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Blind Homologous *R2R3 Myb* Genes Control the Pattern of Lateral Meristem Initiation in *Arabidopsis* [W]

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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 axillary 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 (Stirnberg 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 (Steeves 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, *ls* [Schumacher et al., 1999] and *blind* [Schmitz et al., 2002]; in maize, *barren stalk1* [Gallavotti et al., 2004]; in rice, *monoculm1*

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[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* [Doebley 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 *ls* 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.

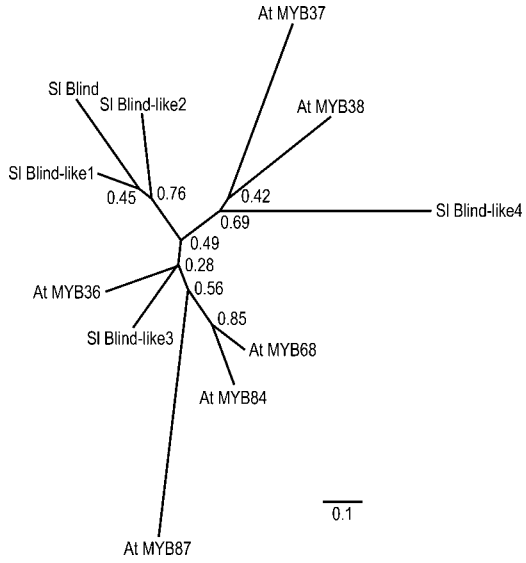

Figure 1. Phylogenetic Tree of Bl-Like Protein Sequences.

The conserved N-terminal domains (amino acids 1 to 118) of the six *Arabidopsis* MYB proteins (MYB36, 37, 38, 68, 84, and 87) showing the highest sequence similarity to the tomato Bl protein (Sl Blind) were compared with three Bl-like sequences from tomato (Sl Blind-like1, 2, and 3) and one sequence from potato (St Blind-like4) using ClustalW software. Multiple sequence alignments were done using the bootstrap neighbor-Joining tree option with 1000 bootstraps. The phylogenetic tree was obtained using Phylodendron software (version 0.8d by D.G. Gilbert) with horizontal tree growth and intermediate node position. At, *Arabidopsis thaliana*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum.*

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

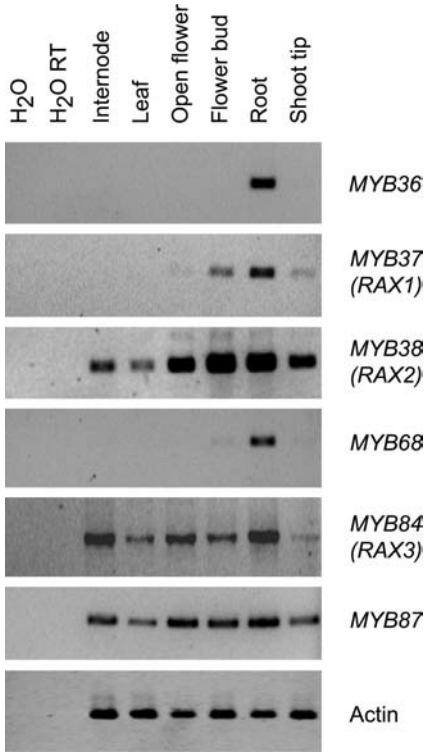

Figure 2. RT-PCR Analysis of *Myb* Gene mRNA Accumulation.

Total RNA from different plant organs was analyzed by RT-PCR using *Myb* gene–specific primer combinations. Amplification of actin cDNA was used to ensure that equal amounts of cDNA were added to each PCR reaction.

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 stereomicroscope 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

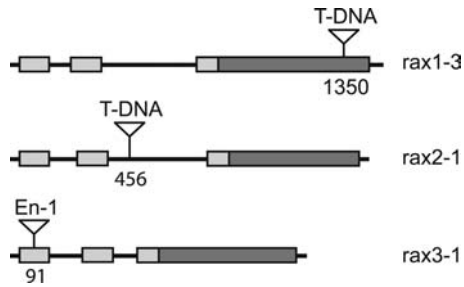

Figure 3. Schematic Structure of *R2R3 Myb* Genes Showing the Relative Positions of T-DNA (*rax1-3* and *rax2-1*) or *En-1* (*rax3-1*) Insertions in the Mutant Alleles *rax1-3*, *rax2-1*, and *rax3-1*.

Numbers indicate the distance of the T-DNA or *En-1* insertion from the start of the open reading frame. Exons are represented by boxes and introns by black lines. The MYB domain is indicated in light gray.

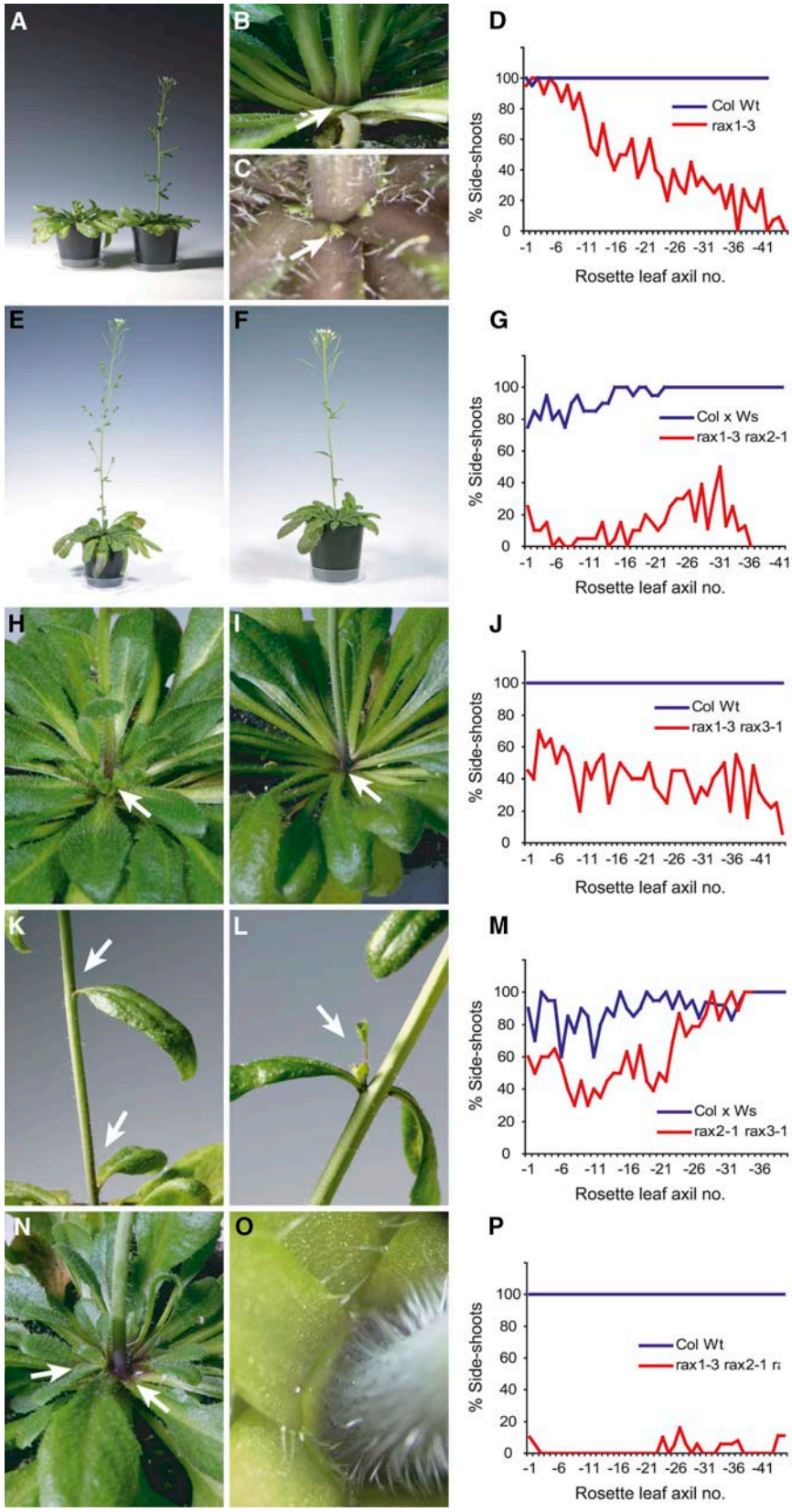

Figure 4. Different Members of the *RAX* Gene Family Redundantly Regulate Axillary Bud Formation.

Table 1. Flowering Time of *rax* Mutants

Genotype	Days to Flowering	Total Leaf Number
rax1-3	80.1 ± 0.7**	60.1 ± 0.8*
rax2-1	71.8 ± 0.6	37.9 ± 0.6**
rax3-1	84.1 ± 0.4	61.8 ± 0.7
rax1-3 rax2-1	75.6 ± 0.9**	46.7 ± 1.1**
rax1-3 rax2-1 rax3-1	69.8 ± 1.3**	43.6 ± 1.8**
Col-0	84.3 ± 0.6	62.7 ± 0.6
Ws	71.6 ± 0.7	40.5 ± 0.5
Col-0 Ws	78.7 ± 0.6	60.2 ± 0.7

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 paraclades 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), and a reduction in male fertility. To correlate expression of the *35S:RAX2* transgene with the observed phenotypic alterations, we selected five independent transgenic lines. Semiquantitative RT-PCR analysis revealed that in comparison with the Col wild type, these transgenic plants showed an increase in *RAX2* transcript level, and the amount of *RAX2* transcript was found to correlate well with the severity of the mutant phenotype (see Supplemental Figure 6 online).

Different Members of the *RAX* Gene Family Redundantly Regulate Axillary Meristem Formation at Different Stages of Shoot Development

To uncover functions of homologous *RAX* genes that might be masked because of genetic redundancy, we constructed double and triple mutants by combining loss-of-function alleles. Combination of the *rax1-3* and *rax2-1* alleles resulted in a strong enhancement of the branching defect observed in *rax1-3* plants. These double mutants did not produce axillary buds in most of their rosette leaf axils (Figures 4F, 4G, and 4I). During reproductive development, axillary shoots were formed in the axils of most cauline leaves. However, the first cauline leaves of the bolting stem very often did not support the formation of axillary shoots (Figure 4K). In other individuals, axillary shoots terminated with leaf-like structures (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 (**[G]** and **[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.

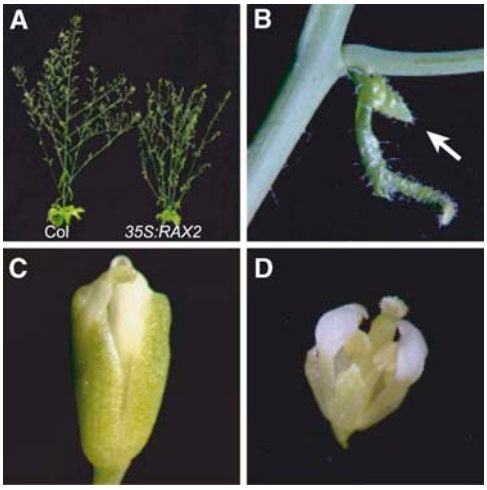

Figure 5. Analysis of *RAX2* Function.

(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.

rax1-3 rax2-1 rax3-1 Mutants Fail to Initiate 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 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 Axils 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 axils 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

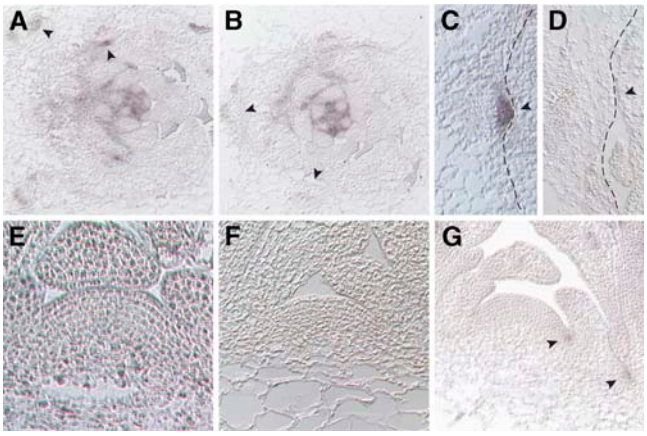

Figure 6. Patterns of *RAX2*, *RAX3*, and *STM* mRNA Accumulation in the Vegetative Shoot Apex.

(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.

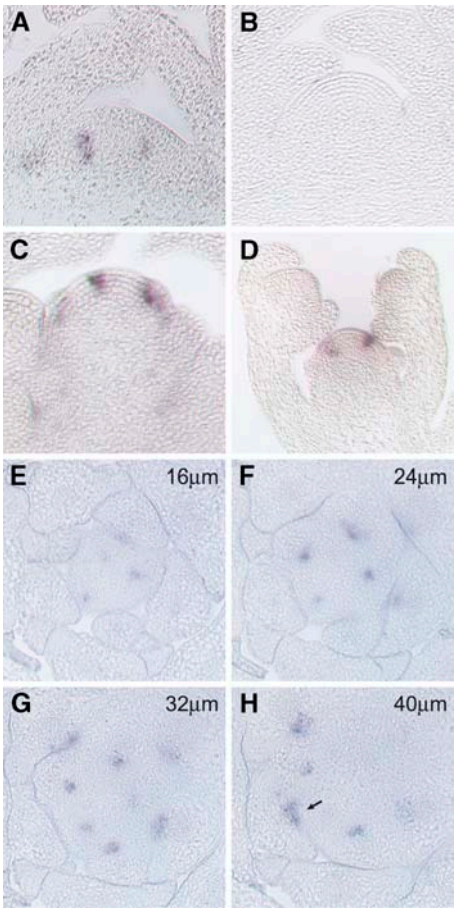

Figure 7. Patterns of *RAX1* Transcript Accumulation during Vegetative and Reproductive Development.

(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]** and **[C]** to **[H]**) or a *RAX1* sense probe **(B)**.

Figure 7 online). 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 8F). 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

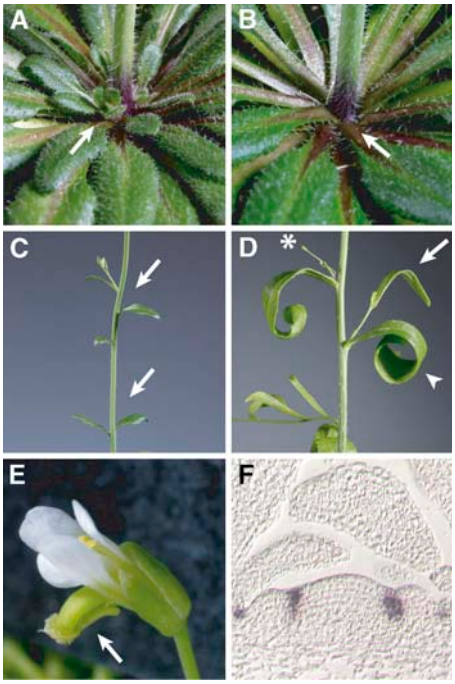

Figure 8. *rax1-3* Enhances the Branching Defect of *las-4*.

(A) to **(E)** In comparison with *las-4* plants **(A)**, *las-4 rax1-3* mutants showed a further reduction in the formation of lateral shoots during the vegetative **(B)** and reproductive phases (**[C]**, arrows) of development. In the double mutant, lateral shoots terminated early (**[D]**, asterisk) or were replaced by leaf-like organs (**[D]**, arrow) or flowers. In addition, *las-4 rax1-3* plants frequently showed a bent growth of cauline leaves (**[D]**, arrowhead) and pistils (**[E]**, arrow).

(F) *LAS* mRNA accumulation in the *rax1-3 rax2-1 rax3-1* triple mutant was like that in the Col wild type.

experiments, we conclude that *LAS* and *RAX1* redundantly influence initiation, maintenance, and identity of axillary meristems.

In *las-4 rax3-1* double mutants, shoot branching during the vegetative phase was almost identical to *las-4*. However, also in these plants we found that shoots in the axils of cauline leaves were frequently replaced by flowers, indicating a contribution of *RAX3* to the regulation of axillary meristem identity/determinacy.

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.

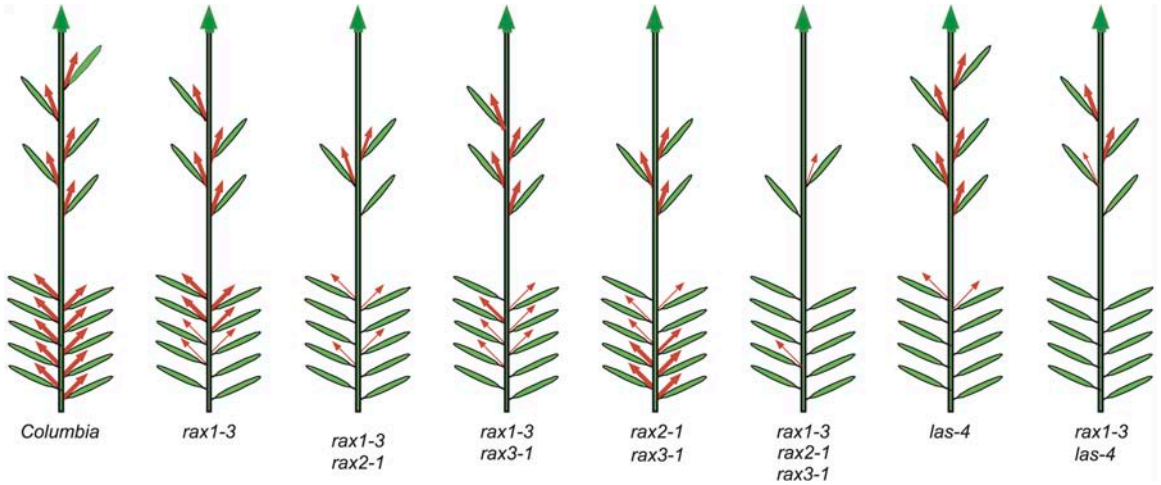

Figure 9. Comparison of the Shoot Branching Patterns of *rax* and *las* Mutants.

Schematic representations of the shoot branching patterns of the Col wild type and plants that were homozygous for *las-4*, *rax1-3*, or different combinations of *rax1-3*, *rax2-1*, *rax3-1*, and *las-4*. The plants have been grown to maturity under short-day conditions. Red arrows indicate lateral shoots originating from rosette or cauline leaf axils. The width of a red arrow indicates the proportion of plants analyzed that developed a side shoot in a specific zone along the shoot axis. The drawings also indicate the degree of cauline leaf formation by the different mutants.

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 *ls bl* 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 axillary 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 axillary region, indicating a specific identity of this tissue, which may be a prerequisite for axillary 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′-CATTGACTTGAGCGTTTCCAATACATAGA-3′) and *M38-3R* (5′-TCAAGTGATGA-ACCTAGCTGATGGATAA-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′-CAAAGACTTTTTGACATCTCCCCCTC-3′) and *Myb84-1937R* (5′-GGATCTAACAAGAGTTGAGAGAAAGAG-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 (Brandstädter 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′-GGAAGAGAACAACAACGACAAGATCAAG-3′) and *M36-2* (5′-CCATCAATAATCCATTATCCATCCC-3′), *M37-1* (5′-AACGAGAGAAATGGGAAGAGC-3′) and *M37-2* (5′-CCTCCCATACCCCATCAAATC-3′) or *M37-2333R* (5′-CCCATTGTACCCTTTTGTCCT-3′), *6922-1* (5′-AGATAGAGAGATGGGTAGGGCTCC-3′) and *6922-2* (5′-TACTCGAGATCAGTAGTACAACATGAAC-3′) or *M38-1359R* (5′-GTCTTCTTCTTCAGTGAAATCTCC-3′), *M68-1* (5′-AAGAATCAAGAAAATGGGAAGAGC-3′) and *M68-2* (5′-CTACCACTCCCTAAAGACACAG-3′), *M84-1* (5′-AGGCCGCTATCAAGAACAAG-3′) and *M84-2* (5′-CCATATATACGTTACGTACCCCC-3′) or *M84-1092R* (5′-GATTTGAGTTTTGCATCTTCCTC-3′), and *M87-2661F* (5′-ACATCATCATCATCGAGTCAGAA-3′) and *M87-3020R* (5′-CTCAAACCCGTCCATAAAGAAC-3′). Amplification of actin cDNA using the primers *ActinAT-for* (5′-TGGTGTCATGGTTGGGATG-3′) and *ActinATrev* (5′-CACCACTGAGCACAATGTTAC-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′-AGATAGAGAGATGGGTAGGGCTCC-3′) and *6922-2* (5′-TACTCGAGATCAGTAGTACAACATGAAC-3′) and cloned behind the cauliflower mosaic virus 35S promoter and Ω-leader of the vector pRT-/Not/Asc (Überlacker and Werr, 1996). The expression cassette was transferred to the plant transformation vector pGPTV-Bar-Asc (Überlacker and Werr, 1996). *Arabidopsis* plants were transformed according to the floral dipping method (Clough and Bent, 1998) using the *Agrobacterium tumefaciens* strain GV 3101 (Koncz and Schell, 1986). Transgenic plants were selected by spraying with 250 mg/L Glufosinat (BASTA; Hoechst).

RNA in Situ Hybridizations

Sample preparations and in situ hybridizations of 8-μm sections were done as described by Coen et al. (1990) with slight modifications. Tween 20 (0.03%) was added to the fixative, and dewatering of the fixed material was done without NaCl. Plant material was embedded in Paraplast+ (Kendall) using the ASP300 tissue processor (Leica). Hybridization probes were specific for the third exon of *MYB37* (base pairs 934 to 1436, relative to the ATG), *MYB38* (base pairs 933 to 1402), and *MYB84* (base pairs 623 to 1154). *LAS* 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 377, 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 ClustalW (1.75) at http://www.es.embnet.org/Doc/phylodendron/clustal-form.html (Thompson et al., 1994) using the bootstrap neighbor-joining tree option with 1000 bootstraps. The phylogenetic tree was obtained using Phylodendron 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 *Blind-like2* is represented by tomato unigene SGN-U226585; the sequence was extended by sequencing the EST cLEW-23-B13. Sl *Blind-like3* is represented by tomato unigene SGN-U224024 and *Solanum 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*), At2g36890 (*MYB38*, *RAX2*), At5g65790 (*MYB68*), 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 *rax1-3* Mutants.

Supplemental Figure 5. Phenotype of *rax2-1* and *rax3-1* 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.

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