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Article

The *Arabidopsis MAX* Pathway Controls Shoot Branching by Regulating Auxin Transport

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Summary

Background: Plants achieve remarkable plasticity in shoot system architecture by regulating the activity of secondary shoot meristems, laid down in the axil of each leaf. Axillary meristem activity, and hence shoot branching, is regulated by a network of interacting hormonal signals that move through the plant. Among these, auxin, moving down the plant in the main stem, indirectly inhibits axillary bud outgrowth, and an as yet undefined hormone, the synthesis of which in *Arabidopsis* requires *MAX1, MAX3*, and *MAX4,* moves up the plant and also inhibits shoot branching. Since the axillary buds of *max4* mutants are resistant to the inhibitory effects of apically supplied auxin, auxin and the MAX-dependent hormone must interact to inhibit branching.
Results: Here we show that the resistance of *max* mutant buds to apically supplied auxin is largely independent of the known, AXR1-mediated, auxin signal transduction pathway. Instead, it is caused by increased capacity for auxin transport in *max* primary stems, which show increased expression of *PIN* auxin efflux facilitators. The *max* phenotype is dependent on PIN1 activity, but it is independent of flavonoids, which are known regulators of PIN-dependent auxin transport.
Conclusions: The MAX-dependent hormone is a novel regulator of auxin transport. Modulation of auxin transport in the stem is sufficient to regulate bud outgrowth, independent of AXR1-mediated auxin signaling. We therefore propose an additional mechanism for long-range signaling by auxin in which bud growth is regulated by competition between auxin sources for auxin transport capacity in the primary stem.

Introduction

Plant shoot systems are generated by a modular growth pattern. The primary shoot apical meristem at the shoot tip produces successive units consisting of a stem segment, a leaf, and a secondary shoot apical meristem in the axil of the leaf. Each axillary meristem has the same developmental potential as the primary shoot apical meristem, and thus secondary shoots can arise from the activity of the axillary meristems. The growth of the secondary shoots is, however, tightly regulated, with many arresting at an early stage as a small bud. The presence of these dormant buds allows plants to modulate their shoot system architecture in response to environmental conditions and developmental stage.

A classic example of this regulation is the inhibition of bud outgrowth by the primary shoot apex—a phenomenon known as apical dominance. The plant hormone auxin (IAA, Indole-3-acetic acid) has long been implicated in the ability of the primary shoot apex to inhibit axillary bud growth [1]. For example, in isolated *Arabidopsis* stem segments carrying a leaf and an axillary bud, application of auxin to the cut surface of the apical stem inhibits the outgrowth of the axillary bud [2]. The in vivo significance of this result is clear from the fact that mutations in the *AUXIN RESISTANT1* (*AXR1*) gene of *Arabidopsis*, which confer a primary defect in auxin-regulated transcription [3, 4], result in increased shoot branching and buds resistant to inhibition by apical auxin [5]. Despite the central role of auxin, it is clear that it acts indirectly, because auxin transported from the apex does not enter the buds [6, 7], and applying auxin directly to buds does not prevent their outgrowth [8]. Furthermore, expression of the wild-type *AXR1* gene in the xylem parenchyma and interfascicular tissue of the stem is sufficient to restore a wild-type branching habit to the *axr1-12* mutant [5]. Thus, auxin in the stem is somehow able to influence the growth of buds some distance away.

Many potential second messengers for auxin action have been suggested (for reviews, see [9, 10]). One particularly good candidate is the plant hormone cytokinin. Direct application of cytokinin to buds promotes their outgrowth, even in the presence of an apex/apical auxin [11], as does cytokinin supplied basally through the main stem [2]. It has also been shown that auxin can regulate the synthesis and export of cytokinin from the root [12, 13] and its synthesis locally in the shoot [14], suggesting that auxin could act by reducing the supply of cytokinin to the buds, thereby inhibiting their growth.

Genetic analyses have provided an additional candidate as a second messenger for auxin. Mutants in three model species have been identified that have increased shoot branching and limited pleiotropic phenotypes. These are the *ramosus* (*rms*) mutants of pea, the *decreased apical dominance* (*dad*) mutants of petunia, and the *more axillary branching* (*max*) mutants of *Arabidopsis* [15–17]. These mutants have very similar phenotypes and represent at least partly orthologous pathways since *RMS1*, *DAD1*, and *MAX4* have been shown to be orthologs [18, 19]. A subset of these genes is required for the production of a graft-transmissible, upwardly mobile signal that inhibits branching [20, 21].

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In *Arabidopsis* these are *MAX1*, *MAX3*, and *MAX4*, and reciprocal grafting experiments have suggested that MAX1 acts downstream of MAX3 and MAX4 in the biosynthesis of the signal [20]. *MAX4* and *MAX3* encode divergent carotenoid cleavage dioxygenases, suggesting that the signal is carotenoid derived, and *MAX1* encodes a cytochrome P450 family member [18, 20, 22]. *MAX2* encodes a leucine-rich repeat F box protein [17], which acts locally in the shoot, is not required for the synthesis of the signal [20], and therefore is proposed to act in the transduction of the signal at the node.

The mechanism by which the MAX pathway acts is as yet unclear. However, the pathway is known to interact with auxin because *max4* and *rms1* mutant buds are resistant to the inhibitory effects of apical auxin [18, 23]. This suggests that the MAX pathway may act as a second messenger for auxin in regulating bud outgrowth. The most obvious mechanism to achieve this would be for auxin to upregulate the synthesis of the MAX-dependent compound, which would move into the buds and directly inhibit their growth. However, although auxin does substantially upregulate *RMS1* expression in pea stems [24], it has no effect on stem expression of *MAX4* [25]. Indeed, grafting experiments have shown that it is possible to separate AXR1-mediated auxin signaling and MAX-compound synthesis into completely different tissues while maintaining wild-type branching patterns [25]. Thus, the point of interaction between the MAX pathway and auxin must be after the synthesis of the MAX signal and/or largely independent of AXR1-mediated auxin signaling.

In this report, we provide evidence that the MAX pathway acts substantially independently of auxin signaling and instead works by regulating auxin transport capacity in the main stem. We show that this is likely to be mediated by changes in expression of *PIN* auxin efflux facilitator genes and that the action of the MAX pathway does not require flavonoids, which are known to regulate auxin transport. Thus, the MAX signal represents a novel regulator of auxin transport, which we propose regulates bud activity at a distance through the modulation of auxin transport capacity in the stem, thus modulating the sink strength of the stem for bud-derived auxin.

Results

The MAX Pathway Acts Largely Independently of AXR1-Mediated Auxin Signaling

Many mutants in the transcription-regulating auxin signaling pathway have shoot-branching defects and auxin-response defects in their buds, demonstrating that this pathway regulates bud outgrowth and apical dominance [3, 26, 27]. Similarly, mutations in the MAX pathway genes result in increased branching, and *max4* buds have been shown to be resistant to apical auxin [18]. Where tested, none of these mutants (MAX or auxin-related) has altered levels or timing of axillary meristem formation, suggesting that they all act primarily at the stage of bud growth regulation [17, 28]. To investigate the relationship between these pathways in more detail, we analyzed branching in the *axr1-12* mutant, which is deficient in auxin signaling, and *max1-max4*. We also constructed double mutants between *axr1-12* and *max1-max4* to further this analysis. We used two assays to analyze bud activity, namely measurements of rosette branching in mature plants and the growth response of buds on excised nodes to apically supplied auxin. We found that branching from the rosette is markedly increased in both *max* and *axr1-12* mutants, compared to wild-type (Col-0), with *max* mutants having higher levels of branching than *axr1-12* plants (Figure 1A). The outgrowth response of buds on excised nodes to apically applied auxin was significantly greater in both *axr1-12* and *max1-max4* than in the wild-type (Col-0), indicative of reduced auxin sensitivity (Figure 1B). However, *axr1-12* buds were more resistant than *max* buds. Thus, while *max* plants have higher branching

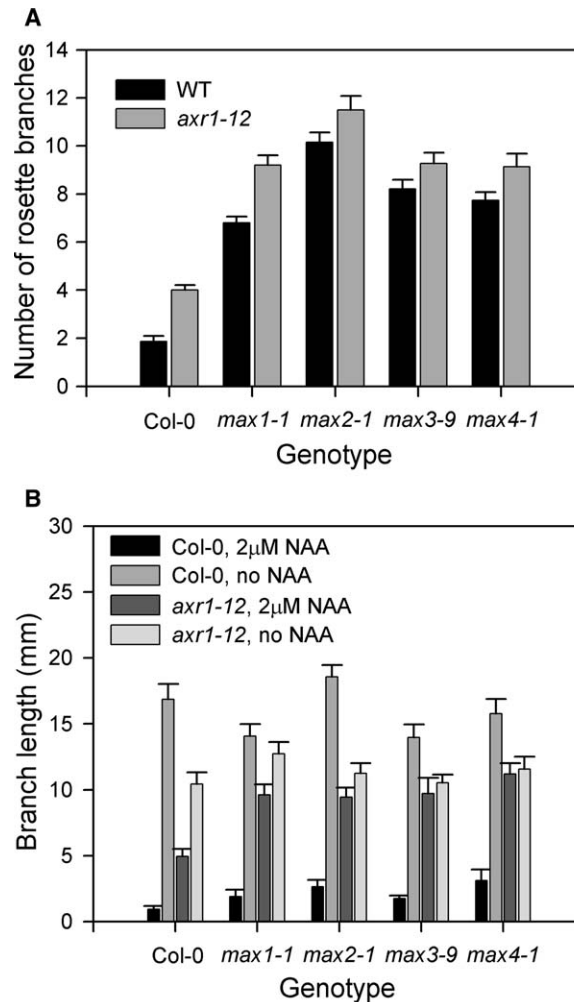

Figure 1. The Interaction between AXR1-Mediated Auxin Signaling and the MAX Pathway

(A) Secondary rosette branch number in the *max* mutants, in either a wild-type (Col-0) or *axr1-12* genetic background. Measurements were made after cessation of primary meristem activity (approximately 7 weeks); n = 14, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(B) Response of buds on nodal segments to apically applied auxin. For each genotype, the effects of background (wild-type versus *axr1-12*) and auxin treatment (2 μM NAA versus no NAA) are shown. Measurements are of mean branch length after 5 days of treatment, n = 9 to 20, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

levels, *axr1-12* buds show less inhibition of bud outgrowth by auxin. These data indicate that a proportion of the increased branching in the *max* mutants cannot be explained by a deficiency in AXR1-mediated signaling (i.e., is AXR1 independent), and likewise there is a proportion of the auxin resistance in *axr1-12* buds that is not due to a deficiency in the MAX pathway (i.e., is MAX independent). Furthermore, when the double mutants were analyzed, both the number of rosette branches (ANOVA, in each case $p < 0.01$; Figure 1A) and the degree of auxin resistance (ANOVA, in each case, $p < 0.01$; Figure 1B) were found to be greater than those observed in either parent. Indeed, the *axr1-12* and *max* phenotypes are substantially additive, with double mutant buds showing little or no auxin response at all. These results indicate that the MAX pathway and AXR1-mediated auxin signaling act largely independently in the regulation of shoot branching. Since the *axr1-12* mutant is defective in a vast array of auxin responses [4], the discovery of an AXR1-independent auxin response is significant.

max Mutants Have Increased Auxin Transport Capacity

Since previous reports have linked auxin transport and shoot branching [7, 29], we investigated whether the MAX pathway might regulate auxin transport. Auxin is transported basipetally down the stem, and this transport is dependent on members of the PIN family of auxin efflux facilitators, which are basally localized in the cells of the xylem parenchyma and mediate directional movement of auxin down the stem [30, 31]. We analyzed bulk transport of radio-labeled auxin in *max* mutants, relative to wild-type. The apical ends of 15 mm excised stem segments were incubated in radiolabel for 18 hr, and the amount transported into the basal 5 mm was then measured (after [31]). The *max* mutants were found to have a marked increase in the ability to transport auxin relative to wild-type (Figure 2A). This assay is demonstrably NPA sensitive and therefore presumably measures only active transport (Figure 3A). To determine whether this result was due to change in the capacity for auxin transport or in the rate of auxin transport, we used a pulse-chase assay. Auxin was loaded into the apical ends of 25 mm *max4-1* and wild-type stem segments for 1 hr. Radiolabeled auxin was then collected as it emerged from the basal ends of the stem segments in 30 min windows. Again, this assay is fully NPA sensitive (not shown), and so presumably measures active transport. The time course of emergence of the loaded auxin was very similar for *max4-1* and wild-type, with a peak in emergence at around 3–3.5 hr, but for each time window more auxin emerged from the *max4-1* stem than the wild-type (Figure 2B). This suggests that the main effect of the *max4* mutation is on auxin transport capacity rather than transport rate. It should be noted that *max* mutant stems have wild-type anatomy (Figures 2J and 4A–4C), so for example, these increases in auxin transport do not arise from differences in the amount of vasculature between genotypes.

If transport capacity is severely limiting in wild-type stems, then the increase in transport capacity in the *max* mutants might allow auxin to flow unimpeded down the stem, resulting in depletion of auxin in the node, reduced activity through the auxin signaling pathway,

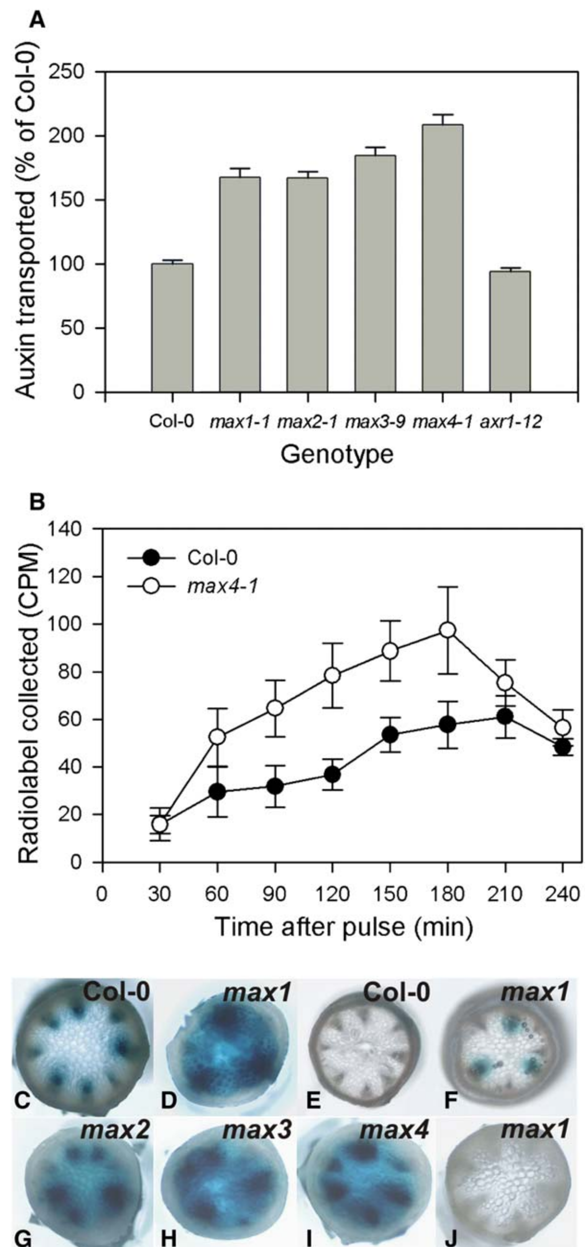

Figure 2. Quantification of Auxin Transport Capacity in *max* Mutants

(A) Bulk levels of auxin transport in *max* mutant and *axr1-12* stem segments. Mean levels of radiolabel transported (in CPM) are shown relative to Col-0; n = 30, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(B) Auxin transport capacity in *max4-1*. The apical ends of wild-type and *max4-1* stem segments were loaded with radiolabeled auxin for 1 hr. Radiolabel emerging from the basal end of the segments was collected in dithiodethylcarbamate buffer. Data points show the mean amount of radiolabel (measured in CPM) collected during the 30 min preceding a time point; n = 8, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(C–J) *DR5::GUS* expression in *max* mutant stems. Staining for GUS activity in basal stem segments of Col-0 (C), *max1-1* (D), *max2-1* (G), *max3-9* (H), and *max4-1* (I); and in apical segments of Col-0 (E) and *max1* (F). Also shown is an unstained *max1-1* basal stem segment (J), showing normal vasculature.

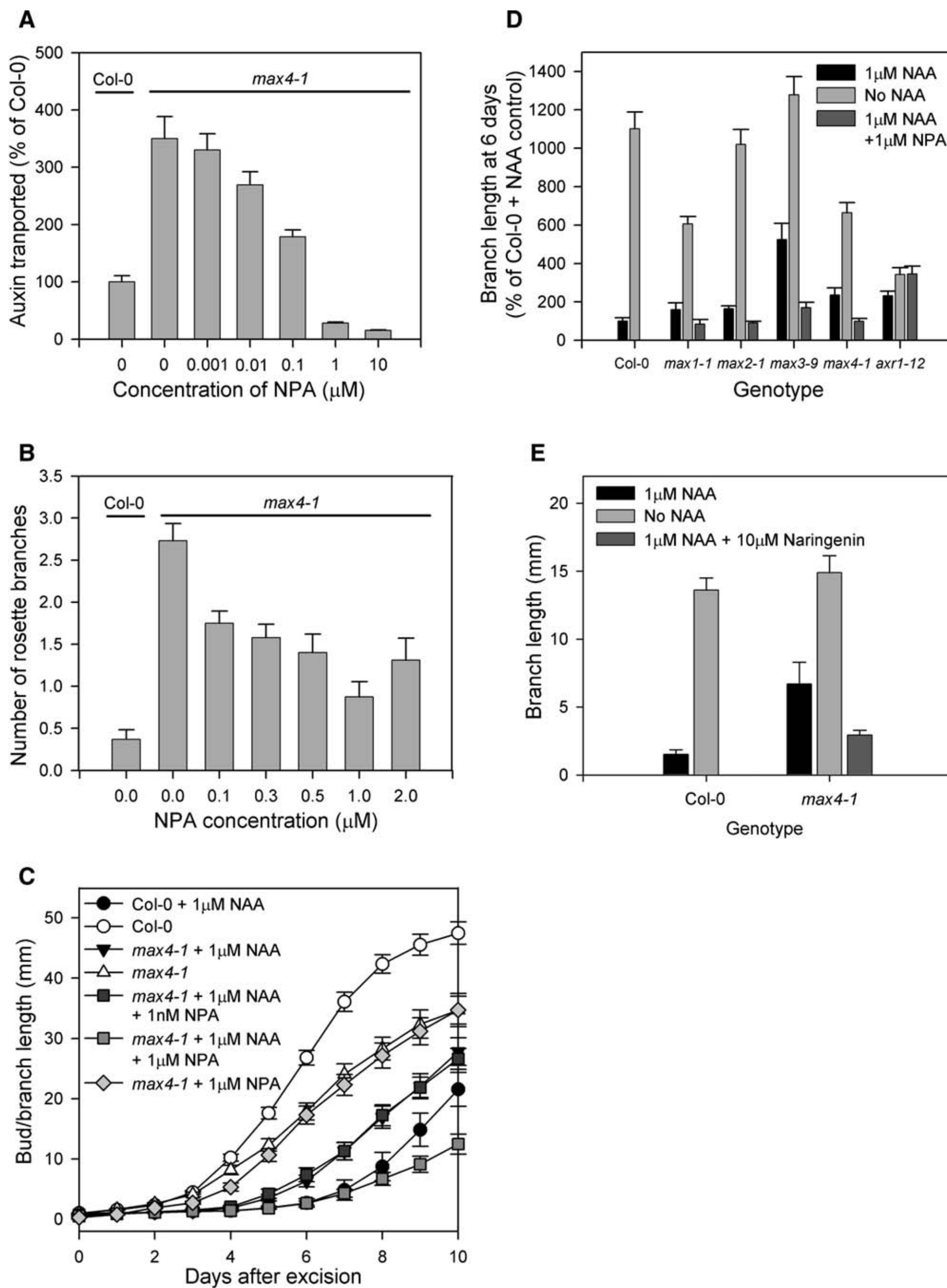

Figure 3. The Effect of Restoring Wild-Type Auxin Transport to *max* Mutants

(A) Reduction of auxin transport in the presence of NPA. Bulk auxin transport was assessed in Col-0 and *max4-1* stem segments, in the presence of increasing NPA concentrations. Wild-type auxin transport is restored in the range 100 nM–1 μM NPA. Measurements are of mean levels of radiolabel transported (n = 30), relative to Col-0; bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(B) Branching in intact plants grown in the presence of NPA. NPA is able to suppress the *max* phenotype. Measurements are of secondary rosette branch number at 4 weeks; n = 15, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(C) Outgrowth kinetics of *max4-1* buds. Apical addition of 1 μM NPA in the presence of 1 μM apical NAA restores the inhibition of outgrowth of *max4-1* buds to wild-type kinetics; 1 μM NPA alone has no effect. Data points show mean branch lengths (n = 16) over a 10 day time course. Bars indicate SEM; some bars are omitted for clarity. Data representative of three independent data sets, all showing the same effect.

(D) Bud responses of *max* and *axr1-12* mutants. Apical addition of 1 μM NPA rescues the *max* but not the *axr1-12* phenotype. Measurements are of mean branch length at 6 days; n = 16, bars indicate SEM.

(E) Outgrowth kinetics of *max4-1* buds. Apical addition of 10 μM naringenin in the presence of 1 μM apical NAA restores the inhibition of outgrowth of *max4-1* buds to wild-type kinetics. Experiment performed as in (C). Measurements are of branch length at 5 days (n = 16), bars indicate SEM. Data representative of two independent data sets showing the same effect.

and thus increased bud outgrowth. To test this hypothesis, we examined activity of the auxin-responsive *DR5*::*GUS* promoter-reporter construct in the *max* mutants. This reporter is a generally reliable indicator of the activity of the *AXR1* auxin signaling pathway and often reflects auxin levels [32]. Directly contrary to the idea of reduced auxin signaling at the node, the *max* mutants have a large increase in *DR5::GUS* activity in the stem vasculature relative to wild-type, both in basal and apical nodes (Figures 2C–2J). These data are consistent with increased auxin levels throughout the transport stream, suggesting that the increased transport capacity in *max* mutant stems results in more auxin in transit through the stem at any one time.

Increased Auxin Transport Capacity Causes the *max* Branching Phenotype

To investigate whether the increased auxin transport capacity is necessary for the branching phenotype of *max* mutants, we tested the effect of pharmacologically inhibiting auxin transport on the *max* phenotype, via the well-characterized inhibitor of auxin transport NPA (1-*N*-Naphthylphtalamic acid). We first confirmed that auxin transport in the *max* mutants is NPA sensitive (see Figure S1 in the Supplemental Data available with this article online) and determined that concentrations in the order of 1 μM restore auxin transport to approximately wild-type levels (Figure 3A). We then tested the effect of this concentration of NPA on shoot branching and bud responses to apical auxin.

Whole plants were treated with NPA by its addition to the agar-solidified medium of plants grown in sterile culture (Figure 3B). Increasing doses of NPA reduced shoot branching up to concentrations of 1 μM. At 2 μM, NPA treatment resulted in increased branching compared to 1 μM NPA. These results suggest that the increased auxin transport of the *max* mutants causes the increased branching phenotype, but that auxin transport levels below wild-type also promote branching. This latter observation corresponds well with classical data showing that inhibiting auxin transport in wild-type plants leads to bud outgrowth, because auxin is prevented from reaching the node [29, 33], and also with the phenotype of the *transport inhibitor response3* (*tir3*) mutant, which has reduced auxin transport and increased branching [34]. We have previously shown that treatment with 1 μM NPA leads to increased bud outgrowth in wild-type plants (which have less auxin transport to start with), which agrees with the long-established idea that too little auxin transport also leads to increased shoot branching [2].

When we tested the effect of NPA on bud auxin response, we found that while low concentrations of NPA do not affect bud outgrowth at all, 1 μM NPA completely restores a wild-type auxin response to *max* buds (Figures 3C and 3D). NPA has no effect on *max* bud outgrowth in the absence of apical auxin (Figure 3C), suggesting that the effect of NPA is on auxin transport in the stem and not on the buds directly. This effect of NPA holds for all the *max* mutants (Figure 3D), but not *axr1-12*, which does not have increased auxin transport (Figure 2A). These data confirm both the causal relationship between increased auxin transport capacity and the *max* branching phenotype and an independent mechanism of action of the MAX and AXR1 pathways.

Increased Levels of PIN Proteins Are Associated with, and Required for, the *max* Branching Phenotype

Since the family of PIN auxin transport facilitator proteins has been shown to mediate the amount and direction of polar auxin transport [30, 35–37], we investigated whether they might be targets of the MAX pathway in the regulation of auxin transport. We examined localization of the well-characterized *PIN1p::PIN1:GFP* translational fusion construct [38] in inflorescence stems of *max* mutants by confocal microscopy. PIN1:GFP protein levels were clearly elevated in the vascular bundles of *max* mutants compared to wild-type. Reporter protein levels were particularly stronger in the xylem tissue adjacent to the cambial region (Figures 4A–4C). In longitudinal sections of *max1-1* stems, the majority of PIN1:GFP showed typical basal localization in xylem parenchyma cells; however, the amount of protein in the basal cell membrane was increased and a significant fraction was clearly not basally localized (Figures 4D and 4E). To test whether these changes in PIN1 levels are due to transcriptional upregulation, we used a *PIN1p::GUS* transcriptional fusion reporter construct. *PIN1p::GUS* activity was noticeably elevated in *max1-1* inflorescence stems compared to wild-type (Figures 4F and 4G). We extended this analysis to other *PIN* genes via semiquantitative RT-PCR, and we found that levels of transcripts from *PIN1* and *3*, and probably *PIN4* and *6*, are increased, although *PIN7* was downregulated in *max* mutants relative to wild-type (Figure 4J). To test whether this elevated level of PIN expression is causally related to the increased branching phenotype of the *max* mutants, we constructed *pin1 max* double mutants and found that they have significantly reduced branching relative to *max* single mutants, showing that *PIN1* expression is important for the *max* phenotype (Figure 4I). Branching was not returned to completely wild-type levels in *pin1 max* double mutants, which we ascribe to the upregulation of other PIN proteins in the *max* mutant backgrounds (Figure 4J). Consistent with this, we found that there is greater residual auxin transport in *pin1 max* compared to *pin1* (data not shown). The altered expression of the PIN genes is still observed in *pin1 max* double mutants (Figure 4J), suggesting that it is not a result of feedback from increased branching or auxin levels in the stem, but rather is a direct effect of lack of MAX signaling. Based on these data, we propose that the MAX pathway regulates branching by modulating auxin transport capacity through control of *PIN* transcript levels.

The MAX Pathway Does Not Regulate Auxin Transport in a Flavonoid-Dependent Manner

Flavonoids are naturally occurring inhibitors of auxin transport [39–41]. We found that, like NPA, the flavonoid naringenin is able to restore wild-type apical auxin responses to *max* mutant buds, although at much higher concentrations than NPA, consistent with its lower activity (Figure 3E). A role for flavonoids in shoot-branching control has previously been reported through the analysis of a flavonoid-deficient mutant. The *transparent testa4* (*tt4*) mutant lacks the enzyme chalcone synthase, and thus makes no flavonoids at all, resulting in a maternal effect, yellow seed phenotype [42]. One allele of this

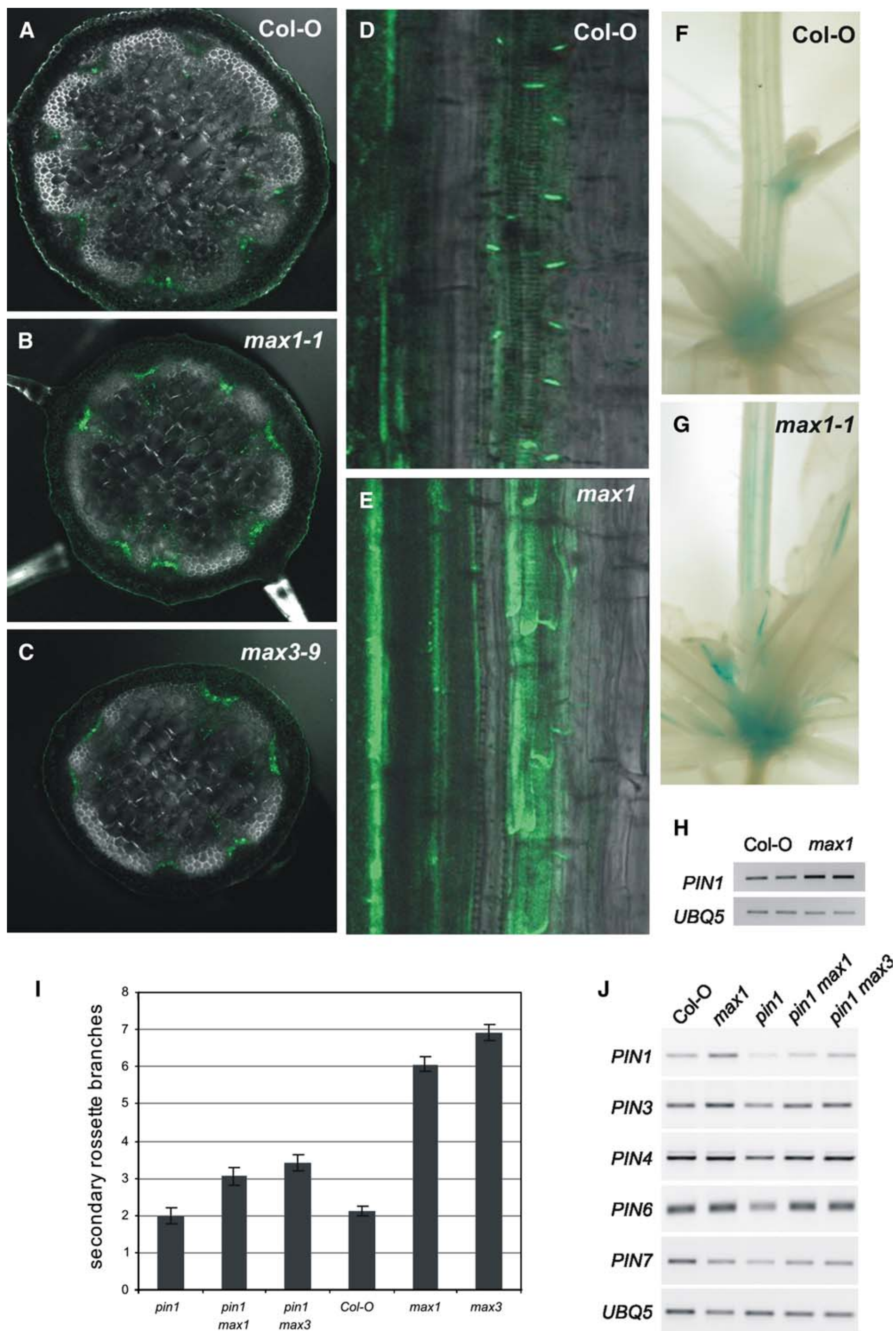

Figure 4. Expression Levels and Localization of PIN Proteins in *max* Mutants and Effect of *pin1* Mutation on *max* Secondary Rosette Branching

(A–C) Localization of PIN1:GFP in transverse cross-sections of 30-day-old wild-type (A), *max1-1* (B), and *max3-9* (C) basal inflorescence stems. Images representative of 25–30 samples.

(D and E) Subcellular localization of PIN1:GFP in radial longitudinal sections of 30-day-old basal inflorescence stems from wild-type (D) and *max1-1* (E) plants. Images representative of 25–30 samples.

(F and G) *PIN1::GUS* activity in basal inflorescence stems of 28-day-old wild-type (F) and *max1-1* (G) plants.

(H) Semiquantitative RT-PCR analysis comparing *PIN1* expression levels in 30-day-old basal inflorescence stems of wild-type and *max1-1* (top). The analysis was performed with parallel samples. Normalization of cDNA was performed with *UBQ5*-specific primers (bottom).

(I) Mean number of second-order rosette branches of single and double mutant combinations of *pin1*, *max1*, and *max3* plants. Branching was scored 45 days after germination, n = 26–53, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

mutant (2YY6) has been reported to confer a bushy phenotype [39], suggesting a link between auxin transport, flavonoids, and shoot branching. To investigate potential interactions between flavonoids and the *max* pathway, we attempted to construct double mutants between *tt4* (2YY6) and *max1*-*max4*. However, during this process we found that the branching phenotype of *tt4* (2YY6) results from a *max4* mutation in *tt4* (2YY6). To confirm this, a backcross between *tt4* (2YY6) and Col-0 was performed, which in the F2 showed independent segregation of the pigment accumulation and branching phenotypes. The *max4* allele from *tt4* (2YY6) (denoted *max4-5*) was sequenced and found to contain a premature stop codon in the second exon (data not shown). These data demonstrate that *tt4* does not confer a branching phenotype, which was confirmed with an independent allele (*tt4-1*; in the *Ler* background), in addition to the one backcrossed out of the *tt4* (2YY6) line (denoted *tt4-2*). Both alleles confer levels of branching not significantly different from wild-type and significantly less than *tt4* (2YY6) (t test, $p < 0.01$; Figure 5A). Since completely flavonoid-deficient plants have wild-type branching, flavonoids cannot be important to produce normal branching patterns. Furthermore, since the *tt4-2 max4-5* double mutant is bushy, flavonoids are also not required for elaboration of the *max* phenotype. This raises questions about the link between increased auxin transport and increased branching observed in the *max* mutants, since *tt4* mutants have been reported to have increased auxin transport and thus would be predicted to have increased branching. To address this question, we compared auxin transport in the stems of *tt4-1*, *tt4-2*, and the *max* mutants. We found modest but significant increases in auxin transport in *tt4-1* (t test, $p < 0.01$), but no real difference from wild-type in *tt4-2* (t test, $p = 0.514$) (Figure 5B). The effects of the *tt4* mutants are therefore much smaller in the stem than the increases observed in the *max* mutants, and thus are likely not large enough to cause detectable branching phenotypes. It should be noted, however, that these data do not contradict previous reports showing larger increases in auxin transport in the seedlings of *tt4* [40, 41].

Discussion

The MAX Pathway and the Regulation of Shoot Branching

Auxin has long been implicated in the regulation of shoot branching, but it has been clear for almost as long that its mechanism of action is indirect, with auxin moving down through the vasculature of the primary stem inhibiting the outgrowth of axillary buds located some distance laterally [10]. Our data suggest that in *Arabidopsis* there are at least two mechanisms by which this occurs, both of which must be active for wild-type levels of bud outgrowth. The first of these mechanisms is AXR1 dependent, the strength of the response presumably increasing with auxin concentration in the stem and presumably perceived by the TIR1/AFB auxin receptors and transduced to changes in gene expression [43, 44]. It is likely that targets for this pathway include genes encoding cytokinin biosynthetic enzymes, which are known to be downregulated at the node (and indeed elsewhere) by auxin in an AXR1-dependent manner [14]. This would reduce cytokinin availability to the bud and hence reduce bud activity.

Our data support a second mechanism for auxin action that is independent of classical signal transduction and is not directly related to auxin concentration in the stem or bud. This pathway involves an influence of auxin transport capacity in the main stem on bud outgrowth. The evidence for the existence of this pathway is strong. In the highly branched *max* mutants, auxin transport capacity is increased, correlating with increased PIN1 accumulation in the stem. If auxin transport is restored to more wild-type levels, either pharmacologically with NPA or naringenin or genetically with the *pin1* mutant, wild-type branching levels are restored and importantly auxin response in the buds is also returned to wild-type. These effects are independent of AXR1. So, based on these data, it is clear that increased auxin transport capacity in the stem causes increased shoot branching by a mechanism that does not directly require the AXR1-mediated auxin signaling pathway. It is of course indirectly required, since auxin signaling through this pathway is needed for the actual growth of the bud.

This finding is somewhat unexpected for two reasons. First, AXR1-independent auxin signaling is extremely unusual, and second, a wealth of existing physiological evidence associates reduced auxin transport with increased shoot branching, precisely the opposite of our observations. It has generally been assumed that inhibiting auxin transport from the shoot apex reduces the concentration of auxin at the node, leading to a derepression of bud activity. The same seems likely to be true in plants from which the shoot apex, and thus the major auxin source, has been removed. We see no reason to challenge this model, but our results necessitate an additional mechanism to explain how increased auxin transport capacity in the stem, associated with increased auxin signaling, as evidenced by *DR5::GUS* expression, results in increased shoot branching. Our model for this additional mechanism centers on another well-characterised phenomenon in the literature: the tight correlation between the ability of buds to grow out and their ability to export auxin into the main stem [7, 45]. Given these data, it is probable that efficient auxin export from the bud is actually required for active bud growth. One possible explanation for this is suggested by the recent demonstration that shoot meristem function depends on removal of auxin from the meristem epidermis by transporting it into the growing stem below [46]. Thus, if the bud cannot export auxin out into the main stem, it may be unable to sustain an active meristem. This would explain why increased auxin transport capacity in the main stem allows increased bud growth. Buds would easily be able to establish auxin efflux into the main stem if the capacity for auxin transport is

(J) Semiquantitative RT-PCR analysis comparing *PIN* expression levels of different members of the *PIN* family in basal inflorescence stems of single and double mutant combinations of *pin1*, *max1*, and *max3* plants. *UBQ5* was used as normalization control. *PIN2* was not analyzed since it is not expressed in inflorescence stems. *PIN5* and *PIN8* represent divergent, poorly characterized members of the *PIN* gene family and were thus not included in the analysis.

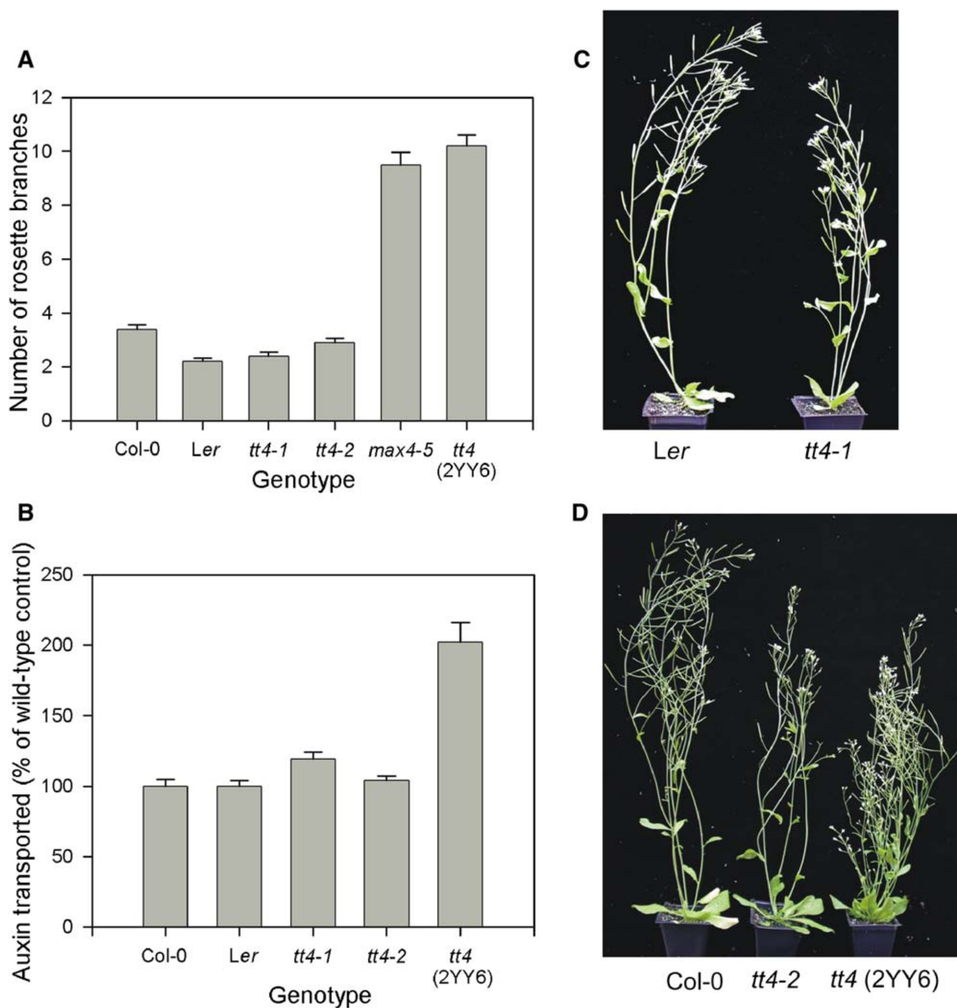

Figure 5. The Role of Flavonoids in the Regulation of Shoot Branching

(A) Secondary rosette branch number in *tt4*. Measurements were made after cessation of primary meristem activity (approximately 7 weeks); n = 10, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(B) Bulk levels of auxin transport in *tt4*. Mean levels of radiolabel transported (in CPM) are shown relative to Col-0; n = 30, bars indicate SEM. Data representative of three independent data sets, all showing the same effect.

(C) Comparison of *Ler* and *tt4-1*, 42 days after germination.

(D) Comparison of Col, *tt4-2*, and *tt4* (2YY6), 42 days after germination.

high there, and therefore if the stem can provide a strong auxin sink.

In this model, in a wild-type situation, auxin exported from the young leaves of the primary apex fills the transport capacity of the main stem, blocking access to auxin from the buds and hence preventing establishment of auxin efflux from the buds, blocking their growth. Removal of the primary apex would remove the auxin source, freeing up transport capacity in the stem to act as a sink for bud-derived auxin. An alternative mechanism to promote bud outgrowth in this scenario is to increase the capacity for auxin transport in stem, allowing simultaneous flow of auxin from the primary apex and axillary buds into the stem, thereby supporting the growth of multiple axes at once in spite of high auxin levels in the stem. This is essentially the situation in the *max* mutants. In this context, it is interesting to note that the pattern of *DR5::GUS* activity in *max* mutant stems is not even between the vascular bundles (Figures 2C and 2E) but appears to reflect the phyllotactic pattern of lateral organs (and their associated buds), consistent with increased active auxin export out of these growing buds into the adjacent vascular bundle in the main stem.

The mechanism that we propose is in many ways analogous to the observations of Sachs [47] investigating vascular differentiation in stem segments. He observed that an auxin source applied laterally on a cut apex would trigger vascular differentiation in the stem to connect the source to the existing vasculature. However, if auxin was applied directly to the preexisting vasculature as well, the vasculature created by the lateral auxin source did not join the original vasculature. In other words, the presence of auxin within the original vasculature prevented further auxin export into that vasculature. This observation can be explained in terms of the canalization hypothesis, wherein auxin sources and sinks are linked by self-reinforcing auxin transport through narrow cell files. The presence of auxin reduces the sink

strength of the vasculature, making it refractory to other auxin sources. Conversely, in the absence of auxin, the vasculature is a strong sink for the lateral auxin source, the two becoming linked by a canalized auxin stream, manifested as new vasculature. This is directly analogous to the model for bud growth regulation that we are proposing here; buds cannot efficiently export auxin in wild-type plants because the stem vasculature is not a strong sink for auxin. However, by removing the auxin or by increasing the transport capacity, the vasculature becomes a better sink for auxin, and buds can export auxin and grow out. It is in fact highly likely that the export of auxin from buds is also necessary to create vascular connections between the bud and the stem, which are necessary for the further development of the bud, thus providing further parallels with Sachs' data.

Perhaps one of the most interesting implications of our model is the ability of the apex to influence the activity of the bud "at a distance," without the movement of any signal between stem and bud [48]. Instead, the growth of the bud is regulated by competition between auxin sources for auxin transport capacity in the stem. This would be, as far as we are aware, the first example of long-distance signaling by such a mechanism, and adds another mode of action through which the intricate auxin distribution system within the plant can regulate development. There are already excellent examples of how the PIN and other auxin transporter systems regulate development by generating differences in auxin concentration across tissues, including cases where the concentration differences are generated by canalization between auxin sources and sinks. Here, regulation is achieved by creating bottlenecks for auxin flow, like a traffic jam. The extent to which this system is used is as yet unclear; however, it is apparent that the MAX pathway operates this way, providing the potential to regulate auxin movement through the plant via the local and/or global changes in MAX pathway activity.

The MAX Pathway Is a Novel Regulator of Auxin Transport

Our results demonstrate that the shoot-branching phenotype of the *max* mutants is caused by increased auxin transport capacity in the main stem. This correlates with increased PIN1 accumulation and increased expression of the *PIN1* gene, as well as several other *PIN*s. This suggests that a primary function of the MAX pathway is to modulate *PIN* expression in the stem. The likely target tissue for MAX action is therefore the xylem parenchyma, which is the main site for polar auxin transport down the stem. Consistent with this, *MAX1*, which is required for a late step in the synthesis of the MAX-dependent compound, is expressed at high levels in the vasculature [19], as is *MAX2*, which is involved in perception of the signal (P. Stirnberg and O.L., unpublished results).

Whether the *PIN* genes represent immediate early targets for the MAX pathway is a matter for future investigation, but it is clear that the link between the MAX pathway and the PINs is independent of known regulators of PIN activity, such as the AXR1-mediated auxin response pathway, which in some circumstances regulates PIN gene expression [49], and the flavonoids, which inhibit PIN function and have been suggested to be involved in MAX action [40, 50]. Thus, the MAX pathway represents

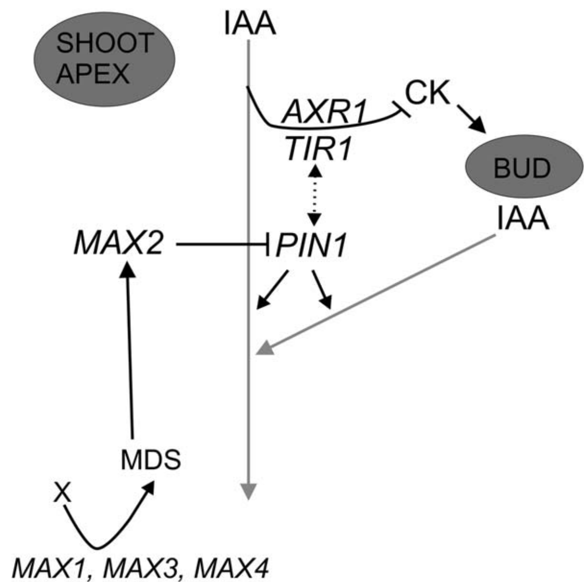

Figure 6. Model of the Regulation of Bud Outgrowth

MAX1, *MAX4*, and *MAX4* act to produce the as yet unidentified long-distance signal MDS (MAX-dependent signal), which is transported up the plant and perceived by *MAX2*-dependent detection and signaling. This results in reduction in *PIN* gene transcription, reducing auxin transport capacity, and blocking export of auxin from the bud. Auxin also acts via a canonical signaling pathway to reduce cytokinin levels at the node, further blocking bud outgrowth.

a third mechanism for regulating auxin transport. Since the *axr1-12* mutant has wild-type auxin transport levels and the *tt4* mutants have wild-type shoot branching, the MAX pathway is the only one of the three involved in branching control by the auxin transport capacity-dependent mechanism. It will therefore be very interesting to investigate further the specific physiological and developmental roles for each of these pathways to determine the extent to which they are each uniquely attuned to function in different circumstances.

Conclusion

We have shown that the MAX pathway of *Arabidopsis* regulates auxin transport capacity in the stem by regulating abundance of PIN auxin efflux facilitator proteins. This in turn allows regulation of shoot branching in plants, and we propose that this is by modulating the ability of buds to export auxin. Thus, auxin may influence shoot branching via multiple pathways (Figure 6), one of which appears to act at a distance from the target tissue by modulating competition by auxin sources in the primary and axillary buds for auxin transport capacity.

Experimental Procedures

Plant Growth

For growth on soil, *Arabidopsis thaliana* seeds were sown on Levington's F2 compost, at a density of one per 16 cm^2. Seeds were cold treated for 3 days after sowing and then grown at 20ºC/15ºC in a 16 hr light/8 hr dark photoperiod, under a light intensity of $\sim$150 $\mu molm^{-2} s^{-1}$. Branching measurements were made after cessation of primary meristem activity.

Plants were grown under axenic conditions for bud hormone response assays and inhibitor studies. Seeds were sterilized in 10% (w/v) chlorine bleach and then washed with 70% (w/v) ethanol (×1) and sterile distilled water (×6). Seeds were then cold-treated for 3

days. Seeds were sown on *Arabidopsis thaliana* salts (ATS)-agar (1% sucrose, 0.8% agar) medium, described by Lincoln et al. [3]. For inhibitor studies, appropriate concentrations of NPA or naringenin were added to the media. Plants were then grown under a 22ºC/18ºC 16 hr light/8 hr dark regime (90 $\mu molm^{-2} s^{-1}$).

The following plants lines were previously described: *max1-1* [17], *max2-1* [17], *max3-9* [22], *max4-1* [18], *axr1-12* [3], *tt4* (2YY6) [39], *tt4-1* [42], *DR5::GUS* [32], and *PIN1p*::*PIN1:GFP* [38]. Plant line SALK_047613 (http://signal.salk.edu/cgi-bin/tdnaexpress) contains a T-DNA insertion in intron 3 of *PIN1* (At1g73590). Plants homozygous for the insertion exhibit the typical pin-formed shoot phenotype and we renamed the line *pin1-613*. No PIN1-specific signal was found in immunolocalization studies of *pin1-613* roots, indicating that it represents a null allele (data not shown).

PIN1p::GUS

To generate PIN1p::GUS, we amplified 2044 bp of PIN1 promoter sequence (−2051 to −7 relative to the start codon) from Col-O genomic DNA by using oligos 5′-GCAGGTCAATATAGATCATAAAGTG-3′ and 5′-TTCGCCGGAGAAGAGAGAGGGAA -3′. The resulting fragment was cloned into the pGEM-T (Promega, Madison, WI) and subsequently transferred into pPZPGUS.1 [51], to give pPIN1::GUS. Col-O plants were transformed with pPIN1::GUS. T2 progeny of several independent transformants were tested for GUS staining and a representative line containing a single transgene was brought to homozygosity and subsequently used for detailed analysis.

Bud Hormone Response Assays

The split plate assay was performed essentially as described in [2]. Plants were grown in axenic conditions for 3 weeks, until bolting occurred. The first cauline nodal section was then excised and placed between two agar blocks in a Petri dish. Hormones etc. were added to either agar block to assess the effect on bud outgrowth. In this study, Naphthylacetic acid (NAA) (Sigma), 1-*N*-Naphthylphtalamic acid (NPA) (Riedel-de-Häen), and Naringenin (Sigma) were used in the indicated concentrations. The length of buds was assessed daily for 10 days.

Auxin Transport Assays

Two types of auxin transport assays were used, both of which were modifications of the protocol described by Okada et al. [31]. In the first, the apical ends of 15 mm stem segments (all from the first cauline internode) were incubated for 18 hr (under constant light conditions) in 30 μL of 0.5× ATS medium (no sucrose), containing 1 μM ^{14}C labeled IAA (American Radiolabeled Chemicals, St Louis, MO). After this time, the basal 5 mm of the stem segment was excised, and the radiolabel was extracted by treatment with 80% (w/v) methanol for 48 hr. The amount of radiolabel was then quantified by scintillation in the presence of Microscint-40 (Perkin-Elmer). In the second assay, bundles of 10 (25 mm) stem segments were used to increase the signal-to-noise ratio. The apical end of the segments were incubated in 300 μL of 0.5× ATS buffer (no sucrose), containing 1 μM ^{14}C labeled IAA, for 1 hr. The basal ends of the segments were then incubated in 160 μL 2.5 mM diethyldithiocarbamate buffer for 30 or 40 min periods, after which they were successively transferred to fresh buffer for 30 or 40 min, seven more times. The radiolabel collected in each period was measured by scintillation in the presence of Microscint-40.

Histochemical Staining for GUS Activity

Histochemical localization of GUS activity was determined via material from 4-week-old (*PIN1::GUS*) or 6-week-old (*DR5::GUS*) soil-grown plants. Tissue was placed in X-Gluc staining solution (0.5 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-glucuronide, 50 mM sodium phosphate [pH 7.0], 0.05% Triton-X-100, 0.1 mM $K_4Fe(CN)_6$, and 0.1 mM $K_3Fe(CN)_6$), and incubated at 37ºC for 16 hr. Tissue was then destained in 70% (w/v) ethanol.

In Situ Expression and Localization Analysis of GFP

PIN1p::PIN1:GFP was crossed into *max1-1* and *max3-9* and doubly homozygous lines were used for analysis. Transverse and longitudinal hand sections were made from basal internodes of inflorescence stems (approximately 1 cm above the rosette) of 30-day-old plants. Longitudinal sections were generated by radial cuts through the center of a vascular bundle performed under a binocular microscope. Sections were mounted in water and GFP fluorescence was immediately inspected on a Zeiss Axiovert 200M-LSM 510 Meta confocal laser scanning microscope. For each genotype, 25–30 samples were examined.

Semiquantitative RT-PCR Analysis

PolyA^{2+} RNA was extracted from the base of inflorescence stems (basal 4 cm) of 30-day-old plants by using the QuickPick mRNA Micro kit (BIO-NOBILE, Turku, Finland) as recommended by the supplier. Extracted RNA was reverse transcribed with Superscript II (Invitrogen, Paisley, UK) according to the manufacturer's instructions. cDNAs were diluted 1:7 in water for subsequent semiquantitative RT-PCR under the following conditions: initial denaturation at 94ºC for 3 min; cycle settings: denaturation for 30 s at 94ºC, annealing for 30 s at 55ºC, and extension for 45 s at 72ºC. PCR with variable cycle numbers was performed and quantified on agarose gels, ensuring that reactions had not reached the plateau phase. *UBIQUITIN5* and *TUBULIN9* expression levels were used as normalization controls. Sequences of primers used in this study will be made available upon request.

Supplemental Data

The Supplemental Figure can be found with this article online at http://www.current-biology.com/cgi/content/full/16/6/553/DC1/.

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References

1. Thimann, K.V., and Skoog, F. (1934). On the inhibition of bud development and other functions of growth substance in *Vicia faba*. Proc. R. Soc. Lond. B. Biol. Sci. *114*, 317–339.
2. Chatfield, S.P., Stirnberg, P., Forde, B.G., and Leyser, O. (2000). The hormonal regulation of axillary bud growth in *Arabidopsis*. Plant J. *24*, 159–169.
3. Lincoln, C., Britton, J.H., and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell *2*, 1071–1080.
4. Leyser, H.M., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J., and Estelle, M. (1993). *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. Nature *364*, 161–164.
5. Booker, J.P., Chatfield, S.P., and Leyser, H.M.O. (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. Plant Cell *15*, 495–507.
6. Hall, S.M., and Hillman, J.R. (1975). Correlative inhibition of lateral bud growth in *Phaseolus vulgaris* L. Timing of bud growth following decapitation. Planta *123*, 137–143.
7. Morris, D.A. (1977). Transport of exogenous auxin in two-branched dwarf pea seedlings (*Pisum sativum* L.). Planta *136*, 91–96.
8. Brown, B.T., Foster, C., Phillips, J.N., and Rattigann, B.M. (1979). The indirect role of 2,4-D in the maintenance of apical dominance in decapitated sunflower seedlings (*Helianthus annus* L.). Planta *146*, 475–480.
9. Cline, M.G. (1991). Apical dominance. Bot. Rev. *57*, 318–358.
10. Napoli, C.A., Beveridge, C.A., and Snowden, K.C. (1999). Reevaluating concepts of apical dominance and the control of axillary branching. Curr. Top. Dev. Biol. *44*, 127–169.
11. Sachs, T., and Thimann, K.V. (1964). Release of lateral buds from apical dominance. Nature *201*, 939–940.
12. Li, C.-J., Guevara, E., Herrera, J., and Bangerth, F. (1995). Effect of apex excision and replacement by 1-naphthylacetic acid on

cytokinin concentration and apical dominance in peas. Plant Physiol. *94*, 465–469.

13. Eklöf, S., Astot, C., Blackwell, J., Moritz, T., Olsson, O., and Sandberg, G. (1995). Auxin/cytokinin interactions in wild-type and transgenic tobacco. Plant Cell Physiol. *38*, 225–235.
14. Nordstrom, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., and Sandberg, G. (2004). Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. Proc. Natl. Acad. Sci. USA *101*, 8039–8044.
15. Beveridge, C.A., Ross, J.J., and Murfet, I.C. (1994). Branching mutant *rms-2* in *Pisum sativum* (grafting studies and endogenous indole-3-acetic acid levels). Plant Physiol. *104*, 953–959.
16. Napoli, C. (1996). Highly branched phenotype of the petunia *dad1–1* mutant is reversed by grafting. Plant Physiol. *111*, 27–37.
17. Stirnberg, P., van De Sande, K., and Leyser, H.M. (2002). *MAX1* and *MAX2* control shoot lateral branching in *Arabidopsis*. Development *129*, 1131–1141.
18. Sorefan, K., Booker, J., Haurogne, K., Goussot, M., Bainbridge, K., Foo, E., Chatfield, S., Ward, S., Beveridge, C., Rameau, C., et al. (2003). *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. Genes Dev. *17*, 1469–1474.
19. Snowden, K.C., Simkin, A.J., Janssen, B.J., Templeton, K.R., Loucas, H.M., Simons, J.L., Karunairetnam, S., Gleave, A.P., Clark, D.G., and Klee, H.J. (2005). The *Decreased apical dominance1*/Petunia hybrida *CAROTENOID CLEAVAGE DIOXYGENASE8* gene affects branch production and plays a role in leaf senescence, root growth, and flower development. Plant Cell *17*, 746–759.
20. Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., Turnbull, C., Srinivasan, M., Goddard, P., and Leyser, O. (2005). *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a carotenoid-derived branch-inhibiting hormone. Dev. Cell *8*, 443–449.
21. Turnbull, C.G., Booker, J.P., and Leyser, H.M.O. (2002). Micrografting techniques for testing long-distance signalling in *Arabidopsis*. Plant J. *32*, 255–262.
22. Booker, J., Auldridge, M., Wills, S., McCarty, D., Klee, H., and Leyser, O. (2004). *MAX3/CCD7* is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signalling molecule. Curr. Biol. *14*, 1232–1238.
23. Beveridge, C.A., Symons, G.M., and Turnbull, C.G.N. (2000). Auxin inhibition of decapitation-induced branching is dependent on graft-transmissible signals regulated by genes *Rms1* and *Rms2*. Plant Physiol. *123*, 689–697.
24. Foo, E., Bullier, E., Goussot, M., Foucher, F., Rameau, C., and Beveridge, C.A. (2005). The branching gene *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. Plant Cell *17*, 464–474.
25. Bainbridge, K., Sorefan, K., Ward, S., and Leyser, O. (2005). Hormonally controlled expression of the *Arabidopsis MAX4* shoot branching regulatory gene. Plant J. *44*, 569–580.
26. Leyser, H.M., Pickett, F.B., Dharmasiri, S., and Estelle, M. (1996). Mutations in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. Plant J. *10*, 403–413.
27. Hamann, T., Mayer, U., and Jurgens, G. (1999). The auxin-insensitive *bodenlos* mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. Development *126*, 1387–1395.
28. Stirnberg, P., Chatfield, S.P., and Leyser, H.M. (1999). *AXR1* acts after lateral bud formation to inhibit lateral bud growth in *Arabidopsis*. Plant Physiol. *121*, 839–847.
29. Tamas, I.A., Schlossberg-Jacobs, J., Lim, R., Friedman, L., and Barone, C. (1989). Effect of plant growth substances on the growth of axillary buds in cultured stem segments of *Phaseolus vulgaris*. J. Plant Growth Regul. *8*, 165–183.
30. Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science *282*, 2226–2230.
31. Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirements of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. Plant Cell *3*, 677–684.
32. Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell *9*, 1963–1971.
33. Prasad, T.K., Hosokawa, Z., and Cline, M.G. (1989). Effects of auxin, auxin-transport inhibition and mineral nutrients on apical dominance in *Pharbitis nil*. J. Plant Physiol. *135*, 472–477.
34. Ruegger, M., Dewey, E., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G., and Estelle, M. (1997). Reduced naphthylphthalamic acid binding in the *tir3* mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell *9*, 745–757.
35. Chen, R., Hilson, P., Sedbrook, J., Rosen, E., Caspar, T., and Masson, P.H. (1998). The *Arabidopsis thaliana AGRAVITROPIC 1* gene encodes a component of the polar-auxin-transport efflux carrier. Proc. Natl. Acad. Sci. USA *95*, 15112–15117.
36. Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002). Lateral relocation of auxin efflux regulator *PIN3* mediates tropism in *Arabidopsis*. Nature *415*, 806–809.
37. Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G., et al. (2002). *AtPIN4* mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. Cell *108*, 661–673.
38. Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591–602.
39. Brown, D.E., Rashotte, A.M., Murphy, A.S., Normanly, J., Tague, B.W., Peer, W.A., Taiz, L., and Muday, G.K. (2001). Flavonoids act as negative regulators of auxin transport *in vivo* in *Arabidopsis*. Plant Physiol. *126*, 524–535.
40. Peer, W.A., Bandyopadhyay, A., Blakeslee, J.J., Makam, S.N., Chen, R.J., Masson, P.H., and Murphy, A.S. (2004). Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in *Arabidopsis thaliana*. Plant Cell *16*, 1898–1911.
41. Buer, C.S., and Muday, G.K. (2004). The *transparent testa4* mutation prevents flavonoid synthesis and alters auxin transport and the response of *Arabidopsis* roots to gravity and light. Plant Cell *16*, 1191–1205.
42. Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koornneef, M., Ausubel, F.M., and Goodman, H.M. (1995). Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. Plant J. *8*, 659–671.
43. Kepinski, S., and Leyser, O. (2005). The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature *435*, 446–451.
44. Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature *435*, 441–445.
45. Li, C.-J., and Bangerth, F. (1999). Autoinhibition of indoleacetic acid transport in the shoots of two-branched peas (*Pisum sativum*) plants and its relationship to correlative dominance. Physiol. Plant. *106*, 415–420.
46. Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature *426*, 255–260.
47. Sachs, T. (1981). The control of patterned differentiation of vascular tissues. Adv. Bot. Res. *9*, 151–162.
48. Bangerth, F., Chun-Jan, L., and Gruber, J. (2000). Mutual interaction of auxin and cytokinins in regulating correlative dominance. Plant Growth Regulation *32*, 205–217.
49. Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development *132*, 4521–4531.
50. Lazar, G., and Goodman, H.M. (2006). MAX1, a regulator of the flavonoid pathway, controls vegetative axillary bud outgrowth in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *103*, 472–476.
51. Diener, A.C., Li, H., Zhou, W., Whoriskey, W.J., Nes, W.D., and Fink, G.R. (2000). Sterol methyl-transferase 1 controls the level of cholesterol in plants. Plant Cell *12*, 853–870.