicate the proportion of plants analyzed (n = 20) that formed an axillary bud in a specific position along the shoot axis.
(E) and (F) Growth habit of a Col wild-type (E) and a rax1-3 rax2-1 (F) plant.
(G) to (I) During vegetative development, wild-type plants (Col × Ws) have developed axillary buds in most of their leaf axils (G) and (H), whereas lateral shoot formation is strongly inhibited in rax1-3 rax2-1 plants (I).
(J) In comparison with rax1-3, rax1-3 rax3-1 double mutants displayed an enhanced shoot branching defect.
(K) and (L) In rax1-3 rax2-1 inflorescences, many cauline leaf axils were empty (K, arrows) or axillary shoots terminated early with leaf-like structures (L, arrow).
(M) and (N) In rax2-1 rax3-1 plants, side shoot formation was partially suppressed along the upper half of the vegetative shoot axis. Arrows in (N) indicate leaf axils with and without axillary buds.
(O) and (P) In the rax1-3 rax2-1 rax3-1 triple mutant, lateral shoot formation during the vegetative phase is almost completely inhibited. (O) shows a close-up of an empty rosette leaf axil.
group of small, densely cytoplasmic cells localized next to the adaxial center of the primordium border (Figures 6A and 6C). These cell groups develop into new meristems. The focused STM expression domains were not observed in the triple mutant (Figures 6B and 6D), suggesting that axillary meristems were not initiated in barren leaf axils of the rax1-3 rax2-1 rax3-1 mutant and that the RAX genes are involved in an early step of axillary bud formation.

**RAX1 and RAX3 Transcripts Accumulate in the Axes of Young Leaf Primordia**

RAX1 transcript accumulation was monitored by in situ hybridization experiments on tissue sections of Col wild-type and rax1-3 mutants. Plants were grown under short-day conditions and fixed 28 d after sowing. In these plants, RAX1 transcripts were detected in the axis of leaf primordia from P0 to P10/11 (Figures 7A and 7E to 7H). Along the longitudinal axis, the RAX1 expression domain was three to five cell layers deep, including always the L3 layer and frequently also the L1 and L2 layers of the SAM. Transverse sections revealed that RAX1 mRNA accumulates in a ball-shaped domain at the adaxial center of leaf primordia, extending approximately three to five cell layers in the adaxial–abaxial and the tangential dimensions (Figures 7E to 7H). Tissue sections of vegetative rax1-3 mutants displayed a very similar pattern of RAX1 transcript accumulation, indicating an unaltered distribution and stability of the shortened rax1-3 transcript (see Supplemental

---

**I. Axillary Meristems**

The strong branching defects observed in the rax1-3 rax2-1 rax3-1 triple mutant indicate that the corresponding genes are the key regulators, and RAX3 seems to have only a minor influence. In long-day conditions, the phenotypic abnormalities of rax1-3 rax2-1 rax3-1 plants were strongly reduced or not detectable.

---

**II. Analysis of RAX2 Function**

- **A** 35S-RAX2 plants (right) show a reduction in height in comparison with Col control plants (left).
- **B** Axil of a 35S-RAX2 plant showing a bud of an accessory paraclade (arrow) between a side shoot and a curled cauline leaf.
- **C** and **D** In comparison with the Col wild type (C), sepals of 35S-RAX2 plants (D) are characterized by a reduction in size.

---

**Figure 5. Analysis of RAX2 Function.**

(A) 35S:RAX2 plants (right) show a reduction in height in comparison with Col control plants (left). (B) Axil of a 35S:RAX2 plant showing a bud of an accessory paraclade (arrow) between a side shoot and a curled cauline leaf. (C) and (D) In comparison with the Col wild type (C), sepals of 35S:RAX2 plants (D) are characterized by a reduction in size.

rax1-3 rax2-1 rax3-1 triple mutants exhibited an almost complete inhibition of axillary bud formation during vegetative development (Figures 4O and 4P). Furthermore, the number of cauline leaves was significantly reduced, and the first leaf axils formed after the floral transition were found to be frequently empty. Deviations in phyllotaxis similar to those in the rax1-3 rax2-1 double mutant were also observed in the triple mutant. Furthermore, the triple mutant flowered significantly earlier than wild-type control plants (Table 1). The above phenotypic analyses suggest that RAX1, RAX2, and RAX3 encode proteins with partially redundant functions regulating the formation of axillary meristems during the vegetative phase and at the beginning of reproductive development. The strong branching defects observed in the rax1-3 rax2-1 double mutant indicate that the corresponding genes are the key regulators, and RAX3 seems to have only a minor influence. In long-day conditions, the phenotypic abnormalities of rax1-3 rax2-1 rax3-1 plants were strongly reduced or not detectable.

**I. Axillary Meristems**

Long and Barton (2000) have shown that STM expression can be used as a marker for axillary meristem development. In this study, STM mRNA accumulation was analyzed to test at which stage axillary bud formation is blocked in the rax1-3 rax2-1 rax3-1 triple mutant. Transverse sections from vegetative plants grown for 28 d under short-day conditions were hybridized to an antisense STM probe. STM transcripts were detected in the SAM and the interprimordial regions of both the Col wild type and the triple mutant (Figures 6A and 6B). In the axils of older leaf primordia of the wild type, STM expression was focused to a

---

**Figure 6. Patterns of RAX2, RAX3, and STM mRNA Accumulation in the Vegetative Shoot Apex.**

(A) to (D) Transverse sections through vegetative shoot tips (28 d) of Col wild-type ([A] and [C]) and rax1-3 rax2-1 rax3-1 ([B] and [D]) plants were hybridized with an STM antisense probe. In wild-type and rax1-3 rax2-1 rax3-1 plants, STM mRNA is detected in the SAM and in interprimordial regions ([A] and [B]). In addition, a focused STM expression domain was detected next to the adaxial center of older leaf primordia in the wild type ([A] and [C], arrowheads) but not in the triple mutant ([B] and [D], arrowheads). (E) and (F) Longitudinal sections through shoot tips of 28-d-old Col plants grown under short-day conditions, which were hybridized with an antisense (E) or a sense probe (F) from the RAX2 gene. (G) Longitudinal section through a vegetative Col shoot tip (28 d) hybridized with a RAX3 antisense probe. The arrowheads point to RAX3 expression domains in leaf axils.
Figure 7. Patterns of RAX1 Transcript Accumulation during Vegetative and Reproductive Development.

(A) and (B) Longitudinal sections through shoot apices of 28-d-old Col plants grown under short-day conditions.

(C) and (D) Longitudinal sections through shoot apices of Col plants grown for 28 d in short days and subsequently for 4 d (C) or 12 d (D) under long-day conditions.

(E) to (H) Successive transverse sections from 28-d-old plants grown in short days. The approximate distance from the top of the meristem to the middle of the section is given in the top right corner of the image. The arrow in (H) points to an almost circular RAX1 expression domain in the center of a leaf axil. Sections were hybridized with a RAX1 antisense ([A] to [C]) or a RAX1 sense probe (B).

In young Col wild-type plants grown for 14 d in short photoperiods, the RAX1 antisense probe did not reveal a signal, whereas this probe detected the RAX1 transcript in the same experiment in apices of 28-d-old plants.

Sections of reproductive shoot tips were obtained from plants grown under short-day conditions for 28 d and then induced to flower by 2, 4, or 12 successive long photoperiods. After the shift to long days, RAX1 transcripts were found in the axils of primordia on the elongating stem (Figures 7C and 7D). In the inflorescence meristem, RAX1 expression was only detected in the axils of stage 0 and stage 1 flower primordia (Smyth et al., 1990).

Cellular distribution of RAX1 mRNA during the reproductive phase was as in the vegetative phase.

In situ hybridizations with RAX2 sense and antisense probes were performed on tissue sections of shoot tips from vegetative Col wild-type shoots. Using two different antisense probes, we detected RAX2 transcripts in all cells of the tissues analyzed (Figure 6E). By contrast, no labeling was observed when a RAX2 sense probe was used (Figure 6F). This result suggests a uniform expression of RAX2 in all cells, which is corroborated by the strong bands observed in RT-PCR experiments with RNA from different tissues (Figure 2).

Longitudinal sections through 28-d-old vegetative shoot apices showed RAX3 transcripts at the adaxial base of leaf primordia (Figure 6G). This expression domain, which was also detected in transverse sections, has an almost spherical shape comprising approximately three to five cell layers in all dimensions. RAX3 mRNA was detected in the axils of vegetative leaf primordia from P2 to P10. During the reproductive phase, RAX3 mRNA was found in the axils of flower primordia originating from the inflorescence meristem (see Supplemental Figure 7 online). Whereas the patterns of RAX3 and RAX1 transcript accumulation were similar, the RAX3 signal intensity was found to be lower.

**RAX1 and LAS Have Partially Redundant Functions**

The recessive las mutant fails to initiate axillary meristems during vegetative development (Greb et al., 2003). LAS is expressed in a band-shaped domain at the adaxial boundaries of leaf primordia and encodes a putative transcription factor belonging to the GRAS family of regulatory proteins. Because both LAS and RAX1 affect the process of axillary meristem formation and show overlapping expression domains, we have tested for a possible interaction between both genes. Examination of LAS transcript accumulation in the rax1-3/rax2-1/rax3-1 triple mutant by RNA in situ hybridization revealed an expression pattern that is indistinguishable from the Col wild type (Figure 6F). On the other hand, RAX1 expression was found to be unaffected in las-4 mutant plants (see Supplemental Figure 7 online). These experiments suggested that the RAX1-RAX3 genes and LAS are not related to each other in a hierarchical order but are components of independent control mechanisms of axillary meristem formation.

To test this hypothesis, we have constructed a las-4 rax1-3 double mutant. In contrast with las-4 mutants, in which between one and five of the uppermost rosette leaves support axillary bud formation (Figure 8A), las-4 rax1-3 plants did not produce any axillary buds from vegetative nodes (Figure 8B). Also, the oldest cauline leaves very often did not support the development of axillary shoots (Figure 8C), resulting in an enhancement of the las-4 branching defect. A detailed comparison of las-4 and las-4 rax1-3 plants revealed additional defects in the double mutant. A high proportion of those side shoots that originated from younger cauline leaf axils terminated growth after formation of one or two leaves, resulting in a strong reduction in length of these lateral shoots (Figure 8D). Secondary branching was strongly suppressed. Toward the tip of the bolting stem we frequently observed the formation of flowers instead of shoots in the axils of cauline leaves. In many las-4 rax1-3 plants, cauline leaves and pistils showed a bent growth (Figures 8D and 8E). From these
LAS and RAX1-3 plants grown in short days do not develop axillary buds from rosette leaf axils formed early in the vegetative phase. Toward the middle of the rosette, the number of axillary buds increased, and almost all leaves formed late in the vegetative phase initiated lateral buds, resulting in an acropetal gradient of axillary bud formation (Figure 9). In addition, axillary shoots always developed from the cauline leaf axils of rax1-3 plants. This phenotype suggests that formation of axillary meristems is highly dependent on RAX1 function during the early phase of vegetative development and becomes less dependent on it as the plant matures. The gradient in axillary bud formation observed in rax1-3 may be the result of redundant gene activities that replace the missing RAX1 function at later stages of vegetative and in reproductive development. Indeed, double mutants combining rax1-3 with either rax2-1 or rax3-1 show an enhanced shoot branching defect (Figure 9).

Plants homozygous for either the rax2-1 or the rax3-1 loss-of-function alleles did not exhibit a defect in axillary bud formation. However, in rax2-1 rax3-1 double mutants, approximately half of the leaves in the middle and at the top of the rosette did not support the formation of axillary buds (Figure 9). From these data, we conclude that the RAX2 and RAX3 functions are more important in the middle and late phases of vegetative development. The triple mutant rax1-3 rax2-1 rax3-1 displayed the most extreme defect in axillary shoot formation (Figure 9), which supports the hypothesis of a redundant control of axillary meristem formation by these three MYB genes.

Taken together, the above observations suggest that the formation of axillary meristems along the shoot axis is not uniformly controlled. Interestingly, the tomato Bl mutant is impaired in axillary meristem formation in specific zones along the shoot axis (Schmitz et al., 2002). Lateral shoots are preferentially formed between the second and the fifth node of the primary shoot and at the two nodes below the inflorescence. In Arabidopsis, the deviations in phenotypes between plants harboring different mutant genes suggest that the three genes analyzed show overlapping patterns of activity at different stages of shoot development. However, we did not observe an obvious corresponding zonation in the distribution of RAX transcripts along the shoot axis. Therefore, we speculate that RAX gene activities are regulated in a zone-specific manner either through quantitative differences at the RNA level or at the protein level by protein modification and/or by interaction with specific partners. The uncovered fine-tuning of shoot branching may enable a plant to react very specifically to changes in environmental conditions. An influence of the environment on the proposed control mechanism is indicated by the fact that the defects observed under short-day conditions were strongly diminished or vanished in long days. Arabidopsis accessions that are impaired in shoot branching in specific zones along the shoot axis, like the Zu-0 ecotype (Kalinina et al., 2002), may carry loss-of-function alleles of one or several of the RAX genes analyzed.

**DISCUSSION**

In tomato, a Myb transcription factor encoded by the Bl gene controls the initiation of axillary meristems during vegetative and inflorescence development. In this study, we have identified the subgroup of Myb genes in Arabidopsis exhibiting the highest similarity to Bl and analyzed the functions of three members of this subgroup in the process of shoot branching. RT-PCR analysis revealed that the genes MYB37 (RAX1), MYB38 (RAX2), MYB84 (RAX3), and MYB87 are expressed in the shoot tip where a functional Bl homolog is expected to exert its function. Knockout mutants have been identified for the genes MYB37, MYB38, and MYB84 and were analyzed further.

**Myb Genes Differentially Regulate the Formation of Axillary Meristems in Different Phases of Arabidopsis Shoot Development**

Phenotypic analysis has demonstrated that homozygous rax1-3 plants grown in short days do not develop axillary buds from rosette leaf axils formed early in the vegetative phase. Toward the middle of the rosette, the number of axillary buds increased, and almost all leaves formed late in the vegetative phase initiated lateral buds, resulting in an acropetal gradient of axillary bud formation (Figure 9). In addition, axillary shoots always developed from the cauline leaf axils of rax1-3 plants. This phenotype suggests that formation of axillary meristems is highly dependent on RAX1 function during the early phase of vegetative development and becomes less dependent on it as the plant matures. The gradient in axillary bud formation observed in rax1-3 may be the result of redundant gene activities that replace the missing RAX1 function at later stages of vegetative and in reproductive development. Indeed, double mutants combining rax1-3 with either rax2-1 or rax3-1 show an enhanced shoot branching defect (Figure 9).

Plants homozygous for either the rax2-1 or the rax3-1 loss-of-function alleles did not exhibit a defect in axillary bud formation. However, in rax2-1 rax3-1 double mutants, approximately half of the leaves in the middle and at the top of the rosette did not support the formation of axillary buds (Figure 9). From these data, we conclude that the RAX2 and RAX3 functions are more important in the middle and late phases of vegetative development. The triple mutant rax1-3 rax2-1 rax3-1 displayed the most extreme defect in axillary shoot formation (Figure 9), which supports the hypothesis of a redundant control of axillary meristem formation by these three MYB genes.

Taken together, the above observations suggest that the formation of axillary meristems along the shoot axis is not uniformly controlled. Interestingly, the tomato Bl mutant is impaired in axillary meristem formation in specific zones along the shoot axis (Schmitz et al., 2002). Lateral shoots are preferentially formed between the second and the fifth node of the primary shoot and at the two nodes below the inflorescence. In Arabidopsis, the deviations in phenotypes between plants harboring different mutant genes suggest that the three genes analyzed show overlapping patterns of activity at different stages of shoot development. However, we did not observe an obvious corresponding zonation in the distribution of RAX transcripts along the shoot axis. Therefore, we speculate that RAX gene activities are regulated in a zone-specific manner either through quantitative differences at the RNA level or at the protein level by protein modification and/or by interaction with specific partners. The uncovered fine-tuning of shoot branching may enable a plant to react very specifically to changes in environmental conditions. An influence of the environment on the proposed control mechanism is indicated by the fact that the defects observed under short-day conditions were strongly diminished or vanished in long days. Arabidopsis accessions that are impaired in shoot branching in specific zones along the shoot axis, like the Zu-0 ecotype (Kalinina et al., 2002), may carry loss-of-function alleles of one or several of the RAX genes analyzed.
At Least Two Pathways Regulate the Formation of Axillary Meristems in Arabidopsis

The Arabidopsis LAS gene (Greb et al., 2003) and its orthologs in tomato (Schumacher et al., 1999) and rice (Li et al., 2003) are key regulators of axillary meristem formation. las-4 mutants are impaired in the formation of axillary meristems during the vegetative phase. Primary branching of the flowering stem is not affected by the las-4 mutation, suggesting that LAS function is only required for the initiation of axillary meristems during vegetative development. Characterization of the rax1-3 mutant has demonstrated that axillary shoot formation is also strongly dependent on RAX1 function in the early phase of vegetative development followed by a decreasing dependence toward maturation. rax1-3 las-4 double mutants did not develop any axillary shoots during the vegetative phase of development and showed a strong reduction in the number of early cauline leaves supporting the formation of a lateral shoot. The complete lack of side shoots in rosette leaf axils and the failure to initiate axillary shoots in the early cauline leaf axils is more than the sum of the defects observed in both single gene mutants. These data suggest that LAS and RAX1 play a role in axillary meristem formation along the whole axis of an Arabidopsis plant. They may be members of completely independent pathways or, if both pathways contribute to the formation of one regulator of axillary meristem initiation, different levels of this regulator may be needed during vegetative and reproductive development. Similar results have been obtained through the analysis of las bli double mutants in tomato (Schmitz et al., 2002).

The hypothesis of two independent regulatory pathways is consistent with the expression patterns of LAS and RAX1 in wild-type and mutant backgrounds. The patterns of transcript accumulation of LAS in rax1-3 rax2-1 rax3-1 and of RAX1 in las-4 plants did not show any deviations from the corresponding wild-type controls. These results demonstrate that LAS and RAX1 are not related to each other in a hierarchical order and, therefore, support the idea that the genes are elements of two independent control pathways of axillary meristem formation.

Furthermore, those axillary shoots that originated from young cauline leaf axils of the las-4 rax1-3 double mutant frequently terminated after formation of one or two leaves, and secondary branching was strongly inhibited. In the axils of the youngest cauline leaves of the double mutant, lateral shoots were frequently replaced by flowers. These observations demonstrate that LAS and RAX1 not only affect initiation but also maintenance and identity of axillary meristems along the flowering stem.

The phenotypes of rax mutants described in this report were observed using plants grown to maturity in short photoperiods. When rax1-3, rax2-1, and rax3-1, as well as the double and triple mutants, were cultivated under long-day conditions, the described defects in shoot branching were greatly diminished or no longer detectable. In long photoperiods, the activities of RAX1, RAX2, and RAX3 may be compensated by related Myb genes of the same subgroup or by yet another pathway. In both cases, we would expect the alternative pathway to be regulated by daylength.

RAX1 and RAX3 mRNAs Accumulate in Axillary Meristem Progenitor Cells

The RAX1 gene is expressed in the axils of leaf and flower primordia. During vegetative development, RAX1 transcripts were detected from P0 to P10/11 in a spherical domain at the adaxial center of leaf primordia. These expression domains had a diameter of approximately three to five cells always including the L3 layer and very often the L1 and L2 layers of the meristem. After floral transition, RAX1 transcripts were found at the adaxial side of young primordia initiated on the elongating stem. The cellular dimensions of these expression domains and the developmental
timing of expression were comparable to the vegetative phase. Like RAX1, the LAS gene is expressed in the zone between leaf primordia and the SAM (Greb et al., 2003). In contrast with LAS, the RAX1 expression domain does not extend along the whole adaxial boundary of a leaf primordium but is focused to the center of the leaf axil where the new meristem will be initiated. Therefore, the RAX1 expression pattern contains positional information that may be crucial to determine the specific position of a lateral meristem. Because the RAX1 expression domain is completely included within the LAS expression domain and mutations in either gene cause defects in the process of auxillary meristem formation, it is tempting to speculate that these two genes may be related to each other in a hierarchical order. However, in situ hybridization experiments revealed no regulatory interdependence of these two genes. Other genes, such as CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, are also expressed in the region between the meristem and developing primordia. Further experiments are needed to determine whether RAX1 and CUC1 or CUC2 are related in a hierarchical order of gene interactions. All these genes seem to contribute to a very distinct transcript profile of the auxillary region, indicating a specific identity of this tissue, which may be a prerequisite for auxillary meristem formation.

In the shoot tip, RAX3 transcripts accumulate in a pattern that is similar to the RAX1 pattern, but the hybridization signals were weaker. In contrast with RAX1 and RAX3, the RAX2 gene is not expressed in a specific pattern in the shoot tip but was found to be transcribed in all cells of the tissues analyzed. However, the phenotypic defects seen in rax1-3 rax2-1 and rax2-1 rax3-1 plants suggest a specific role of RAX2 during the late period of vegetative development. This discrepancy would be explainable if a second gene, which may be expressed in a specific pattern, is needed for RAX2 function.

RT-PCR analysis demonstrated that all three genes analyzed show the highest levels of transcript accumulation in the root. MYB68, another member of the same Myb gene subfamily, was recently shown to be strongly expressed in root pericycle cells and to regulate lignification (Feng et al., 2004). The rax1-3 mutant analyzed in this study did not show an alteration in root morphology. It remains to be tested whether or not a specific function of the Bl-related Myb genes in root development can be uncovered in mutants harboring different combinations of mutant alleles.

**METHODS**

**Plant Materials and Growth Conditions**

Seeds of Arabidopsis thaliana ecotypes Col-0 and Landsberg erecta were obtained from the Nottingham Arabidopsis Stock Centre. For cultivation under short-day conditions Arabidopsis plants were grown in a controlled environment room with 8 h light (16 h dark), 20°C day temperature, 18°C night temperature, and 50% relative humidity. Short-day plants were grown to maturity under the conditions described. Cultivation under long-day conditions was done in the greenhouse with additional artificial light when needed.

**Identification of Mutants**

Screenings for knockout mutants for Arabidopsis genes MYB37 (RAX1), MYB38 (RAX2), MYB68, MYB84 (RAX3), and MYB87 were performed in several Arabidopsis populations. A RAX1 T-DNA insertion line (SALK_071748, Col-0 background) was identified in the SALK T-DNA insertion line collection (Alonso et al., 2003) at the Jonas Salk Institute for Biological Studies. A RAX2 T-DNA insertion line (Ws background) was found in the population available at the University of Wisconsin–Madison Biotechnology Center Arabidopsis Knockout Facility (Krysan et al., 1999). PCR screens were performed according to the Arabidopsis Knockout Facility protocol in the population transformed with the T-DNA vector pD991. Gene-specific primers used for the screening were M38-2F (5’-CATTGCTTTGACGGCTTCT-3’) and M38-3R (5’-TCAAGTGATGACCTAGTGTG-3’). The RAX3 En-1 insertion line (Col-0 background) was identified in an En/Spm transposon mutagenized population of Arabidopsis plants (Wisman et al., 1998) following the protocol of Steiner-Lange et al. (2001). Microarrays with PCR products of transposon-flanking sequences were hybridized with gene-specific probes. The probe for RAX3 was generated with the primers Myb84-818F (5’-CAAGACTTTTTGACCATCCCTCCCTC-3’) and Myb84-1937R (5’-GGATTCTAAGAGGATTGAGAAGAGG-3’). insertions of the T-DNAs or the En-1 transposon were verified by DNA sequencing and DNA gel blot analysis, respectively.

**DNA Isolation and DNA Gel Blot Analysis**

Plant DNA preparation and DNA gel blot analysis have been performed as described previously (Brandstätter et al., 1993). Standard techniques were used according to Sambrook and Russell (2001).

**RNA Isolation and RT-PCR Analysis**

Total RNA was isolated using the RNeasy plant mini kit (Qiagen) following the manufacturer’s instructions. For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using SuperScript II polymerase (Life Technologies) according to the manufacturer’s instructions. The product of the first-strand cDNA synthesis reaction was amplified by PCR using the gene-specific primers M36-1 (5’-GGAAAGAAGACACAAAACAGCGAAGATC-AAG-3’) and M36-2 (5’-CCATCAATATCATCTTCCATC-3’), M37-1 (5’-AAGAGAGAAGAATGGAAGAGG-3’) and M37-2 (5’-CTCCCTCCATCAGGTTTGGGTTCCTGTTCT-3’), 692-1 (5’-AGATAGAGGATGAGGATGCTC-3’) and 692-2 (5’-TACTCGAGATCTAGTACAGAATGAC-3’) or M38-1398R (5’-GTTCCTTCTCTAGTGAATCTCC-3’), M68-1 (5’-AGAGGATGAGGAATGGAAGAGG-3’) and M68-2 (5’-CTTCCACCTCCAAGACACAG-3’), M84-1 (5’-AGGCCGCTATCAAGAAGAC-3’) and M84-2 (5’-CCATATCTGTGTACAGACCC-3’) or M84-1309R (5’-GATTCAATTCTTGATCCCTC-3’), and M87-2681F (5’-ACATGTAATCTGAGATGCTGAA-3’) and M87-3209R (5’-CTCCAACCTGTCATATAAAGAC-3’). Amplification of actin cDNA using the primers ActinATrev (5’-TGTTGCTTGTTGGAAGAT-3’) and ActinATfwd (5’-CACCAGCTACGACAATGTAC-3’) was performed as a control to ensure that equal amounts of cDNA were added to each PCR reaction.

**Analysis of Shoot Branching**

Branching of rosettes and inflorescences was analyzed with the help of a binocular 2 weeks after the onset of flowering. Rosettes were held upside down, and the rosette leaf axils were examined one by one beginning with the oldest leaf axil. Analyzed leaves were removed successively so that the younger leaf axils became accessible. For each experiment, 20 plants were analyzed; each experiment was repeated at least once.

**Analysis of Flowering Time**

For determination of the flowering time, seeds were imbibed in water at 4°C for 4 d. Subsequently, only germinated seeds were transferred to soil to ensure an equal state of development. Flowering time was recorded when the first flower had opened.
DNA Construct and *Arabidopsis* Transformation

The *RAX2-1* cDNA was amplified with the primers 6922-1 (5′-AGATA-GAGAGATGGTAGGCTGC-3′) and 6922-2 (5′-TACTGAGATCG-TAGTACAAGTA-3′) and cloned behind the cauliflower mosaic virus 35S promoter and ß-1,2,4-glucuronidase reporter gene (Kendall) using the ASP300 tissue processor (Leica). Hybridization probes were specific for the third exon of At1g55580 (base pairs 934 to 1436, relative to the ATG), MYB38 (base pairs 933 to 1402), and MYB84 (base pairs 623 to 1154). LASS probes contained the nucleotides 2 to 1348 of the open reading frame. STM probes included base pairs 78 to 1122 relative to the ATG. The sequences were cloned into pGEM vectors in sense and antisense orientations relative to the T7 promoter. Linearized plasmids were used as templates for probe synthesis with T7 RNA polymerase. Probes were not hydrolyzed. After the color reaction, slides were mounted in 30% glycerol and photographed using differential interference contrast microscopy.

DNA Sequencing and Analysis

DNA sequences were determined by the DNA Core Facility of the Max Planck Institute for Plant Breeding Research on ABI Prism 317, 3730 XL, or 3130 XL sequencers (Applied Biosystems) using BigDye terminator chemistry. Premixed reagents were from Applied Biosystems. Oligonucleotides were purchased from Life Technologies or Metabion. Analysis of sequences was performed using the Wisconsin Package (version 10.0-UNIX; Genetics Computer Group). Database searches were performed using BLAST programs (Altschul et al., 1990) against National Center for Biotechnology Information sequence databases.

Multiple sequence alignments were done with ClustaW (1.75) at http://www.es.baconet.org/doc/phyloendron/clustal-form.html (Thompson et al., 1994) using the bootstrap neighbor-joining tree option with 1000 bootstraps. The phylogenetic tree was obtained using Phyloendron software (version 0.8d by D.G. Gilbert) with horizontal/vertical tree growth and intermediate node position. *Solanum lycopersicum* Blind-like1 is represented by the overlapping tomato unigenes SGN-U222803 and SGN-U234684; the sequence was extended by sequencing the EST cTOE-17-F19. *Sl MYB68* is represented by tomato unigene SGN-U222804 and *Sl RAX2-1* by potato unigene SGN-U224024 and *Sol anum tuberosum* Blind-like4 by potato unigene SGN-U280553.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource database (http://www.arabidopsis.org) with Arabidopsis Genome Initiative locus identifiers At5g57620 (MYB36), At5g23000 (MYB37, RAX1), At2g38880 (MYB38, RAX2), At5g65790 (MYB84), At3g49690 (MYB84, RAX3), At4g37780 (MYB87), and At1g55580 (LAS).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Alignment of Blind-Like Protein Sequences.

**Supplemental Figure 2.** RT-PCR Analysis of *rax* Alleles.

**Supplemental Figure 3.** Scanning Electron Microscopy Analysis of *rax* Mutants.

**Supplemental Figure 4.** Root Development in *rax* Mutants.

**Supplemental Figure 5.** Phenotype of *rax*-1 and *rax*-3 Plants.

**Supplemental Figure 6.** Semiquantitative RT-PCR Analysis of 35S::RAX2 Plants.

**Supplemental Figure 7.** In Situ Hybridization Analysis of RAX1 and RAX3 mRNA Accumulation.

**ACKNOWLEDGMENTS**

We thank the Nottingham Arabidopsis Stock Centre for providing seed stocks, the Automatic DNA Isolation and Sequencing group of the Max Planck Institute for Plant Breeding Research for DNA sequencing, and U. Pfordt and U. Tartler for excellent technical assistance. We also thank G. Coupland, H.M.O. Leyser, and M. Koornneef for critical reading of the manuscript and R.-D. Hirtz for help with the scanning electron microscopy. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 572 of the University of Cologne (Germany).

Received October 13, 2005; revised December 23, 2005; accepted January 17, 2006; published February 3, 2006.

**REFERENCES**


Greb, T., Clarenz, O., Schäfer, E., Müller, D., Herrero, R., Schmitz, D., U. Pfordt and U. Tartler for excellent technical assistance. We also thank G. Coupland, H.M.O. Leyser, and M. Koornneef for critical reading of the manuscript and R.-D. Hirtz for help with the scanning electron microscopy. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 572 of the University of Cologne (Germany).


