MAX4 and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea

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Shoot branching is inhibited by auxin transported down the stem from the shoot apex. Auxin does not accumulate in inhibited buds and so must act indirectly. We show that mutations in the *MAX4* gene of *Arabidopsis* result in increased and auxin-resistant bud growth. Increased branching in *max4* shoots is restored to wild type by grafting to wild-type rootstocks, suggesting that *MAX4* is required to produce a mobile branch-inhibiting signal, acting downstream of auxin. A similar role has been proposed for the pea gene, *RMS1*. Accordingly, *MAX4* and *RMS1* were found to encode orthologous, auxin-inducible members of the polyene dioxygenase family.

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Variation in shoot branching is an important cause of diversity in plant form. Individual species have a characteristic branching pattern, which can change through the life cycle in response to developmental cues and to environmental conditions (Cline 1991; Beveridge et al. 2003). Branching control therefore requires the integration of many signals, both known and unknown.

Shoot branches arise from axillary meristems that form in the axils of leaves on the primary shoot axis. The axillary meristems themselves initiate leaves to form a bud. Bud growth can arrest but has the potential to reactivate to produce a shoot branch. Removal of the primary shoot apex results in activation of arrested axillary buds. The ability of the shoot apex to repress axillary bud growth is termed apical dominance. Thimann and Skoog (1933) reported that a compound, derived from the shoot apex, and later identified as auxin (indole-3-acetic acid), could inhibit the growth of lateral buds when applied to the stump of a decapitated plant. Subsequent work has provided multiple lines of evidence in support of auxin-mediated bud inhibition in planta. However, a second messenger must relay the auxin signal into the bud because apically derived auxin is not transported into buds (Morris 1977) and exogenous auxin applied directly to buds does not inhibit their growth (Cline 1996).

One model proposes that the effect of auxin on bud growth is mediated by cytokinin. Cytokinin can directly promote bud growth (Cline 1991); transgenic plants with increased auxin levels have reduced cytokinin levels (Eklöf et al. 2000), and cytokinin export from roots increases after decapitation, with this increase being abolished by application of auxin to the decapitated stump (Bangerth 1994). However, there is also good evidence for novel regulators of bud growth downstream of auxin. The *ramosus* mutants (*rms1* to *rms5*) of pea (for reviews, see Beveridge 2000; Beveridge et al. 2003) have increased lateral branching, but this phenotype can be almost completely rescued by grafting a wild-type (WT) rootstock to an *rms1*, *rms2*, or *rms5* mutant scion. Such grafting studies show that *RMS1* and *RMS5* are required for the production of a graft transmissible signal that moves from root to shoot and inhibits branching (Foo et al. 2001; Morris et al. 2001). This mobile signal is unlikely to be auxin or cytokinin because, as well as increased branching, the *rms1* and *rms5* mutants have reduced root-derived cytokinin and have at least WT auxin levels and transport (Beveridge et al. 1997; Morris et al. 2001). This is exactly the opposite of the prediction for a bushy plant and may be the result of feedback regulation of auxin and cytokinin levels. It is possible that the *RMS1/RMS5*-dependent long distance signal is a second messenger for auxin. The lateral buds of *rms1* shoots can only respond to the inhibitory effects of apical auxin when grafted to WT rootstocks (Beveridge et al. 2000).

To identify genes that regulate bud growth in *Arabidopsis*, we screened mutagenized populations for plants with increased branching and have identified four loci, mutations at which result in more axillary growth, named *max1* to *max4* (Stirnberg et al. 2002). In this paper, we describe the phenotype of the *max4* mutant and the cloning of the *MAX4* gene, and show that this gene is orthologous to *RMS1*.

Results and Discussion

Isolation and genetic characterization of the max4 *mutants*

We have identified a class of *Arabidopsis* mutants with more axillary branches and placed them in four complementation groups named *max1* to *max4* (Stirnberg et al. 2002). Four independent recessive alleles were found at the *MAX4* locus in the Columbia (Col) ecotype. Two alleles (*max4-1* and *max4-2*) were isolated from the Sainsbury Laboratory Arabidopsis Transposant (SLAT) collection (Tissier et al. 1999), and two alleles (*max4-3*

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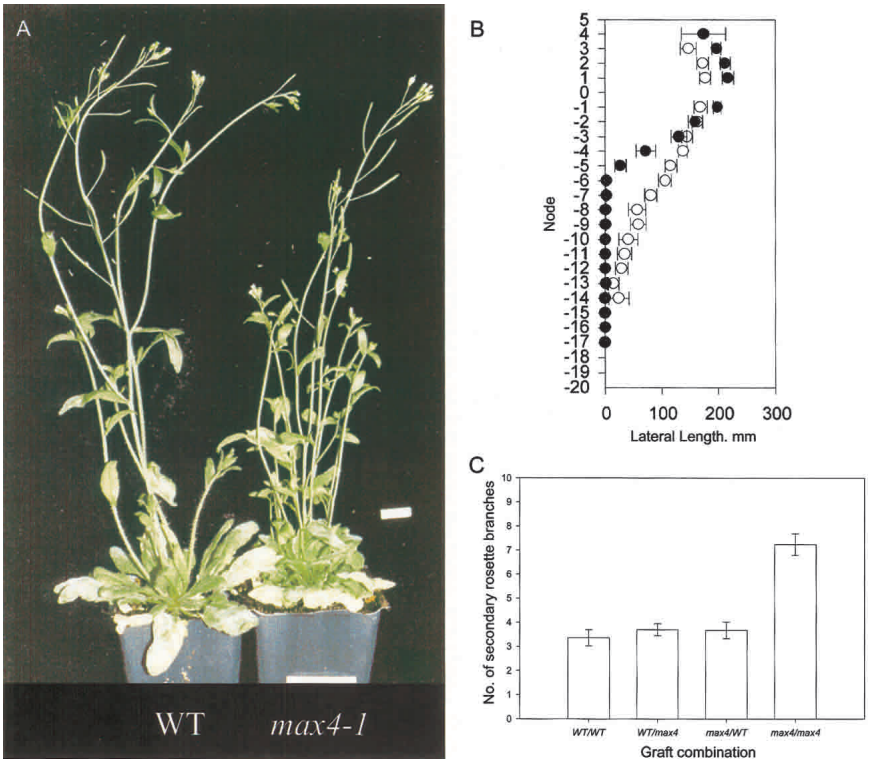

Figure 1. Shoot phenotype of *max4-1* mutant plants. (*A*) Wild-type (WT) and *max4-1* plants were grown under a 16-h long-day photoperiod for 3 wk. Bar, 1 cm. (*B*) Lateral bud and inflorescence lengths of WT (closed circles) and *max4-1* (open circles). Lateral lengths were measured when the primary shoot apex had ceased activity. Cauline nodes are numbered positively (5 youngest, 1 oldest) and rosette nodes are numbered negatively (–1 youngest, –20 oldest). Node number is aligned so that node 1 is the oldest cauline node. Error bars represent the standard errors of the mean; $n = 10$. (*C*) Mean branch numbers originating from the rosette of plants at maturity produced by hypocotyl grafting between WT and *max4-1* plants (shoot genotype/root genotype). Error bars represent the standard errors of the mean; $n = 4$–11.

and *max4-4*) were isolated from the AMAZE population (Wisman et al. 1998). There were no apparent differences in the severity of phenotype conferred by these alleles, and the *max4-1* allele was chosen for detailed phenotypic analysis, following two rounds of back-crossing to WT.

The max4-1 *mutant has increased shoot branching*

Mature *max4-1* mutant plants grown under a 16-h photoperiod have a bushy appearance at maturity as a result of increased growth of the buds in rosette leaf axils (Fig. 1A). In WT plants, all the cauline nodes and the most apical rosette nodes produced buds that developed into lateral inflorescences and, on average, the uppermost 5.1 ± 0.2 rosette nodes produced an elongated inflorescence >4 mm in length (Fig. 1B). A basipetal gradient of inflorescence lengths was observed, with branches arising in the youngest leaf axils having the greatest mean branch lengths. This gradient continued into older rosette nodes, where buds remained smaller than 4 mm in length. In most plants ($n = 7/10$) a weak acropetal gradient of bud growth was also observed, although these buds remained very small throughout the life of the plant. Between the acropetal and basipetal gradients were one to three nodes that carried tiny buds, or no bud visible to the naked eye (data not shown).

As in WT, all the cauline nodes of mature *max4-1* plants produced buds that developed into elongating inflorescences with the same basipetal gradient (Fig. 1A). However, a greater number of rosette buds developed into inflorescences compared with WT, on average 8.5 ± 0.4 (Fig. 1B). An acropetal gradient of bud growth was also observed in most of the *max4-1* plants ($n = 8/10$), but in contrast to WT, these buds grew out to form short inflorescences. Therefore, both the basipetal and acropetal patterns of bud activity are similar to WT in long-day-grown *max4-1* mutants, but the mutant buds are more likely to grow out.

We found no evidence that, unlike the *supershoot* mutant (Reintanz et al. 2001; Tantikanjana et al. 2001), but similar to the *Arabidopsis max1* and *max2* mutants (Stirnberg et al. 2001) and the *axr1* auxin-resistant mutant (Stirnberg et al. 1999), the *max4* mutations affect the number of axillary meristems formed at each node. Rather, the defect appears to be specifically in bud outgrowth. Furthermore, like *max1*, *max2*, and *axr1*, *max4* affects both the acropetal and basipetal gradients in a similar way.

MAX4 *expression in the roots is sufficient for WT shoot branching*

To determine the site of action of *MAX4*, we performed reciprocal hypocotyl grafting experiments (Turnbull et al. 2002). The self-grafted control plants reproduced branching phenotypes similar to intact controls, indicating that the grafting process does not affect branching (data not shown). Graft combinations with either a WT scion or a WT rootstock showed WT shoot branching patterns (Fig. 1C), indicating that, although the *MAX4* gene can act in the shoot to inhibit branching, expression in the root is sufficient for WT shoot branching levels. These data suggest that the *MAX4* gene is required for the production of a graft-transmissible inhibitor of shoot branching.

Auxin responses in the max4-1 *mutant*

We assayed the response of *max4-1* axillary buds to auxin using a split plate assay (Chatfield et al. 2000). Bud outgrowth from excised cauline nodes placed between divided agar sections in a Petri dish is inhibited by apically applied auxin. In this system, without hormone treatment, buds of WT and *max4-1* grew out with similar kinetics, with elongation commencing 2 d after node excision (Fig. 2A). Apical auxin inhibited WT bud outgrowth for an average of 6 d, whereas *max4-1* mutant buds were partially resistant to apical auxin, being inhibited for an average of only 4 d.

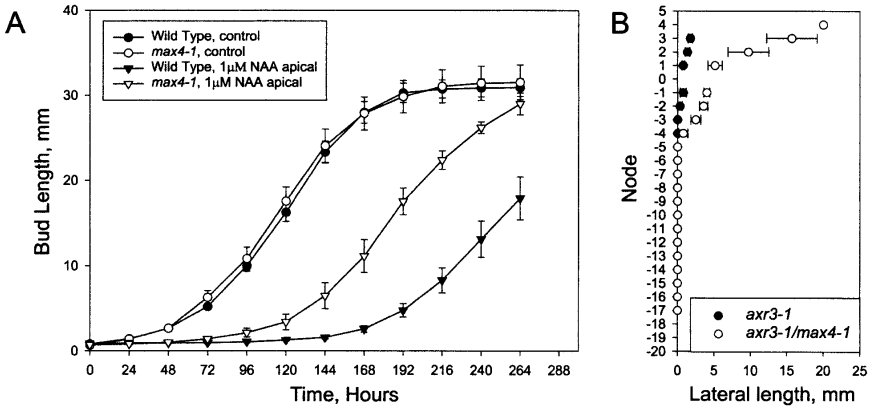

Figure 2. Altered auxin responses in *max4-1* mutants. (*A*) Lateral inflorescence outgrowth of excised *max4-1* and WT nodes in response to the synthetic auxin 1-NAA. The oldest cauline node was excised from WT (closed symbols) or *max4-1* (open symbols), and inserted between two agar blocks. The apical agar block contained either no 1-NAA (circles) or 1 μM 1-NAA (triangles). Bud outgrowth was measured every 24 h. Error bars represent standard error of the mean; $n = 11$ to 19. (*B*) Lateral bud and inflorescence lengths of *axr3–1* (closed circles) and *axr3-1*, *max4-1* double-mutant plants (open circles). Lateral lengths were measured 55 d after sowing, and the mean primary inflorescence height of the *axr3-1* and the *axr3-1*, *max4-1* double-mutant plants were not significantly different. Cauline nodes are numbered positively and rosette nodes are numbered negatively. Node number increases with proximity to the shoot apex. Node number is aligned so that node 1 is the oldest cauline node. Error bars represent standard error of the mean; $n = 7$.

To test further the role of auxin in the *max4-1* phenotype, we constructed double mutants between *max4-1* and *axr3-1*. The *axr3-1* mutation is a semidominant gain of function mutation resulting in an overresponse to auxin and reduced shoot branching (Cline et al. 2001). The *axr3-1*, *max4-1* double mutant was found to have increased lateral branch lengths in comparison with those of *axr3-1* mutant plants (Fig. 2B). This suggests that some of the dominant branch suppressing effects of *axr3-1* require *MAX4*, consistent with the idea that the graft-transmissible MAX4-dependent signal acts downstream of auxin to inhibit branching. In further support of this model, when double mutants were constructed between the loss-of-function auxin-resistant *axr1-12* mutant and *max4-1*, branching levels were no higher than those of the single mutants (data not shown).

MAX4 is not generally required for auxin response because, other than in shoot branching assays, the *max4-1* mutation had little or no effect on responses to exogenous auxin or *axr3-1* phenotypes (data not shown).

Molecular characterization of the MAX4 *gene*

The *max4-1* and *max4-2* lines were found to contain single transposon insertions that cosegregated with the mutant phenotypes (data not shown). DNA flanking the transposon in *max4-1* and *max4-2* was isolated by inverse PCR and both amplified fragments were found to be identical in sequence to parts of the same predicted gene (At4g32810; Fig. 3). When At4g32810 was sequenced from the *max4-3* and *max4-4* alleles, it was found to contain a four-base insertion and a two-base deletion, respectively (Fig. 3). These changes are predicted to result in premature termination of the encoded protein and are consistent with transposon excision footprints (Cardon et al. 1993). The fact that four independent *max4* alleles have mutations in this one gene provides strong evidence that this is the *MAX4* gene.

We isolated the *MAX4* cDNA by reverse transcription PCR (RT–PCR) of purified polyadenylated mRNA extracted from WT shoot tissue. The resulting cDNA contains a single open reading frame predicted to encode a protein of 570 amino acids (Fig. 3). The cDNA is identical to the coding region of an expressed sequence tag (EST) in the database.

The cDNA was introduced into *max4-1* mutant plants under the control of the cauliflower mosaic virus 35S promoter. Seventeen independent T1 plants from these transformations had a WT phenotype, which was stably inherited in the T2 generation (data not shown), confirming that *MAX4* is At4g32810. Interestingly, overexpression of *MAX4* from the 35S promoter in WT plants had no obvious phenotypic effect (data not shown). This suggests that either MAX4 is not the rate-limiting step in the synthesis of the graft-transmissible substance, or the levels of the substance are regulated posttranscriptionally, or the substance cannot inhibit branching below WT levels.

MAX4 belongs to the polyene chain dioxygenase family

Database searches using the predicted protein sequence of MAX4 (Altschul et al. 1990) show that it is a member of the polyene chain dioxygenase superfamily, and is likely to be localized to plastids (Emanuelsson et al. 2000). A phylogenetic analysis of family members from plants, animals, and bacteria is shown in Figure 4A (constructed using ClustalX 1.8 program; Thompson et al. 1997). MAX4, RMS1 (see below), and a rice sequence (*Os*MAX4) form a well-supported clade. The abscisic acid (ABA) biosynthetic protein, VP14, falls within another strongly supported clade that includes *Arabidopsis* carotenoid cleaving dioxygenases (CCDs, also called NCEDs) probably involved in ABA biosynthesis (*At*CCD2, *At*CCD3, *At*CCD5, *At*CCD6, and *At*CCD9; for review, see Seo and Koshiba 2002). The representatives of the animal RPE65, BETA DIOX1 and BETA DIOX2 proteins, form a third well-supported clade; and a fourth discrete group of proteins with similarity to bacterial lignostibene dioxygenases is represented here by two *Sphingomonas paucimobilis* proteins. The *At*CCD1 protein, which is not involved in ABA biosynthesis but cleaves beta carotene (Schwartz et al. 2001), and *At*CCD7 and *At*MAX4 are grouped on long branches.

These results suggest the attractive hypothesis that *MAX4* encodes a carotenoid-cleaving dioxygenase involved in the synthesis of a mobile branch-inhibiting substance. This substance is very unlikely to be ABA because of the lack of ABA-related phenotypes in *max4* mutants (data not shown) and the lack of requirement for ABA in auxin-mediated inhibition of bud outgrowth (Chatfield et al. 2000). Taken together, these data support the hypothesis that MAX4 is involved in the syn-

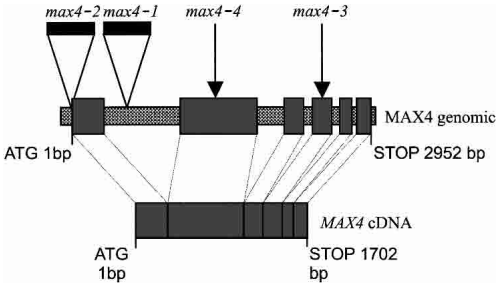

Figure 3. Diagram of *MAX4* genomic and *MAX4* cDNA showing the relative positions of the transposon-induced mutations. The *MAX4* cDNA consists of six exons (larger gray bars) separated by five introns in the genomic DNA (smaller hatched bars). The *max4-1* and *max4-2* mutations are caused by transposon insertions (black bars) and the *max4-3* and *max4-4* mutations (arrows) are caused by transposon footprints.

thesis of a novel hormone that acts downstream of auxin to inhibit shoot branching.

The MAX4 *gene is orthologous to the* RMS1 *gene of pea*

The similar phenotypes conferred by *max4* and the pea *rms* mutants prompted us to test whether any of the *RMS* genes were orthologous to *MAX4*. We isolated the pea ortholog of *MAX4* using degenerate primers based on amino acid alignment between MAX4 and the deduced amino acid sequences from two *Medicago truncatula* ESTs showing high homology with MAX4 [60% (77/127) and 62% (60/96) identity]. A pea gene was isolated encoding a 561 amino acid protein showing 68% identity with MAX4 across the 518 amino acids at the C terminus. Comparison of the RT–PCR-amplified cDNA and the corresponding genomic DNA revealed five introns for *PsMAX4* at the same position as in the *Arabidopsis* gene and of comparable sizes (740/918; 263/122; 88/110; 78/91; 70/89).

We mapped the *PsMAX4* sequence using a recombinant inbred line mapping population (Laucou et al. 1998). *PsMAX4* was found to map to the top of linkage group III at same position as *RMS1* (Blixt 1976). In an F2 population of 95 individuals (M3T-884 × Torsdag) segregating for *rms1*, complete cosegregation was observed between *PsMAX4* and *RMS1*. Southern analysis using *PsMAX4* as a probe revealed a 12-kb band present for WT progenitors Weitor and Raman and absent for mutant alleles *rms1-2* and *rms1-3* (Fig. 4B). The map position of *PsMAX4*, its deletion in two independent *rms1* alleles, and the similar phenotypes of *max4* and *rms1* mutants provide strong evidence that *RMS1* and *MAX4* are true orthologs.

Expression of the MAX4 *and* RMS1 *genes*

Transcripts for *MAX4* and *RMS1* are present at very low levels and are not readily detectable on Northern blots. However, they can be amplified by PCR from all tissues tested (data not shown). These widely distributed but very low expression levels for *MAX4* transcripts are supported by Affymetrix gene chip data, publically available from the Nottingham Arabidopsis Stock Centre. To determine more precisely the location of *MAX4* transcription, we constructed promoter–GUS fusions using 2.7 kb of DNA upstream of the MAX4 translational start site. This construct was introduced into WT Col plants, and GUS expression was analyzed using the chromogenic substrate X-Gluc. Strong expression was consistently observed in root tips (Fig. 5A). Other tissues such as hypocotyls and petioles occasionally showed very weak expression in some plants from each line (data not shown). Similarly, weak expression was variably observed in nodal sections associated with young axillary buds (Fig. 5C). No GUS staining was observed in the buds themselves, consistent with the remote site of action of MAX4 predicted by the grafting studies.

Because *MAX4* and *RMS1* are predicted to act downstream of auxin, we tested whether the transcription of either gene is affected by auxin. We used RT–PCR to investigate the expression of the *RMS1* gene in pea using the classical apical dominance test involving decapitation and replacement of the apex by exogenous auxin (Fig. 5D). Decapitation caused a substantial drop

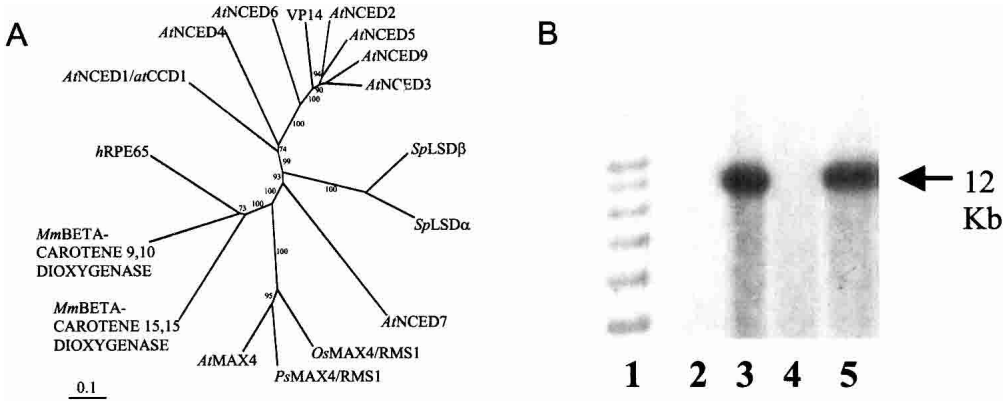

Figure 4. (*A*) Phylogenetic analysis of polyene chain dioxygenases. The unrooted phylogenetic tree was generated from multiple sequence alignments, using ClustalX and modified by eye. Positions with gaps were excluded. Numbers at branch forks represent bootstrap values as a percentage of 10,000 bootstraps, and give a confidence limit for grouping together the sequences. Proteins are labeled with a prefix that represents the species origin of the sequence: *At*, *Arabidopsis thaliana*; *Ps*, *Pisum sativum*, pea; *Os*, *Oryza sativa*, rice; *Sp*, *Sphingomonas paucimobilis*; *h*, human; *Mm*, Mus musculus, mouse. (*B*) Southern blot analysis of the *PsMAX4* gene in WT and isogenic *rms1* pea lines. Lanes *2–5* contain *Eco*R1-digested genomic DNA from *rms1-2*, Weitor, *rms1-3*, and Raman, respectively. Lane *1* contains a 1-kb ladder.

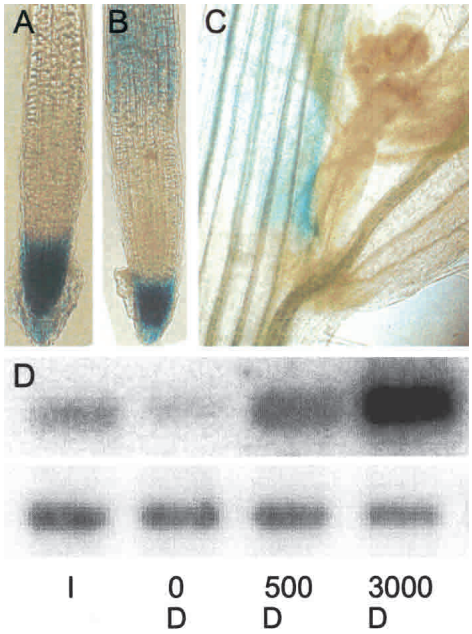

Figure 5. Root tip expression of the GUS reporter protein driven by 2.7 kb of DNA upstream of the *MAX4* gene. (*A*) Untreated 5-day-old seedlings. (*B*) Seedlings transferred to 1 µM 1-NAA for 24 h. (*C*) Nodal section showing expression close to a young bud, but not in the bud. (*D*, *top* panel) *RMS1* gene expression in internode 4 of 14-day-old plants, with 5 leaves expanded, that were intact (I) or 6 h after decapitation (D), treated with 0, 500, or 3000 mg/L^{-1} IAA to the decapitated stump. (*Bottom* panel) Actin gene expression was monitored as a control.

in *RMS1* expression within 6 h after treatment. This reduction was not only prevented by replacement of the apex with 500 and 3000 mg/L exogenous IAA, but *RMS1* expression was up-regulated compared with intact controls. In contrast, similar experiments in *Arabidopsis* using both RT–PCR and the promoter::GUS reporter lines failed to detect any up-regulation of *MAX4* transcript levels in stem sections in response to apical auxin (data not shown). However, 24 h after transfer of promoter::GUS seedlings to auxin-containing media (1 µM NAA), up-regulation of GUS expression was observed in regions of the root distal to the apparently constitutive root tip expression pattern (Fig. 5B). No such up-regulation was detected following 6 h exposure (data not shown).

These data suggest that auxin may regulate shoot branching partly through transcriptional up-regulation of *RMS1/MAX4*. In pea, this up-regulation occurs at the node and may be sufficiently rapid to inhibit bud growth in response to apical auxin. In *Arabidopsis*, however, no up-regulation was detected at the node, despite the fact that this is a major site for *AXR1* and auxin action in the regulation of bud growth (Booker et al. 2003). This, combined with the observation that *MAX4* action in the root is sufficient for WT branching, suggests an additional role for auxin downstream of the synthesis of the RMS1/MAX4-dependent signal. For example, auxin may regulate the transport of the RMS1/MAX4-dependent signal into axillary buds. A full test for this hypothesis awaits the identification of the novel MAX4/RMS1 dependent, branch-inhibiting signal.

Materials and methods

Plant growth conditions

Arabidopsis plants were grown in 4-cm square compartments (P40, Cookson Plantpak) containing F2 compost treated with Intercept 70WG (both Levington Horticulture) in a growth room with 16 h light (white light at 70 µmole/m^{-2}s^{-1}). Pea seedlings were grown under glasshouse conditions extended to 18 h light (incandescent light providing ~3 µmole/m^{-2} s^{-1} at pot top), as described by Morris et al. (2001).

Phylogenetic analysis

Sequences for phylogenetic analysis were retrieved from the NCBI database (see Supplemental Material). Sequences were aligned with ClustalX software (Thompson et al. 1997). The alignment was analyzed by eye, and regions with a low confidence of alignment were removed using Bioedit software (Hall 1999). The phylogenetic tree was generated using Neighbour Joining and a distance matrix with correction for multiple substitutions. Bootstrapping values were generated with n = 10,000. TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to visualize the tree.

Gene cloning

The *MAX4* gene was isolated using inverse PCR (iPCR) of *max4-1* and *max4-2* DNA (see Supplemental Material for details). The CaMV35S::*MAX4* cDNA construct was made using standard techniques (Sambrook et al. 1989; Supplemental Material). The *MAX4* promoter–GUS fusion construct was generated by PCR amplification of a 2.7-kb region upstream of the ATG including *Bam*H1 and *Xba*1 restriction sites in the primers. These sites were used to clone the fragment into the pBI101.1 vector. This plasmid was transformed into *Agrobacterium* and then into plants using the floral dip method of Clough and Bent (1998). Two typical lines containing a single site of transgene insertion were taken to homozygosity and used for detailed analysis.

Isolation of the pea MAX4 *homolog* PsMAX4

Degenerate primers were designed in consensus regions between the *Arabidopsis* MAX4 protein and the deduced amino acid sequence of two *Medicago truncatula* genes showing high homology with MAX4 (see Supplemental Material). These primers were used to amplify *PsMAX4* from both cDNA and genomic DNA. The amplification products were used to determine the sequence of the pea cDNA and the positions of introns in the gene (Brunel et al. 1999).

Mapping of PsMAX4

For mapping the *PsMAX4* sequence, a cleaved amplified polymorphic sequence (CAPS) marker was designed to detect a *Dra*I restriction site polymorphism between the two parents of our mapping population of 139 recombinant inbred lines (Laucou et al. 1998); Térèse and line K586 (isogenic to Torsdag; see Supplemental Material). This CAPS marker was also used for cosegregation analysis between *PsMAX4* and the pea branching gene *RMS1* in a population of 95 F2 individuals from a cross between the *rms1-10* mutant line M3T-884, obtained from Térèse by EMS mutagenesis (Rameau et al. 1997; Symons and Murfet 1997), and Torsdag. Linkage analysis was carried out using the MAPMAKER/EXP 3.0 computer program (Lincoln et al. 1992). Southern blot analysis of the two radiation-induced *rms* alleles was carried out using standard techniques (see Supplemental Material).

Ps-MAX4 expression study in pea

Fourteen-day-old cv. Torsdag plants (five leaves expanded, counting acropetally from the cotyledonary node as zero) were left intact or were decapitated below the apex (internode 5) and IAA in lanolin was applied to the decapitated stump, as described by Beveridge et al. (2000). Six hours after treatment, internode 4 was collected. Total RNA was extracted using a modification of the hot-phenol method (Kreig 1996). RT–PCR and Southern blotting were used to determine the abundance of *RMS1* transcripts in these samples. For details, see Supplemental Material.

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