Shoot Branching and Leaf Dissection in Tomato Are Regulated by Homologous Gene Modules

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Aerial plant architecture is predominantly determined by shoot branching and leaf morphology, which are governed by apparently unrelated developmental processes, axillary meristem formation, and leaf dissection. Here, we show that in tomato (Solanum lycopersicum), these processes share essential functions in boundary establishment. Potato leaf (C), a key regulator of leaf dissection, was identified to be the closest paralog of the shoot branching regulator Blind (Bl). Comparative genomics revealed that these two R2R3 MYB genes are orthologs of the Arabidopsis thaliana branching regulator REGULATOR OF AXILLARY MERISTEMS1 (RAX1). Expression studies and complementation analyses indicate that these genes have undergone sub- or neofunctionalization due to promoter differentiation. C acts in a pathway independent of other identified leaf dissection regulators. Furthermore, the known leaf complexity regulator Goblet (Gob) is crucial for axillary meristem initiation and acts in parallel to C and Bl. Finally, RNA in situ hybridization revealed that the branching regulator Lateral suppressor (Ls) is also expressed in leaves. All four boundary genes, C, Bl, Gob, and Ls, may act by suppressing growth, as indicated by gain-of-function plants. Thus, leaf architecture and shoot architecture rely on a conserved mechanism of boundary formation preceding the initiation of leaflets and axillary meristems.

INTRODUCTION

Shoot architecture of seed plants is characterized by multiple repetitions of a basic module called the phytomer, which consists of an internode, a leaf, and an axillary meristem. Studies on leaf evolution suggest that seed plant leaves evolved independent of other megaphylls from branched undifferentiated axes (Stewart and Rothwell, 1993; Rothwell, 1999; Tomescu, 2009). At about the same time in evolution, axillary branches appeared (Galtier and Holmes, 1982; Galtier, 1999; Meyer-Berthaud et al., 2000), providing the basis for the enormous plasticity observed in seed plant shoot architecture (Galtier, 1999).

Leaves are initiated in a regular pattern from cells at the flank of the shoot apical meristem (SAM). Recent studies have shown that seed plants use similar mechanisms to regulate leaf primordium initiation at the SAM and to elaborate leaf form, especially in plants with complex leaves (Blein et al., 2008; Rosin and Kramer, 2009). Leaf initiation at the SAM depends on regulated auxin flux creating auxin maxima at the sites of primordium formation (Reinhardt et al., 2000). Whereas loss of polar auxin transport results in an arrest of primordium formation (Vernoux et al., 2009), ectopic auxin application causes ectopic leaf development (Reinhardt et al., 2000). Similarly, the outgrowth of leaflets requires the formation of auxin maxima along the rachis and subsequent activation of auxin signaling (Barkoulas et al., 2008; Koenig et al., 2009). In tomato (Solanum lycopersicum) compound leaf development, auxin maxima are found at positions where leaflets initiate (Koenig et al., 2009), whereas auxin signaling needs to be repressed at positions where the rachis, a part of the leaf axis without leaf blade, will form.

Class I KNOX gene activity is needed to maintain the SAM as a population of pluripotent cells (Hay and Tsiantis, 2010). Furthermore, KNOX expression during leaf development is causally linked to the elaboration of different leaf forms: In simple leaves, KNOX genes are kept in a repressed state, but in many species with complex leaf development, KNOX gene expression is reactivated in a patterned fashion (Hay and Tsiantis, 2010). Loss of AUXIN/INDOLE-3-ACETIC ACID gene activity, as in the tomato entire (e) mutant (Zhang et al., 2007; Berger et al., 2009), leads to ectopic activation of auxin responses throughout the tomato leaf margins. As a consequence, ectopic leaf blade develops along the rachis and leaflets are fused. Ectopic lamina growth on the rachis can also be induced by auxin application to young leaf primordia (Koenig et al., 2009). Expression of KNOX genes in ectopic positions, as in the mutant Mouse ears (Me; Parnis et al., 1997) or when using transgenic approaches (Hareven et al., 1996), results in highly complex leaves with hundreds of leaflets. However, KNOX gene expression can trigger leaflet formation only when it is induced at the right developmental time (Shani et al., 2009).

NAC transcription factors of the CUC/NAM subfamily play an important role in SAM function and organ separation. During leaf development, CUC genes are expressed at positions where leaf...
blade indentations and leaflets will form (Blein et al., 2008; Berger et al., 2009; Hasson et al., 2011). Expression of CUC genes is detectable before indentations or leaflets are initiated at the leaf margin, where CUC activity is needed to repress growth. A mutation in the tomato Goblet (Gob) gene, the homolog of Arabidopsis thaliana CUP-SHAPED COTYLEDON1 (CUC1)/CUC2, results in leaves with a reduced number of leaflets showing smooth margins and downregulation of KNOX gene transcription (Blein et al., 2008; Berger et al., 2009).

After germination, new meristems initiate in the axils of leaves; these develop into buds and finally into side-shoots, establishing new axes of growth. In tomato, as well as in other species, auxillary meristem (AM) formation requires Lateral suppressor (Ls) (Schumacher et al., 1999; Greb et al., 2003; Li et al., 2003), which is expressed in the boundary region between the leaf primordium and the stem. Tomato blind mutants are compromised in AM formation during vegetative and reproductive development (Schmitz et al., 2002). Blind and the homologous Arabidopsis RAX genes (Keller et al., 2006; Müller et al., 2006) encode R2R3 MYB transcription factors that regulate AM formation in overlapping zones along the shoot axis. In Arabidopsis, recent experiments have demonstrated that CUC1, CUC2, and CUC3 also play a role in AM establishment (Hibara et al., 2006; Raman et al., 2008). CUC expression in the boundary regions of both leaf and leaflet primordia indicates a functional similarity between the two zones.

The genomes of most seed plants show signs of ancient genome duplications. Most or all eudicots are derived from a hexaploid ancestor (Van de Peer et al., 2009). During subsequent evolution, many groups of dicot plants underwent whole-genome duplications (WGD); for example, the Arabidopsis genome shows signs of two rounds of WGD and tomato shows evidence for one round of WGD. WGDs were followed by massive loss of duplicated genes and by chromosome rearrangements, which are at least partially connected to a reduction in chromosome number. Despite these changes in chromosome organization, conservation of gene arrangement can be detected over large evolutionary distances and helps to identify evolutionary conserved functions.

In this study, we elucidate the functions of members of the Blind/RAX gene families in tomato. We show that the branching regulator Blind and the regulator of complex leaf development Potato leaf (C) are coorthologs of Arabidopsis RAX1 and that they differ in their expression patterns. We connect side-shoot development to complex leaf development by demonstrating that the regulation by GOB and two homologous MYB proteins is essential in both processes. These results are discussed in the light of the current view of leaf and shoot evolution in seed plants.

RESULTS

Blind-like2 and Blind Are Coorthologs of RAX1

To find additional factors regulating tomato shoot architecture, we used database searches to identify eight tomato homologs of the R2R3 MYB transcription factor Blind (Bl). We named these homologs Blind-like1 (Bl1) to Bl8. In addition to the nine genes in tomato, we found six homologs in Arabidopsis (Müller et al., 2006) and seven in grape (Vitis vinifera). As shown by phylogenetic analysis, this group is clearly separate from other R2R3 repeat MYB genes (Stracke et al., 2001; Wilkins et al., 2009). Alignment of the amino acid sequences of these proteins showed that the N-terminal part containing the two MYB repeats is highly conserved (see Supplemental Figure 1 online), but sequence similarity in the C-terminal domains can be detected only between specific pairs of sequences (Bl to Bl2 and Bl6, RAX3 to MYB68, and MYB36 to Bl8) and is not significant between other pairs of genes. Bl2 and Bl6 have the highest degree of sequence similarity to each other and are separated by only 6373 bp in the genome, indicating that they originated by tandem duplication. However, the presence of at least five substitutions of highly conserved amino acid residues in the MYB domain indicates that Bl6 is most probably a pseudogene (see Supplemental Figure 1 online). This conclusion is corroborated by the phenotype of a mutant carrying a deletion of both Bl2 and Bl6 (c-2; see below), which is indistinguishable from that of other Bl2 loss-of-function mutants.

Phylogenetic analysis of either the full-length amino acid sequences or the MYB domains alone did not show any reliable topology beyond the above described pairs, and bootstrap values for most of the nodes were low. A one-to-one correlation between tomato and Arabidopsis was detected for only one pair of genes (MYB36 and Bl8). Another method to elucidate the relationships among genes employs our knowledge of the evolutionary history of both plant species. Most eudicots originated from an ancient hexaploid progenitor (Van de Peer et al., 2009). During subsequent evolution, the Brassicaceae underwent two rounds of WGD (Simillion et al., 2002), tomato had one WGD (Fawcett et al., 2009) and some species (e.g., grape) had no additional WGD, so their genomes still represent the ancient hexaploid state (Figure 1C). Following each WGD, chromosomal rearrangements, loss of gene copies, local gene amplification, and other events led to differentiation of the duplicated chromosomes. To elucidate orthology relationships, we determined collinearity of the genomic regions spanning the nine tomato and the six Arabidopsis Bl genes to the grape genome. This was done by running BLASTP (Altschul et al., 1990) comparisons of the Bl gene-containing tomato and Arabidopsis chromosome segments to grape proteins and selecting the top five hits for each protein (see Supplemental Data Set 1 online). Hits between all chromosome pairs were plotted, and collinear regions were visually detected as diagonals (Figure 1A; see Supplemental Figure 2 online). In each case, a single grape genomic region clearly had the highest density of conserved gene hits, and in most cases, two additional regions with lower conservation could be identified (Figure 1B; see Supplemental Figure 2 online). Similar recent rearrangements in closely related species have been reported, for example, in the genus Solanum (Livingstone et al., 1999; Doganlar et al., 2002).

These comparisons suggest that the progenitor of the core dicots before hexaploidization possessed four Bl genes. Amplification during the subsequent rounds of genome duplication was coupled to frequent gene losses. Four collinear groups with diverse gene copy numbers could be identified in each species.
Within the first group, RAX1 is the single Arabidopsis gene derived from the same progenitor as Bl, Bl/2, and Bl/6; therefore, RAX1 is the ortholog of these three paralogous tomato genes (Figures 1A and 1C). Furthermore, RAX2 and Bl/1 are orthologs and originated from a gene that was a paralog of Bl/2/Bl/6/RAX1 in the hexaploid ancestor. The fact that the genomic segments harboring Bl and Bl/2/Bl/6 share a higher density of conserved genes with each other than with genomic regions of Arabidopsis or grape, respectively, indicates that they originated during the genome duplication predicted for the Solanaceae (Figure 1C). In a similar way, orthology relations of the other genes could be resolved.

Analyses of microsynteny suggest that Bl/2 is the strongest candidate to have a redundant function to Bl. Additionally Bl/1, as the ortholog of RAX2, along with Bl/3 and Bl/7 as orthologs to RAX3, are candidates for shoot branching regulators.

The Classical Tomato Trait C Is Caused by a Mutation in Bl/2

To unravel the function of Bl’s closest paralog, Bl/2, two independent mutants (bl/2-1 and bl/2-2) were isolated by TILLING (McCallum et al., 2000). Both mutant alleles affected the codon for the conserved Trp-58 in the MYB domain, which was changed to either TTG (coding for Leu) or TAG (stop codon), most likely
causing knock out mutations (Figure 2A). In populations segregating for either mutation, plants with simpler leaves cosegregated with the homozygous mutant allele. This leaf morphology was reminiscent of the phenotype of the classical mutant C (for cut leaf; Price and Drinkard, 1908; Figures 2B to 2D).

To test the hypothesis that mutations in Bli2 cause the C phenotype, we sequenced the Bli2 gene in different C alleles. Indeed, all tested alleles carry lesions in Bli2 (Figure 2A; see Supplemental Table 1 online). In the classical allele c-1, a Rider/Kielia retrotransposon insertion disrupts the 3’ region of the Bli2 open reading frame. The complete 4867-bp sequence of the transposon is identical to the elements indentified in the Sun1642 fruit shape, fe inefficient, and yellow flesh mutants (Cheng et al., 2009). The c-2 allele contains a 40,580-bp deletion that removes

Figure 2. Bli2 Is C, a Key Regulator of Leaf Architecture.

(A) Mutations (indicated in red) in Bli2 in different c alleles. Coding sequences are represented by boxes, and introns are represented by lines. The MYB domain is shown in blue.

(B) Number of leaflets initiated in leaves along the primary shoot of the c-2 mutant (green) and the wild type (Moneymaker; purple), demonstrating the heteroblasty of both genotypes and a strong reduction of complexity in c leaves. Position 1 corresponds to the first leaf formed after the cotyledons (error bars: confidence interval = 0.05; n = 22).

(C) Penultimate leaf of the primary shoot of the wild type (wt) (Moneymaker) and c-2, showing reduced leaflet formation, fusion of the distal three leaflets, and lack of lobing and serration in the c-2 leaf.

(D) Scanning electron micrograph of a leaf primordium of the c-2 mutant showing the fusion (arrow) of the lateral leaflet primordium with the leaf blade of the terminal leaflet.

(E) and (F) Close-up of the leaf tip of a c-2 mutant, showing a fusion of a terminal leaflet and lateral leaflets from the top (E) and the bottom (F).

(G) and (H) Scanning electron micrographs showing that formation of lateral leaflets in leaf primordia (P4/P5) of c-2 mutants (H) is severely delayed compared with leaf primordia of equal length of wild-type (Moneymaker; [G]) plants. Lateral leaflet primordia (1 and 2) and leaf lobes (LB) are indicated. Bars = 2 cm in (C) and (E), 1 mm in (D), and 100 μm in (G) and (H).
the complete C gene and two neighboring genes. In the c-3 allele, the splice acceptor site of the second exon is mutated, and the start codon in c-4 is changed to ATA. The c-5 and c-integerrima alleles carry changes of conserved amino acids in the MYB domain. Furthermore, the simple leaf mutant coalita (cIt) (Stubbe, 1972) also carries an amino acid substitution in the MYB domain of Bli2. Finally, genetic analyses confirmed that bli2-1 (c-z1) and bli2-2 (c-z2) as well as cIt are allelic to c. F1 plants obtained by crossing these three mutants to c1 or c2 displayed the potato leaf phenotype.

C was a well-known trait in tomato breeding used in the 19th and 20th centuries. Today, potato-leafed cultivars, like the popular beefsteak cultivar Brandywine and the old elite cultivar Matina, are still used by home growers and sold at farmers’ markets. Analyzing the c gene in 10 of these cultivars (see Methods) revealed that they carry the classical allele c-1, confirming that C had its origin in breeding. Additionally, we identified a new allele of C in the cultivar Yellow Submarine (c-ys), a 286-bp deletion in the 3’ region of the gene (Figure 2A; see Supplemental Table 1 online). In total, 10 C alleles were characterized molecularly, identifying seven single nucleotide polymorphisms, two deletions, and one transposon insertion. Thus, C, Bli2, cIt, and potato-leafed heirloom cultivars are allelic mutants.

C Regulates Leaf Complexity

The complexity of tomato leaves (Kessler and Sinha, 2004) is a plastic trait dependent on growth conditions and cultivar background. The number of leaflets per leaf can vary drastically between isogenic plants grown in different experiments (cf. Figure 2B and Supplemental Figure 3A online). Additionally, cultivars such as the greenhouse tomato Moneymaker produce much simpler leaves than the field tomato M82. However, most importantly, the complexity depends on the position of a leaf along the shoot axis, where complexity increases acropetally from the first leaf to the penultimate leaf of the primary shoot (Figure 2B; Shani et al., 2010). c mutants show reduced leaf complexity compared with the wild type, with ~70 to 80% fewer leaflets, and the leaf blade margins are smooth, lacking lobes and serrations. As in the wild type, the basal leaves of c mutants are simpler than the more apical leaves (Figures 2B and 2C; see Supplemental Figures 3A and 3B online). For example, in a c mutant plant, the first two leaves after the cotyledons are usually entire, with no indentations at all; however, the adult leaves do make some leaflets and indentations, although many fewer than the wild type. Thus, heteroblasty is still present in c mutant plants, but the dissection of leaves is severely reduced at all stages. All mutant alleles caused a similar reduction in leaf complexity, except c-int, whose defects were milder (see Supplemental Figure 3B online). Differences were also observed depending on the cultivar background and the growth conditions but were always similar relative to the control (see Supplemental Figure 3A online). Besides the defects in leaf development, c plants develop normally, as would be expected for a trait used in breeding.

Early development of c leaf primordia was analyzed using a stereomicroscope and scanning electron microscopy. Leaflets of c mutant leaf primordia emerge and develop with a delay compared with equally sized wild-type leaf primordia (Figures 2G and 2H; see Supplemental Figure 3C online). Alternatively, c leaf primordia elongate faster than those of the wild type. A similar defect was found in the simple leaf mutant solanifolia (sf; Sinha, 1999). Furthermore, c mutants exhibit fusions of the terminal leaflet with the two distal lateral leaflets, which are already visible at early stages of development (Figures 2D to 2F). Taken together, the c mutant phenotypes of fusions, reduced leaflet number, and smooth margins are very similar to the defects seen in gob mutant leaves (Figures 6A to 6C; Berger et al., 2009).

Bli1, Bli2, and Bli3 Regulate Shoot Branching

To learn more about the functions of the Bli/C gene family, we used RNA interference (RNAi) to reduce the expression of two genes, Bli1, the closest paralog of Bli and C, and Bli3, a close relative of the Arabidopsis branching regulator RAX3 (Müller et al., 2006). The resulting Bli1 and Bli3 RNAi plants frequently lacked side-shoots, the branches that form in the leaf axis; this phenotype was most pronounced between the second and sixth leaf of the primary shoot (Figure 3; see Supplemental Figure 4A online). Leaf axils of wild-type plants almost always harbored auxiliary shoots (99.4%, n = 350 leaf axils). For the RNAi lines, phenotype penetrance varied between experiments, a characteristic that was also described for the branching phenotype of bli (Schmitz et al., 2002). In three independent experiments, transgenic populations lacked 2 to 10% of the side-shoots within the first seven leaves for Bli1 RNAi and 2 to 5% for Bli3 RNAi (n = 12 to 80 plants/population). Double Bli1/Bli3 RNAi plants (three populations, n = 15, 21, and 24) exhibited an enhanced branching defect with 5 to 40% of the first seven leaf axes lacking side-shoots, indicating that Bli1 and Bli3 act redundantly. In the same experiments, bli and Bli RNAi (Schmitz et al., 2002) populations lacked side-shoots in 40 to 70% of the first seven leaf axils (n = 12 to 18). Except for the most basal node, the relative distribution of barren leaf axils in bli and Bli RNAi populations was complementary to that observed in Bli1 and Bli3 RNAi plants (Figure 3; see Supplemental Figure 4B online).

Besides leaf axils completely lacking auxiliary buds, some bli mutants as well as Bli1 and Bli3 RNAi plants developed determinate organs: pin-like structures, reduced leaves, or normal leaves (Figure 3; see Supplemental Figure 4C online). Such terminating auxiliary structures were not observed in the wild type. Furthermore, bli also affects organ separation, similar to c. For example, bli mutants developed concaulescent fusions of side-shoots, mainly affecting the fast developing sympodial shoot (see Supplemental Figures 5A to 5D online; Schmitz et al., 2002). Thus, Bli, Bli1, and Bli3 regulate the initiation of AMs in a zonal and partially complementary fashion, similar to RAX1, 2, and 3 in Arabidopsis (Müller et al., 2006).

Bli and C Are Expressed in Prospective and Actual Boundaries

The Bli and C genes originated from a recent duplication event but have distinct mutant phenotypes; this contrast raises the question of their expression patterns. Quantitative RT-PCR (qRT-PCR) analysis revealed that both genes are expressed in...
the shoot apex but not, or at very low levels, in mature leaves (see Supplemental Table 2 online). RNA in situ hybridizations on tomato shoot tips detected C transcripts in young leaf primordia (Figures 4A to 4F; see Supplemental Figure 6 online), with expression strongest in P2 to P4 and no hybridization signal observed outside of leaf primordia. C was expressed in three well-defined domains: (1) at incipient lateral leaflet primordia preceding organ emergence (Figures 4A to 4F), (2) at the proximal and distal boundaries of young leaflet primordia (Figures 4A to 4E; see Supplemental Figure 6 online), and (3) in the sinuses of developing leaf lobes (Figure 4A; see Supplemental Figure 6 online). The different expression domains were ball- to band-shaped with one to six cells of strong expression in the largest expanse and were just below the outermost cell layer. Thus, C expression marks prospective and actual boundaries during compound leaf development. Therefore, both the phenotype of c and the pattern of C expression in leaf primordia coincide with that of Gob (Blein et al., 2008; Berger et al., 2009).

Bli1 and Bli3 transcripts could not be detected by RNA in situ hybridization, probably due to the detection limits of the technique. Bli1 expression was detected in prospective and actual boundaries in the shoot apex, just like C expression in leaf primordia. During the vegetative phase, Bli1 mRNA accumulated at the position of incipient leaf primordia (P0), preceding organ emergence (Figures 4G and 4H; see Supplemental Figure 7 online). This expression domain usually comprised two to six cells in all three dimensions. Furthermore, Bli1 was strongly expressed adaxially of the leaf primordia. In transverse sections, this oval- to band-shaped domain covered at least half of the boundary between the SAM and the leaf primordium (Figure 4H; see Supplemental Figure 7 online). The hybridization signal usually extended from the L1 through all cell layers of the SAM, fading out in the region where cells start to become vacuolated (Figure 4G; see Supplemental Figure 7 online). Expression of Bli1 was detected from P0 to the oldest leaf axil analyzed (P6) (see Supplemental Figure 7 online). Bli1 expression was also detected on the adaxial side of young AMs, separating them from the parental shoot, which correlates with the function of Bli1 to prevent concaulescent fusions (see Supplemental Figure 5E online). In summary, C and Bli1 and Bli3 are expressed in different organs according to their functions but show similar domains of activity predicting boundaries and organ formation in leaf primordia, shoot apices, and leaf axils.

The fact that Gob and the MYB genes Bli1 and C are expressed in the boundary zones essential for the formation of AMs and leaflets prompted us to test whether the branching regulator Ls is also expressed in leaves. Although mutations in the Ls gene manifest only in a lack of AMs, Ls mRNA accumulates in distinct domains of the leaf axil and at the distal boundary of leaflets (Figures 4I to 4L). This finding supports the view that the two domains have a similar identity.

**Bli1 and C Show Functional Conservation**

The expression studies suggest that Bli1 and C have undergone sub- or neofunctionalization due to promoter differentiation. The almost identical amino acid sequence of the Bl and C DNA binding domains (see Supplemental Figure 1 online) indicates that both proteins target similar downstream genes. To examine Bli1 and C function, we made transgenic tomato plants overexpressing Bl1 or C and found that plants with enhanced expression of Bli1 or C displayed similar developmental defects characterized by retarded growth and delayed development (Figures 5A and 5B; see Supplemental Figure 8 online). All organs remained smaller throughout development. Scanning electron microscopic analysis of the dwarf 3SS:Bli1 plants showed that epidermal cells were either reduced in length (rachis cells) or in number (internode cells; Figures 5C to 5F). Leaf lobes and serrations were more elaborated with sharper lobe and serration tips in both C- and Bl1-overexpressing lines, suggesting that Bl1 can also regulate leaf dissection (Figures 5A and 5B). A lack of functional promoter constructs for both Bl1 and C hindered successful promoter swap
RNA in situ hybridizations with C [A] to [F], Bl [G] and [H], and Ls [I] to [L] antisense probes. Longitudinal [A], [C], [D], [G], [I], and [K] and transverse [B], [E], [F], [H], [J], and [L] sections through tomato shoot apices. Arrows mark the earliest expression domains detected for C and Bl, preceding the formation of lateral leaflets and AMs, respectively. Arrowheads point to expression domains in the distal (black) and proximal (red) boundaries of developing leaflets. The blue arrowhead in (A) points to the weak expression of C at an initiating leaf lobe groove. Asterisks indicate the SAM. Bars = 100 μm in (A) to (D), (G), and (H) to (K) and 50 μm in (E), (F), and (L).

(C) and (D) Two consecutive partial sections through an old leaf primordium showing a large lateral leaflet primordium. (E) and (F) Two cross sections through a stage 3 primordium (P3) separated by 56 μm.

(G) and (H) Bl mRNA accumulates in the axils of leaf primordia.

(I) to (L) Ls is expressed in the axis of leaf primordia [I] and [J] and at the distal boundary of leaflets [I] to [L].

C and Bl Act in Parallel to the Gob/KNOX Pathway

Tomato has a single ortholog of the Arabidopsis CUC1 and CUC2 genes, named Gob (Blein et al., 2008; Berger et al., 2009), and lacks the partially redundant CUC3 function. gob mutants show very similar leaf development phenotypes as c mutants: Both mutants exhibit loss of most second-order and intercalary leaflets and of leaf lobes and serrations (Figures 6B and 6C). Furthermore, the expression pattern of Gob (Blein et al., 2008; Berger et al., 2009) resembles the combined patterns of C and Bl (Figure 4). In addition, Gob also functions in organ separation. Identical to c, the three distal most leaflets of gob leaves are usually fused (Berger et al., 2009; Figure 6C). Because of these similarities, two questions arose: Do gob mutants have the same developmental defects as bl mutants, namely, the lack of AMs, and does Gob act in one pathway with the MYB genes C and Bl? In a total of 80 regenerated gob-3 shoots analyzed, all completely lacked axillary shoots with the exception of infrequently formed sympodial shoots. Leaf axils remained barren throughout the life span of these plants and did not produce any axillary structures (Figure 6D). This result demonstrates that Gob combines the regulation of two different processes: leaflet formation and AM initiation.

To test whether C and Gob act redundantly or in a hierarchical order, we performed double mutant analysis. Whereas c and gob single mutants display acropetally increasing leaf complexity (Figure 6I), c gob double mutants produced simple leaves throughout development, drastically altering the appearance of these plants (Figures 6G to 6I). The lack of lateral leaflets demonstrates that the two boundary genes are partially redundant, and, although expressed only in a very limited number of cells, their functions are essential for compound tomato leaf development. In agreement with the double mutant results, qRT-PCR analysis of C expression in leaf primordia of Gob-4d, a microRNA-resistant gain-of-function mutant (Berger et al., 2009), as well as of Gob in c tissues, did not reveal any regulatory interaction between the two genes (see Supplemental Table 3).
mutants displayed simple c-like basal leaves (Figure 6J); however, from leaf four onwards leaf complexity increased, similar to Me/+ (Figure 6K; see Supplemental Figures 9A to 9D online). The double mutants initiated many higher order leaflets, but the leaflets showed characteristics of c mutant leaves, like fusions of leaflets and smooth margins (Figure 6K; see Supplemental Figure 9B online). In summary, the Me/+ c double mutants showed pheno-
typic characteristics of both single mutants, suggesting that C and Me/TKn2 act independently to influence leaf complexity. This is supported by qRT-PCR analyses showing that expression of Me/ TKn2 was not significantly altered in c leaves (see Supplemental Table 3 online). Finally, we observed that Me/+ enhanced branch-
ing. In contrast with the wild type, Me/+ plants frequently produced accessory side-shoots, indicating that together with Gob, Me also plays a role in both leaf development and AM initiation (see Supplemental Figure 9E online).

C Acts in an Independent Pathway Regulating Leaf Dissection

To check for genetic interactions of c with other known leaf complexity regulators, double mutants with e, trifoliata (tf), and Lanceolate (La), were analyzed. e is characterized by a reduced number of distinct leaflets due to an excess of lamina outgrowth (Zhang et al., 2007; Berger et al., 2009; Figures 7A and 7B). The different defects in e and c mutants are already obvious in basal leaves (Figure 7A). In double mutants, basal leaves showed no lobing and no serration; also, leaflets were further reduced compared with both single mutants (Figure 7A). Similarly, adult c e double mutant leaves were simpler than leaves of both parents, lacking most leaflets, lobes, and serrations, which resulted in an enhanced simple leaf defect (Figure 7B).

tf leaves usually develop a barren rachis with two lateral leaflets at the distal end (Figure 7C). c tf plants mostly produced completely simple leaves, lacking leaflets, lobes, and serrations (Figure 7C). Fifth and sixth leaves of c-z2 tf and c-2 tf double mutants (n = 12 and 20) produced no lateral leaflets, whereas tf leaves (n = 19) initiated 2.0 ± 0.4 lateral leaflets. Few adult leaves of the double mutants developed rudimentary lateral leaflets.

Heterozygous and homozygous La mutants develop leaves with only a few small leaflets and lobes (Ori et al., 2007). In La/+ c (Figure 7D) and La/La c (see Supplemental Figure 9F online) double mutants, these small leaflets and lobes were missing, resulting in an even simpler leaf phenotype. In summary, c enhanced the simple leaf phenotypes of gob, e, tf, and La and modified the defects of Me/+ . The lack of epistasis demonstrates that C acts in a previously unknown pathway regulating leaf dissection, independent of other described leaf complexity reg-

DISCUSSION

Uncovering the Molecular Cause of a Classical Trait

“Potato leafed” is an old and well known trait in tomato breeding, discussed in the scientific literature by White (1901) and de-
scribed as a recessive Mendelian gene by Price and Drinkard

![Figure 5. Protein Function Is Conserved between Bl and C.](image-url)
In this work, we show that C is an R2R3 MYB transcription factor and the closest paralog of the branching regulator Blind. We identified a Rider retrotransposon disrupting the C gene in the classical allele c-1 and lesions in nine other alleles of C. Rider elements originated from a horizontal gene transfer into tomato (Cheng et al., 2009). Outside of the Lycopersicon section, the closest homologs are found in Beta vulgaris and Arabidopsis, but not in other Solanaceae or in any other Asterids, as determined by searching the National Center for Biotechnology Information (NCBI) nr, htgs, gss, and wgs databases (http://blast.ncbi.nlm.nih.gov). Interestingly, the element found in c-1 is identical in sequence (4867 bp) to the Rider elements responsible for mutations in the genes Sun, Fer, and R (Cheng et al., 2009). This active Rider element (Jiang et al., 2009) may also be responsible for some of the many spontaneous mutants collected at the Tomato Genetics Resource Center (TGRC).

Doganlar et al. (2002) proposed C as a quantitative trait locus candidate gene influencing the difference in leaf morphology between the simple leaved eggplant (Solanum melongena) and its wild relative Solanum linnaeanum, which is strongly lobed and serrated. Furthermore, it was speculated that C might cause the leaf phenotype of potato (Solanum tuberosum) plants, as the gene name indicates. However, we found intact copies of C in eggplant and in potato, contrary to this hypothesis (see Supplemental Figure 1 online). Furthermore, C is not likely to be the gene responsible for these leaf form differences, considering the fusion phenotypes seen in c mutants but not in potato or eggplant leaves. The tomato gene Sf, with yet unknown molecular identity, might be a much better candidate, as sf mutant leaves are indeed highly similar to those of potato plants.

**Initiation of Leaflets and Shoot Branches Depends on Homologous Gene Modules**

This work revealed that identical and homologous boundary genes are essential for the initiation of AMs and the initiation of

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**Figure 6. Gob and Me Act Redundantly to C and Bl.**

(A) to (C) Adult leaves of wild-type (wt) (M82), c-z1, and gob plants.

(D) gob mutant plant lacking axillary shoot formation (arrows).

(E) to (H) Plants of indicated genotypes. Leaflets are formed in c-2 and gob mutants but are completely lacking in the c-2 gob double mutant (E), (F), and (H), top-down views; (G), side view.

(I) Leaflets initiated in gob (magenta), c-2 (blue), and c-2 gob (green) mutants. The “1” reflects the first leaf formed after regeneration from hypocotyl (n = 16, 15, and 34, for gob, c, and double mutants, respectively; the length of the lines corresponds to the average leaf number before flowering for each genotype, error bars, confidence interval = 0.05).

(J) Second leaf after cotyledons of wt, c-2, Me/+; and c-2 Me/+ plants. c is epistatic over Me/+ at this stage.

(K) Sixth leaf of Me/+; c-2 Me/+, and c-2 mutant plants displaying an epistasis of Me/+ in the double mutant.

Bars = 2 cm in (A) to (D) and (J) and 5 cm in (E) to (H) and (K).
lateral leaflets (Figure 8), which was unexpected considering the
obviously distinct nature of leaflets and axillary shoots. Collin- 
earity analyses revealed that a recent genome duplication in
tomato evolution led to the establishment of the two paralogous
genes, \(B_l\) and \(C\), regulating branching and leaflet initiation,
respectively. Functional differentiation between \(B_l\) and \(C\) 
appears to be based on different promoter activities, as our data
demonstrate that \(B_l\) and \(C\) have distinct mRNA expression
patterns but conserved protein functions. We can only speculate
about whether the ancestral gene had both functions (i.e.,
whether the duplicated genes have undergone subfunctional-
ization or whether one underwent neofunctionalization).

Indeed, another boundary gene unites both promoter activities
and gene functions, indicating that \(B_l\) and \(C\) may have become
subfunctionalized. This other gene, \(Gob\), is a leaf complexity
regulator (Blein et al., 2008; Berger et al., 2009), and we showed
that \(Gob\) is also essential for axillary shoot formation, \(gob\)
mutants have severe defects in leaflet initiation and completely
fail to initiate vegetative AMs. \(Gob\) orthologs in the \(NAM/CUC\)
family play a conserved role in compound leaf development in
Aquilegia caerulea, Cardamine hirsuta, Pisum sativum, and
tomato (Blein et al., 2008). In addition, this gene family also
regulates the formation of axillary shoots in several species
besides tomato. \(NAM\) together with its paralogs in Petunia
hybrida (Soyer et al., 1998), \(CUP\) in Antirrhinum majus (Weir
et al., 2004), and \(CUC1, CUC2,\) and \(CUC3\) in Arabidopsis (Hibara
et al., 2006; Raman et al., 2008) are all crucial for the formation
of AMs. \(Gob\) is the first example where a \(NAM/CUC\) orthologous
function simultaneously regulates AM formation and compound
leaf development within the same species.

The \(B_l/C\) gene pair and the \(Gob\) gene provide evidence for the
presence of homologous functions regulating AM and leaflet
initiation. Additional observations support this link between the
two processes and will therefore be the subject of future
research, as they suggest that a larger set of genes is involved
in this common mechanism. For example, \(Ls\), a major regulator
of axillary branching, is expressed in leaflet axils, similar to
\(Gob\) and \(C\). Furthermore, we observed several correlations between
leaflet and axillary branch formation, although we do not know
the molecular basis for this phenomenon. For example, the
tomato cultivar M82 produces both more accessory side-shoots

![Figure 7. C Action Is Independent of e, tf, and La.](image)

(A) and (B) Additive phenotypes of c and e in second (A) and adult leaves
(B). (C) and (D) Leaves of tf single and tf c double mutants ([C], fifth leaves)
and of heterozygous La ([La/+] single and La/+ c double mutants ([D],
adult leaves). Both double mutants showed strongly enhanced simple
leaf phenotypes, almost completely lacking lateral leaflet initiation.
Bars = 2 cm.

Figure 8. Boundary Genes Regulate Both AM and Leaflet Initiation.
The transcriptional regulators \(B_l, C, Gob,\) and \(Ls\) are expressed in regions
where AMs (\(B_l, Gob,\) and \(Ls\)) and leaflets (\(C, Gob,\) and \(Ls\)) will be initiated.
Loss of \(B_l, Gob,\) or \(Ls\) function compromises AM formation. Lack of \(C\) or
\(Gob\) expression leads to a reduction in leaf complexity; \(gob\) double
mutants have almost completely simple leaves.
and more leaflets compared with the cultivar Moneymaker. Additionally, we were able to identify a number of tomato mutants, such as tf, that display reduced leaf complexity and reduced AM formation simultaneously. Molecular analyses of these observations will further extend the understanding of the commonalities of AM and leaflet initiation.

All three types of boundary genes, Gob, Bl/C, and Ls, may have at least one function in common: growth repression. Gob-4D mutants, expressing a microRNA-resistant transcript, exhibit retarded growth (Berger et al., 2009) similar to plants misexpressing Bl and C (see Supplemental Figure 8 online) and similar to 35S: Ls plants (G. Schmitz and K. Theres, unpublished). Repression of growth is an obvious requirement at boundary regions, which separate two areas of high growth rates (Hussey, 1971). Interestingly, the expression of the boundary genes Bl/C, Gob, and Ls precedes the physical formation of boundaries and probably defines these areas of inhibited growth, enabling organ separation. All the respective mutants display organ fusions. Furthermore, this repression seems to keep cells in an undifferentiated state with the potential for new morphogenesis, as bl, c, gob, and Ls lose organogenic potential, lacking leaflet and/or AM initiation.

A New Branch in the Leaf-Shoot Comparison

Our observations add a new branch to the recently discussed idea of leaf and shoot holomorphologies and to the leaf shoot continuum hypothesis (Sinha, 1999; Rosin and Kramer, 2009; Blein et al., 2010; Canales et al., 2010; Floyd and Bowman, 2010). Recent publications emphasize the homology between leaf primordium initiation at the shoot apex and leaflet primordium initiation at the marginal blastozone of young leaves: Polar PIN localization leads to auxin response maxima, inducing outgrowth of leaves and leaflets and adjacent CUC expression (reviewed in Rosin and Kramer, 2009). Our data demonstrate parallels between shoot formation and leaflet formation. The initiation of lateral meristems in leaf axils and the initiation of leaflets at the border of existing leaflets share common gene modules (Figure 8).

Even though the boundary genes Gob and Bl are expressed at prospective axillary positions before leaf primordia are detectable, no obvious defect in early leaf development were observed in the respective single mutants and in gob bl double mutants. They have normal phyllotaxis, and young leaf primordia largely resemble wild-type primordia. Defects are observed only later, affecting the initiation of new shoots from the boundary region, just like the defects of c and gob mutants affect leaflet initiation (Figure 8), suggesting that Gob and Bl are not involved in leaf primordium initiation.

Deep Homology or True Homology?

These data raise the question why two different structures like leaflets and AMs employ homologous modules in their morphogenesis. At least two possibilities are conceivable. First, a boundary gene set could have evolved for one of the processes and later during evolution been recruited for the other. This would be called deep homology, the co-option of a preexisting regulatory program or circuit (Gaunt, 1997; Shubin et al., 2009).

Homology is limited to the involved genes, but the resulting organs or structures do not share common ancestry. This seems very likely in the case of leaflets and AMs.

However, although highly speculative, another scenario is supported by the fossil record. True axillary branching is unique for seed plants (Galtier, 1999; Tomescu, 2006). Equally, seed plants evolved leaves independently from other plant lineages (Sanders et al., 2009; Tomescu, 2009; Floyd and Bowman, 2010). The development of both structural novelties may even have been intimately linked, as both probably appeared simultaneously, 375 to 345 million years ago, in the late Devonian and lowermost Carboniferous (cf. Stewart and Rothwell, 1993; Galtier, 1999; Meyer-Berthaud et al., 2000; Beerling and Fleming, 2007; Sanders et al., 2009). The earliest seed plants had leafless three dimensionally branched shoot systems generated by overtopped dichotomous branching from which leaves with leaflets have evolved. Although the fossil record is limited and difficult to interpret, axillary shoots probably coevolved with leaves and may also be derived from overtopped dichotomous branches. The idea that, like leaves and leaflets, axillary shoots were derived from unequal and overtopped dichotomous branches is supported by the fact that Gob, Bl, and Ls, like their orthologs in Arabidopsis, are all expressed already within the SAM. However, no matter what the exact origin of AMs might be, the likely common origin of leaves with leaflets and axillary shoots in ancestral seed plants indicates a homology of these structures at an ancient, very basal level. This would be true homology due to descent from a common ancestral structure, the branches of the leafless ancestor of seed plants.

METHODS

Plant Materials and Growth Conditions

Seeds of c-1 (LA3168), c-2 (3-345), c-3 (3-604), c-4 (3-609), c-5 (3-626), c-int (LA0611 and LA3728A), c-clt (LA2026), bl-2 (LA0980), La (LA335), Me (LA 324), and sf (2-311) were obtained from the TGRC (Davis, CA); c-z1 (e2978), c-z2 (e2986), gob-3 (n5126-m1), gob-4d (e0042), e-2 (n0741), tf-z (e0761), and M82 cultivar from http://zamir.agn.comell.edu/mutants (Menda et al., 2004); and MM from Kiepenkerl. gob-3, Gob-4d, e-2, e-2 c-z1, and e-2 c-z2 mutants were kindly provided by Naomi Ori (Rehovot, Israel). Seeds of the c-1, heirloom tomatoes Brandywine “Suddh Strain,” Oxacan Jewel, Stupice, and Malinowy Ozarowski were obtained from Tomatenundanderes (Eltendorf, Austria); Bloody Butcher, Black Sea Man, Black Pear from Semillas La Palma (El Paso, Spain); Matina, Olena Ukrainian, Brandywine Sherry, and seeds of the c-ys Yellow Submarine were obtained from Reinsaat KG.

Plants were grown under standard glasshouse conditions with additional artificial light (16-h photoperiod) when needed. Shoots of gob-3, c-2 gob-3, and bl gob-3 mutants were regenerated from wound-induced callus (Berger et al., 2009).

Phenotyping

Parts of the leaf, consisting of a continuous leaf blade and separated from other parts of the leaf by a piece of rachis, were counted as leaflets. Leaflet sizes ranged from a few millimeters to 20 cm. Whenever “initiated” or “lateral” leaflets are mentioned, this by definition excludes the terminal leaflet. “Adult leaves” indicates leaves that were generated three plastochrons prior to flowering or later. For phenotypic comparisons, sister
plants from segregating populations were used when possible. In addition, different alleles and/or backgrounds were used. In all experiments, at least one biological replicate was performed. bl-2 and wild-type plants in Figure 3 are from segregating F2 populations (cv LUXMM), and Bil1 Bil3 double transgenic plants are from an F1 population. gob-3 mutations in Figures 6E to 6H had a mixed background (MM:M82, 1:1), and gob c-2 double mutants were from an F3 population of c-2 (MM) crossed with gob-3 (M82). Cultivar abbreviations are used as in TGRC databases. Scanning electron microscopy was performed on a DSM 940 (Zeiss) using fresh tissue.

TILLING

To perform the screening by TILLING (McCallum et al., 2000), we exploited the tomato ethyl methanesulfonate–mutagenized population of M82 (Menda et al., 2004). Screening for mutations in the coding sequence of Bil2 was performed as described by Piron et al. (2010).

Characterization of C Alleles

The Rider element insertion in c-1 was identified by inverse PCR (iPCR) using C-specific primers (see Supplemental Table 4 online). Sequence analysis revealed that the Rider element in c-1 is identical to the SUN-Rider element (accession number EU195798). A tandem transposon insertion cannot be excluded, as a single PCR spanning the complete insertion was not possible. Transposon sequences were amplified using Advantage GC 2 polymerase (Clontech Laboratories).

The genomic flanking sequence of c-2 was obtained by iPCR and fosmid and BAC sequencing. Publically available genome sequence data enabled the final identification of the 40,580-bp deletion.

The provisional alleles c-prov2, -3, -4, and -5 were confirmed as c alleles. 3-631 (c-prov6) was found not to be allelic to c-4.

DNA Sequencing and Analysis

Plant DNA was prepared as described (Brandstädt et al., 1994) or using the DNeasy 96 plant kit and BioSprint 96 automated DNA extraction apparatus (Qiagen). Standard techniques were performed according to Sambrook and Russel (2001), unless otherwise stated. PCR and iPCR products were treated with ExoSAP-IT (USB Corporation) prior to sequencing. PCR sequences were determined by the Max Planck Genome Centre Cologne on Applied Biosystems ABI Prism 377, 3100, and 3730 sequencers using BigDye-terminator v3.1 chemistry. Planck Genome Centre Cologne on Applied Biosystems ABI Prism 377, 3100, and 3730 sequencers using BigDye-terminator v3.1 chemistry.

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RNA in Situ Hybridization

Sample preparations and in situ hybridizations were performed as described by Müller et al. (2006). The C and Bil antisense probes contained the nucleotides +909 to +338 and +948 to –2, respectively, relative to the ATG. The C probe was transcribed using T7 RNA polymerase (Ambion) from Affil-linearized pCR-Blunt-II-TOPO vector (Invitrogen) and Bl probe from Ndel-linearized pGEM-T-Easy (Promega). A PCR product containing the nucleotides +112 to +1224 relative to the ATG was used as template for synthesis of an Ls antisense probe (primers Lsfo and LserX; see Supplemental Table 4 online). Slides were imaged using bright-field microscopy or differential interference contrast microscopy.

RNAi and Overexpression Constructs and Tomato Transformation

The T-DNA cassette of pJawohl17 (provided by Imre Somsich and Beir Ulker, Cologne, Germany) was cloned as a Pmel-RsrII fragment into the binary plant transformation vector pP2P212 backbone (GenBank accession number U10462; Hajdukiwicz et al., 1994), resulting in pJaZP. cDNA fragments of Bil1 (~54 to +892) and Bil3 (~56 to +1025) were cloned in reverse orientation into pDONR201 (Invitrogen) and subsequently into pLaZP using the Gateway cloning system.

For overexpression of Bi (Bi-ox), the Bi open reading frame was cloned as a NotI-Ascl fragment into pRT--/Not/Asc (Überlacker and Werr, 1996) between the cauliflower mosaic virus 3SS promoter/TMV- leader and the cauliflower mosaic virus 3SS polyadenylation sequences. This cassette was introduced into the Ascl site of plant transformation vector pGPTV-Kan-Asc (Überlacker and Werr, 1996). For overexpression of C, a genomic fragment starting 2456 bp upstream of the ATG and ending 746 bp downstream of the stop codon was introduced into the SalI/Sacl site of pGPTV-Kan (Becker et al., 1992). A double 3SS-enhancer element (327 bp each; Benfey et al., 1989) was cloned in reverse orientation into the SalI/SbfI site 5′ of the C promoter.

Agrobacterium tumefaciens–mediated transformation of tomato (Solanum lycopersicum) leaf explants of cultivar MM was performed as described (Knapp et al., 1994). At least four independent transgenic lines exhibiting similar developmental defects were characterized further. For double RNAi experiments, individual T-DNA insertion lines were crossed.

Collinearity Analyses

Genome-wide pairwise BLASTP alignments (Altschul et al., 1990) were performed using protein sequences from Arabidopsis thaliana (ftp://ftp.Arabidopsis.org/home/tair/Genes/TAIR9_genome_release/TAIR9_sequences/TAIR9_pep_20090619.tar), tomato (ftp://ftp.solenomics.net/geneomes/Solanum_lycopersicum/wgs/assembly/build_1.00/S.lycopersicum_scaffolds.1.00.fa.gz), and grape (Vitis vinifera; ftp://ftp.psb.ugent.be/pub/plaza/plaza_public_01/Fasta/). The five best BLAST hits for each protein were selected, irrespective of the e-values. Chromosomal regions containing members of the Bi subfamily in tomato (complete scaffolds) and in Arabidopsis (starting around 200 genes upstream of the corresponding MYB gene and ending −200 genes downstream; e.g., for RAX1, At5g23000, the region between At5g21000 and At5g25000) were used as queries. For each tomato or Arabidopsis region, the three grape chromosomes with the highest number of hits were selected (see Supplemental Data Set 1 online). Collinearity was visualized by plotting the positions of query and target genes.

qRT-PCR

Primordia of fourth and fifth leaves after cotyledons (at a size of 1 to 2 mm and 2 to 5 mm), 2-cm leaves (second leaf after cotyledons), and vegetative shoot apices were harvested. RNA was extracted with the RNeasy plant mini kit (Qiagen), including on-column DNase treatment (DNA-free kit; Applied Biosystems/Ambion) or a subsequent DNasel treatment (Roche Diagnostics). One microgram of RNA was used for cDNA synthesis with the RevertAid H Minus M-MuLV reverse transcriptase kit (Fermentas). qPCR was performed using the PowerSYBR-Green PCR Master Mix (Applied Biosystems). Primers are described in Supplemental Table 4 online. Relative quantification was done using internal standard curves and correcting by the use of reference genes (TIP41, GAPDH1, and CAC; Expósito-Rodríguez et al., 2008).

Accession Numbers

Sequence data from this article can be found in the Sol Genomics Network data library under the following accession numbers: Bi,
REFERENCES


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AUTHOR CONTRIBUTIONS


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