

# The Branching Gene *RAMOSUS1* Mediates Interactions among Two Novel Signals and Auxin in Pea

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In *Pisum sativum*, the *RAMOSUS* genes *RMS1*, *RMS2*, and *RMS5* regulate shoot branching via physiologically defined mobile signals. *RMS1* is most likely a carotenoid cleavage enzyme and acts with *RMS5* to control levels of an as yet unidentified mobile branching inhibitor required for auxin inhibition of branching. Our work provides molecular, genetic, and physiological evidence that *RMS1* plays a central role in a shoot-to-root-to-shoot feedback system that regulates shoot branching in pea. Indole-3-acetic acid (IAA) positively regulates *RMS1* transcript level, a potentially important mechanism for regulation of shoot branching by IAA. In addition, *RMS1* transcript levels are dramatically elevated in *rms3*, *rms4*, and *rms5* plants, which do not contain elevated IAA levels. This degree of upregulation of *RMS1* expression cannot be achieved in wild-type plants by exogenous IAA application. Grafting studies indicate that an IAA-independent mobile feedback signal contributes to the elevated *RMS1* transcript levels in *rms4* plants. Therefore, the long-distance signaling network controlling branching in pea involves IAA, the *RMS1* inhibitor, and an IAA-independent feedback signal. Consistent with physiological studies that predict an interaction between *RMS2* and *RMS1*, *rms2* mutations appear to disrupt this IAA-independent regulation of *RMS1* expression.

## INTRODUCTION

The timing of axillary bud outgrowth and the ensuing growth of axillary shoots is one of the key determinants of shoot architecture. Before the last decade, decapitation was used almost exclusively as a means to study shoot branching. The term apical dominance was coined to describe the suppression of axillary bud outgrowth by the shoot tip (e.g., Cline, 1996). In many species, replacement of the shoot apex with exogenous indole-3-acetic acid (IAA) can maintain branching inhibition (e.g., Cline, 1996). Several studies have suggested that IAA may suppress axillary shoot branching via an acropetally moving second messenger. For example, in pea (*Pisum sativum*) plants with two decapitated shoots, replacement of one shoot tip with exogenous IAA can lead to inhibition of branching in both shoots even though very little auxin moves between shoots (Morris, 1977). Furthermore, in bean (*Phaseolus vulgaris*), IAA supplied to

the shoot tip fails to enter axillary buds below (Hall and Hillman, 1975), and direct IAA application to axillary buds was unable to inhibit their growth (Yeang and Hillman, 1982). These and other studies have led to the hypothesis that apically derived auxin maintains apical dominance via a second signal, such as cytokinin (Bangerth, 1994; Li et al., 1995).

Characterization of relatively nonpleiotropic increased branching mutants in pea (*ramosus* [*rms*]; e.g., Beveridge et al., 1997b), *Arabidopsis thaliana* (*more axillary growth* [*max*]; Stirnberg et al., 2002; Turnbull et al., 2002; Sorefan et al., 2003), and petunia (*Petunia hybrida*) (*decreased apical dominance* [*dad*]; Napoli, 1996; Napoli and Ruehle, 1996) has highlighted the influence of tissues outside of the shoot apex on axillary branching. Grafting studies with these mutants indicate that root and stem tissue can influence branching in the shoot via long-distance signal(s) (Napoli, 1996; Turnbull et al., 2002; Beveridge et al., 2003; Leyser, 2003; Sorefan et al., 2003; Booker et al., 2004). The long-distance signal(s) involved may be potent because just 5 mm of wild-type epicotyl or stem grafted between an *rms1* or *dad1* rootstock and scion can inhibit branching in scions even to the extent of an entire wild-type rootstock (Napoli, 1996; Foo et al., 2001).

Hormonal characterization of the *rms* mutants of pea has revealed that the long-distance signal(s) are most likely novel (Figure 1; Beveridge, 2000; Morris et al., 2001). *RMS1*, *RMS2*, and *RMS5* regulate mobile branching signal(s) produced in shoot and rootstock tissue. Branching in *rms1*, *rms2*, and *rms5* scions can be suppressed by grafting to respective wild-type rootstocks and is not promoted in reciprocal grafts of wild-type

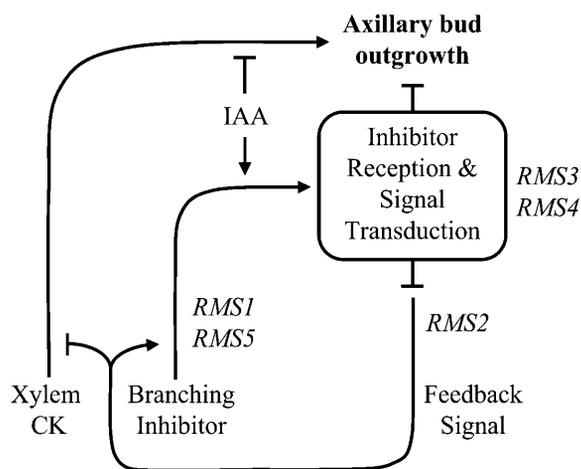
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**Figure 1.** Working Model of Branching Control in Pea.

Adapted from Beveridge (2000), Morris et al. (2001), and Beveridge et al. (2003). The model includes a branching inhibitor and a feedback signal that are novel long-distance signals produced in shoot and rootstock. The feedback signal is induced under conditions of low levels or reduced response to the branching inhibitor and upregulates branching inhibitor synthesis and downregulates xylem cytokinin (CK) content. IAA depresses cytokinin both in xylem sap and shoot and increases branching inhibitor levels. Lines with flat ends indicate suppression; arrows represent promotion.

scions with *rms* mutant rootstocks (reviewed in Beveridge et al., 2003). *RMS1* and *RMS5* are likely to be associated with the same signal (Morris et al., 2001), and experiments with two-shoot grafts indicate that this signal is a branching inhibitor that may move only acropetally in shoots (Foo et al., 2001). Intact *rms1* and *rms5* mutant plants do not display increased xylem sap cytokinin levels or altered IAA level or transport (Beveridge, 2000; Morris et al., 2001). Based on this physiological evidence, the novel long-distance branching signal regulated by *RMS1* will be referred to as the branching inhibitor (Figure 1).

In addition to physiological evidence, the recent identification of *RMS1* and its predicted protein sequence supports the hypothesis that the branching inhibitor is a novel hormone-like substance (Sorefan et al., 2003). The *RMS1* protein belongs to a family of enzymes that includes carotenoid cleavage dioxygenases (CCDs) found in plants and animals (Sorefan et al., 2003) and is homologous to *MAX4* from Arabidopsis (Sorefan et al., 2003) and *DAD1* from petunia (Snowden et al., 2005). The Arabidopsis *MAX4* protein has been shown to cleave specific carotenoids when expressed in bacterial cells and may act with another CCD, *MAX3*, to produce the branching inhibitor in Arabidopsis (Booker et al., 2004; Schwartz et al., 2004). Carotenoids and their derivatives are a diverse group of secondary metabolites, some of which, most notably abscisic acid (ABA); Schwartz et al., 1997), have hormone-like activity. In the Arabidopsis genome, nine *CCD* genes have been identified, and of these at least five encode 9-*cis*-epoxycarotenoid dioxygenase enzymes involved in ABA biosynthesis (Iuchi et al., 2001; Schwartz et al., 2003; Tan et al., 2003). The phenotypes of

mutant *rms1* and *max4* plants are not consistent with ABA deficiency, and gas chromatography–mass spectrometry (GC-MS) analysis of ABA concentration in three pools ( $n > 20$ ) of xylem sap of *rms1* and wild-type plants has given no indication that the branching inhibitor is ABA (C.A. Beveridge, J. Ross, and C. Ngo, personal communication). Recently, another branching gene in Arabidopsis that might regulate the branching inhibitor has been cloned. *MAX1* encodes a putative cytochrome P450 that acts downstream of *MAX3* and *MAX4* (Booker et al., 2005).

In pea, IAA may suppress axillary shoot branching, at least partly, via the branching inhibitor (Figure 1). Exogenous IAA is unable to suppress branching in decapitated *rms* plants (Beveridge, 2000; Beveridge et al., 2000). This response to exogenous auxin after decapitation is regained in *rms1* and *rms2* shoots grafted to wild-type rootstocks, indicating that the long-distance signal(s) regulated by *RMS1* and *RMS2* are required for IAA action in the shoot (Beveridge, 2000; Beveridge et al., 2000). Furthermore, a brief analysis indicates that changes in IAA level in the stem by decapitation and auxin application may influence *RMS1* gene expression in pea (Sorefan et al., 2003). In Arabidopsis, *MAX4* is also required for auxin response in *in vitro* bud outgrowth assays, although auxin-regulated *MAX4* gene expression appears to be limited to root tips (Sorefan et al., 2003). Unlike pea, it is difficult to inhibit branching in Arabidopsis by exogenous auxin after decapitation (Cline, 1996), indicating the possibility of different functions for auxin in pea and Arabidopsis in decapitated plants.

In pea and Arabidopsis, branching genes that act primarily in the shoot rather than via mobile signals have also been identified. This includes the *max2* mutant of Arabidopsis (Leyser, 2003) and *rms3* and *rms4* mutants of pea (Figure 1; Beveridge et al., 1996), whose branching phenotypes cannot be suppressed by grafting to wild-type rootstocks. It is possible that some of these genes may act to regulate the perception of the branching inhibitor. *MAX2* encodes a protein that is a member of the F-box protein family (Woo et al., 2001; Stirnberg et al., 2002). This family contains members involved in targeting proteins for ubiquitin-mediated degradation, a process integral to many hormone signaling pathways (Gray et al., 2001; Xu et al., 2002; Potuschak et al., 2003).

In addition to the branching inhibitor associated with *RMS1*, a novel feedback signal may play a role in shoot branching control in pea. Xylem sap cytokinin export from the roots is dramatically reduced in *rms1*, *rms3*, *rms4*, and *rms5* plants (Beveridge et al., 1997a; Beveridge, 2000; Morris et al., 2001). This downregulation of cytokinin export from the roots appears to be due to altered production of a shoot-derived mobile signal. For example, in reciprocal grafts between *rms4* and the wild type, cytokinin export from wild-type roots is suppressed by grafting to *rms4* scions and is normalized in *rms4* roots grafted to wild-type scions (Beveridge et al., 1997a). A role for IAA in suppressing cytokinin export from the roots has been proposed (Bangerth, 1994); however, the long-distance feedback signal described here is probably not IAA because IAA levels and transport rates are not greatly elevated in the *rms1*, *rms3*, *rms4*, or *rms5* mutant plants (Beveridge, 2000; Morris et al., 2001; S. Morris and C.A. Beveridge, unpublished data). Furthermore, decapitation may not lead to a restoration of

cytokinin export from these mutant roots (C.A. Beveridge, unpublished data).

Feedback regulation of xylem sap cytokinin levels may be *RMS2* dependent (Figure 1). *rms2* and *rms1 rms2* double mutant plants do not display reduced xylem sap cytokinin concentrations (Beveridge et al., 1997b; Dodd et al., 2004). Indeed, *rms2* plants display a small but significant increase in xylem sap cytokinin concentration but because of lower xylem sap flow rates do not show enhanced xylem sap delivery to the shoot compared with the wild type (Dodd et al., 2004). Because *rms2* plants display normal IAA transport and increased rather than depleted shoot IAA levels, it is clear that the feedback signal disrupted in *rms2* plants is unlikely to be IAA (Beveridge et al., 1994, 2000). Therefore, like the branching inhibitor associated with *RMS1*, the feedback signal appears to be a novel hormone-like signal.

In pea, the feedback signal may stimulate activity of the branching inhibitor. Branching is inhibited in *rms1* scions grafted to *rms2* rootstocks but not in *rms2* scions grafted to *rms1* rootstocks (Beveridge et al., 1997b). Although a simple explanation for this result is that *RMS1* and *RMS2* act on sequential steps in the production of the branching inhibitor, additional studies indicate that *RMS2* probably acts before *RMS1*. As mentioned above, xylem sap cytokinin levels are elevated in *rms1 rms2* double mutant plants and depleted in *rms1* plants (Beveridge et al., 1997b), indicating that *RMS2* probably acts before *RMS1* in the control of xylem sap cytokinin levels. *RMS1* and *RMS2* action on different pathways is also supported by pleiotropic traits observed in *rms2* mutants but not *rms1* plants, such as modified pod shape (Murfet and Symons, 2000a; I. Dodd and C.A. Beveridge, unpublished data).

Collective analysis of these results has led to a working model of branching control in pea (Figure 1; Beveridge et al., 2003). In this model, a feedback signal stimulates production of a branching inhibitor and suppresses cytokinin export from the roots (Beveridge, 2000). *RMS1* and *RMS5* are required for synthesis of the branching inhibitor, *RMS2* is required for the feedback pathway, and *RMS3* or *RMS4* act in the shoot, possibly influencing perception of the branching inhibitor. Perturbation of this network, such as by mutation of the *RMS3*, *RMS4*, or *RMS5* genes would result in activation of the feedback signal and upregulation of the branching inhibitor.

In this study, *RMS1* gene expression was employed as a tool to dissect the hormonal and genetic regulation of shoot branching

in pea. Our studies show that IAA stimulates expression of the *RMS1* gene, a potentially important mechanism for auxin action in branching control. We also present evidence that *RMS1* expression is stimulated by a mobile feedback signal that is largely independent from IAA and appears to require *RMS2*.

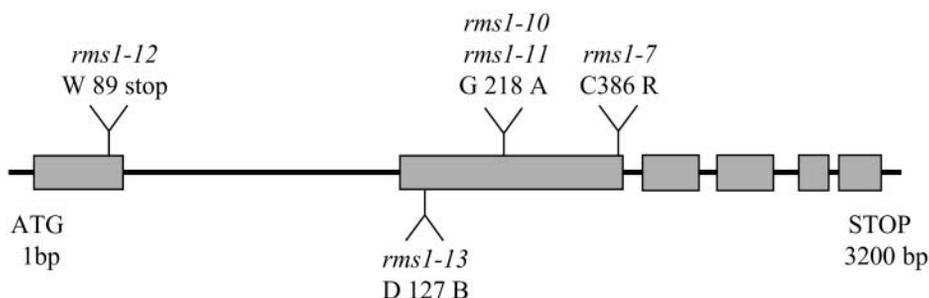
## RESULTS

### The *RMS1* Gene Is Mutated in 10 Independent *rms1* Lines

We reported previously that a pea homolog of *MAX4* from *Arabidopsis* cosegregated with *RMS1* in an F2 mapping population and was encompassed by a large deletion in *rms1-2* and *rms1-3* mutant alleles (Sorefan et al., 2003), suggesting that *RMS1* is the ortholog of *MAX4* in pea. We confirmed the identity of *RMS1* by investigating an additional eight *rms1* mutant alleles. Genomic deletions that encompass the putative *RMS1* open reading frame were detected by DNA gel blot analysis and PCR in *rms1-1*, *rms1-8*, and *rms1-9* mutant alleles (data not shown). Base pair changes that are predicted to result in disrupted *RMS1* protein sequence were detected in *rms1-7*, *rms1-10*, *rms1-11*, *rms1-12*, and *rms1-13* mutant alleles (Figure 2). Mutant alleles containing genomic deletions were induced by x-rays or  $\gamma$ -rays, whereas substitution *rms1* mutant alleles containing base pair changes were induced by either ethyl methanesulfonate or  $\gamma$ -rays (Symons and Murfet, 1997; C. Rameau, personal communication). The *RMS1* gene in pea contains six exons (Figure 2) and encodes a conceptual protein of 562 amino acids that belongs to a family of polyene chain dioxygenase proteins, including the *MAX4* gene product from *Arabidopsis* (Sorefan et al., 2003).

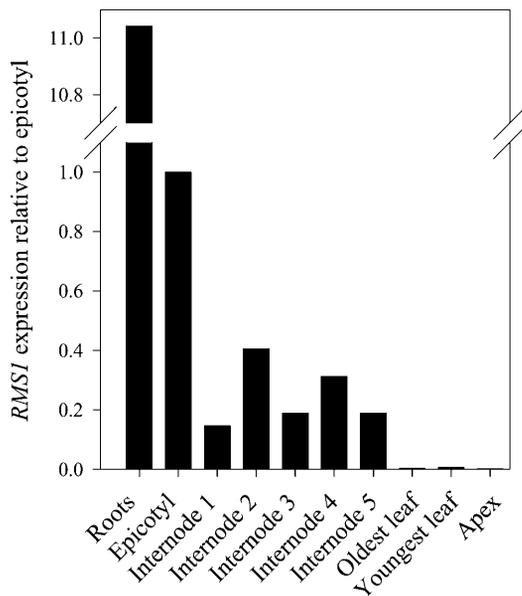
### *RMS1* Expression in the Wild Type

*RMS1* transcript level was monitored in wild-type tissues by real-time RT-PCR (Figure 3) and by RNA gel blot analysis (data not shown). The highest levels of *RMS1* transcript were observed in root tissue. In comparison with root tissue, *RMS1* transcript levels were  $\sim 10$ -fold lower in epicotyl tissue and were  $\sim 100$ -fold lower in internode tissue. Very low *RMS1* transcript levels were observed in leaves and the shoot tip (Figure 3). Epicotyl tissue, which is the stem tissue between the cotyledons and the first node, was included in several subsequent analyses because



**Figure 2.** Structure of the *RMS1* Gene, Showing the Relative Positions of Mutations in Various *rms1* Alleles.

Exons are represented by shaded boxes and introns by black lines.



**Figure 3.** *RMS1* Gene Expression in Different Tissues of Wild-Type Plants.

previous epicotyl interstock grafting studies demonstrated that *RMS1* action in this tissue can suppress bud outgrowth in mutant *rms1* scions (Foo et al., 2001).

#### ***RMS1* Expression Is Altered in *rms* Mutant Plants**

*RMS1* expression in the epicotyl of wild-type and various *rms* single and double mutant seedlings was monitored by real-time RT-PCR (Figure 4). *RMS1* transcript level is expressed relative to the level observed in wild-type epicotyl tissue, which is given the value of 1. Epicotyls from mutant *rms3*, *rms4*, and *rms5* plants accumulated *RMS1* transcript to levels up to three orders of magnitude greater than wild-type plants (Figure 4; see also Figures 7 and 8). Similarly, in *rms1-10* and *rms1-11* mutant plants, which contain a single base pair change in the *RMS1* sequence (Figure 2), *rms1* transcript levels were increased up to two orders of magnitude compared with their respective wild-type progenitors (data not shown). Upregulation of *RMS1* expression in these *rms* mutants suggests that *RMS1* transcript levels may be under feedback control. In contrast with other *rms* mutants, *rms2* plants exhibited a small but reproducible reduction in *RMS1* expression compared with the wild type (Figure 4; see also Figures 7 and 8). Similar patterns of *RMS1* expression were observed in independent *rms* mutant alleles derived from different genetic backgrounds (data not shown).

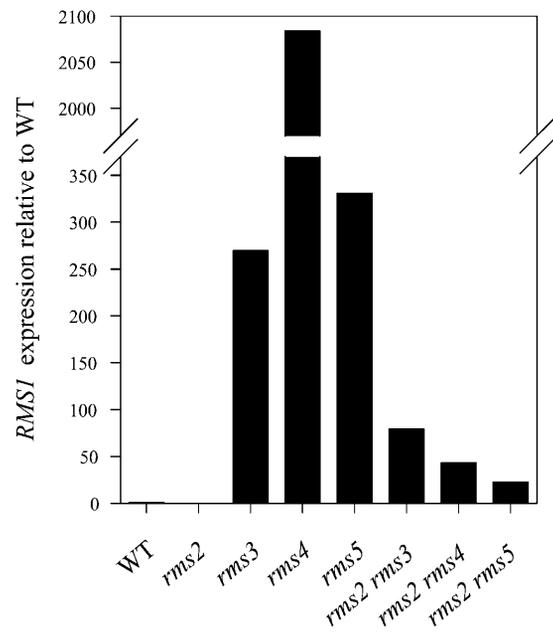
To test whether feedback upregulation of *RMS1* gene expression in *rms3*, *rms4*, and *rms5* plants requires *RMS2*, *RMS1* expression was monitored in *rms2 rms3*, *rms2 rms4*, and *rms2 rms5* double mutants. *RMS1* transcript levels in *rms2* double mutant seedlings were substantially reduced when compared with the respective *rms3*, *rms4*, and *rms5* single mutants (Figure 4), consistent with a role for *RMS2* in the feedback regulation of *RMS1* expression. However, because *RMS1* transcript levels in

these *rms2* double mutant seedlings were still much higher than observed in wild-type and *rms2* mutant seedlings, *rms2* clearly does not completely prevent feedback upregulation of *RMS1* expression.

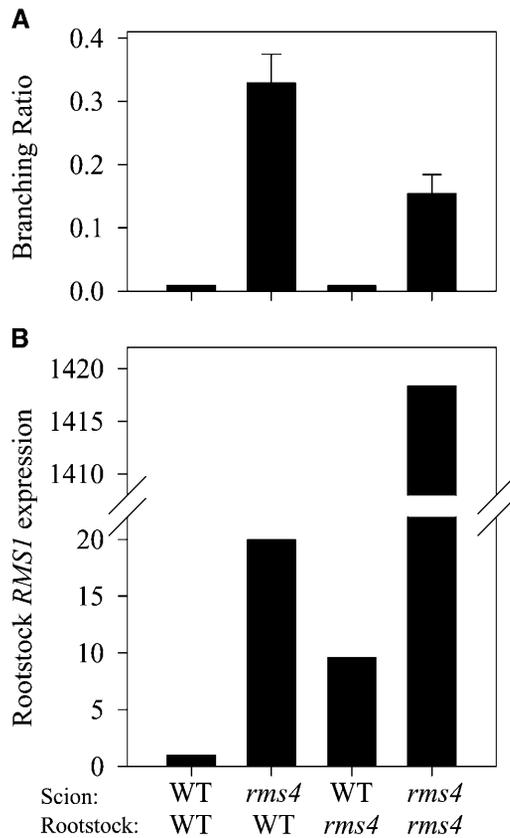
#### ***RMS1* Regulation by a Mobile Feedback Signal**

The elevated *RMS1* transcript levels in several *rms* mutants (e.g., Figure 4) are consistent with physiological studies that indicate that the *RMS1* branching inhibitor may be stimulated by a feedback signal (Figure 1; Beveridge et al., 1997a; Beveridge, 2000). The postulated feedback signal (Figure 1) is mobile and appears to move in the direction of shoot to root. If *rms4* mutant plants exhibit elevated *RMS1* expression because of altered production of a shoot-to-root feedback signal, we would expect that *RMS1* expression in wild-type rootstock tissue could be influenced by grafting to *rms4* scions. In pea, grafts are generally performed in the epicotyl; a portion of epicotyl remains in both scion and rootstock. By monitoring *RMS1* expression in the rootstock epicotyl of reciprocal grafts between *rms4* and wild-type plants, we tested the possibility that *rms4* scions produce altered levels of a shoot-derived feedback signal (Figure 5). The branching phenotype of these grafts was consistent with previous reports that the genotype of the rootstock has little effect on branching in the scion of these genotypes (Figure 5A; Beveridge et al., 1996).

As observed in the epicotyl of intact plants (Figure 4), *RMS1* transcript levels were elevated more than 1000-fold in the rootstock epicotyl of *rms4* self-grafted plants relative to comparable wild-type tissue (Figure 5B). This high level of *RMS1* expression in *rms4* rootstocks was greatly reduced by grafting to



**Figure 4.** *RMS1* Gene Expression in Epicotyl of Wild-Type, Single *rms* Mutant (*rms2* to *rms5*), and Double *rms* Mutant (*rms2 rms3* to *rms2 rms5*) Plants.



**Figure 5.** Branching Phenotype and *RMS1* Gene Expression in Reciprocal Grafts between Wild-Type and *rms4* Plants 46 d after Grafting.

(A) Ratio of total lateral length to main stem length ( $n = 10$  to 26).

(B) *RMS1* expression in rootstock epicotyl expressed relative to the wild-type self-grafts.

wild-type scions (Figure 5B). Conversely, wild-type rootstocks grafted to *rms4* scions exhibited higher *RMS1* expression than those of wild-type self-grafts. This influence of scion genotype on *RMS1* expression in rootstock of grafted plants is consistent with *rms4* scions producing altered levels of a feedback signal.

It is clear that the *RMS4* gene also acts in rootstocks to suppress *RMS1* expression. Although *RMS1* expression is up-regulated in the epicotyls of wild-type rootstocks grafted to *rms4* scions, the level of expression was still substantially less than observed with *rms4* self-grafts (Figure 5A). In addition, grafting to wild-type scions fails to completely suppress *RMS1* expression in *rms4* rootstock epicotyls, indicating that mutation of the *RMS4* gene in rootstock influences *RMS1* expression in that tissue.

### Auxin Regulation of *RMS1* Expression

Several approaches were taken to investigate the interaction between IAA and *RMS1*. Temporal and spatial changes in *RMS1* expression and endogenous IAA levels were monitored in stem tissue after decapitation and replacement of the apex with

exogenous IAA (Figure 6, Table 1). Previous studies have shown that application of similar doses of IAA to decapitated wild-type plants can substantially reduce branching (Beveridge et al., 2000; data not shown). Decapitation resulted in a substantial decrease in *RMS1* expression ranging several orders of magnitude. After decapitation, the first decrease in *RMS1* expression was observed in the uppermost internode (internode 5) at 3 h, at 6 h in internode 4, and at 12 h in epicotyl tissue, which is at the base of the shoot (Figure 6). IAA application to the cut stump prevented the decline in *RMS1* transcript level in all tissues tested over the 12-h time course. Moreover, this IAA treatment was correlated with a small increase in *RMS1* expression at some time points, relative to intact controls.

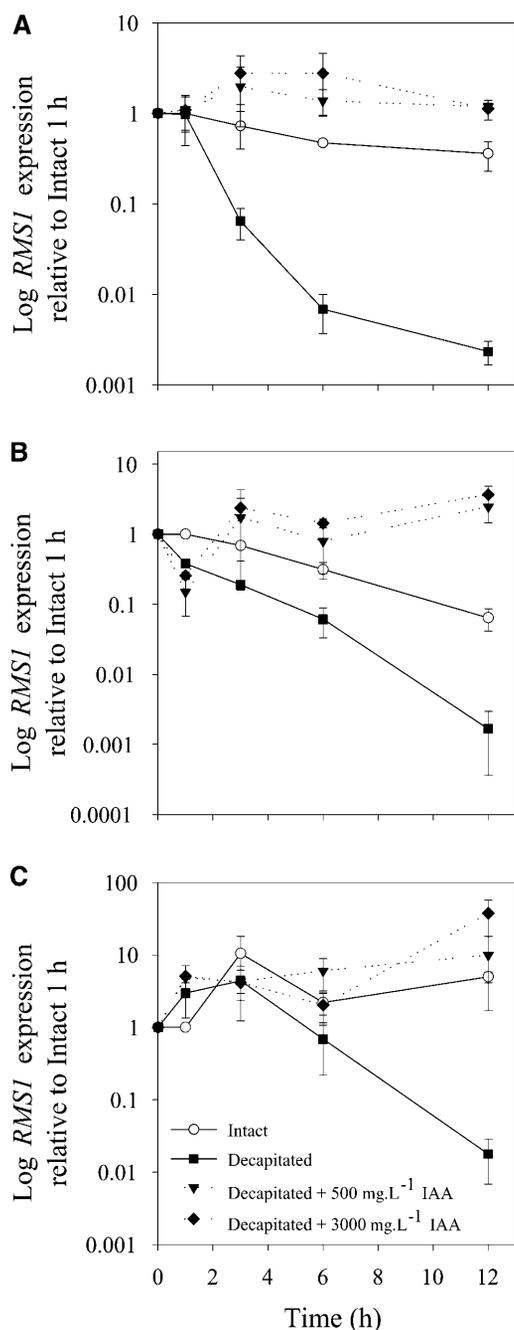
IAA levels were monitored in replicate tissue pools from the same experiment described above (Table 1). IAA levels in the uppermost internode fell by  $\sim 40\%$  within 3 h of decapitation (Table 1), the same time period that an  $\sim 90\%$  drop in *RMS1* expression was observed for this tissue (Figure 6A). An  $\sim 600\%$  increase in stem IAA levels was recorded in decapitated plants treated with  $500 \text{ mg L}^{-1}$  IAA for 3 h (Table 1) and was accompanied by a doubling in *RMS1* transcript levels in this tissue (Figure 6A). Relatively small decreases in stem IAA content therefore appear associated with large decreases in *RMS1* transcript level, whereas large increases in stem IAA content are associated with relatively small increases in *RMS1* transcript level.

The possibility that IAA regulation of *RMS1* transcript level is specific to decapitated plants was investigated by monitoring the level of the *RMS1* transcript in internode 4 of intact plants after treatment with exogenous IAA or 2,3,5-triodobenzoic acid (TIBA), an auxin transport inhibitor, in a ring around internode 5. Exogenous IAA application to intact plants caused a small increase in *RMS1* expression levels (Figure 7), whereas TIBA caused a massive decrease compared with intact untreated plants (Figure 8).

### Auxin-Regulated *RMS1* Expression in *rms* Mutants

The altered *RMS1* expression in *rms2*, *rms3*, and *rms4* plants (Figures 4 and 5) does not appear to be due to altered endogenous IAA levels or transport (Beveridge et al., 1994, 1996, 2000; Morris et al., 2001). As outlined previously, these mutants have reduced ability to suppress branching in response to exogenous IAA (Beveridge, 2000; Beveridge et al., 2000). Because IAA clearly stimulates *RMS1* expression (Figures 6 to 8), the possibility that IAA regulation of *RMS1* expression is disrupted in the *rms* mutants was explored.

The expression of the *RMS1* gene in *rms* mutants was monitored in response to various treatments that alter auxin level in the stem, including decapitation and TIBA and auxin treatment (Figures 7 and 8). Both decapitation and TIBA dramatically reduced *RMS1* expression in wild-type, *rms3*, and *rms4* plants (Figures 7 and 8). Indeed, TIBA was as effective as decapitation in causing a two to three order of magnitude decrease in *RMS1* expression in *rms3* and *rms4* plants (Figure 8). Decapitation or treatment with TIBA had no effect on *RMS1* transcript levels in *rms2* plants, which already exhibited very low *RMS1* expression levels before treatment (Figures 7 and 8).



**Figure 6.** *RMS1* Expression in Wild-Type Plants over the Course of a Decapitation Experiment.

*RMS1* gene expression at different times in intact plants or plants decapitated in internode 5 treated with 0, 500, or 3000  $\text{mg}\cdot\text{L}^{-1}$  IAA to the decapitated stump; internode 5 (A), internode 4 (B), and epicotyl (C). Values are average  $\pm$  SE of two or three pools.

The decapitation-induced reduction in *RMS1* transcript level in wild-type, *rms3*, and *rms4* mutant plants could be largely or wholly prevented by replacement of the apical bud with exogenous IAA (Figure 7). IAA application to intact *rms3* and *rms4* plants resulted in a relatively small increase in *RMS1* expression

compared with the already high expression levels in intact mutant plants (Figure 7). IAA application to intact *rms2* plants restored *RMS1* expression to a level similar to that observed in untreated wild-type plants (Figure 7). Importantly, IAA application to wild-type plants never resulted in a more than 10-fold increase in expression compared with intact untreated wild-type plants (Figures 6 and 7), whereas up to 1000-fold increases in *RMS1* transcript levels were observed in untreated *rms3* and *rms4* plants (Figures 4, 5, 7, and 8).

## DISCUSSION

The expression studies presented here indicate that *RMS1*, which regulates levels of a novel hormone-like branching inhibitor, plays a central role in the signal cross talk that regulates shoot branching. We propose that IAA interacts with *RMS1*, at least partly, by stimulating expression of the *RMS1* gene. We also provide molecular evidence for an additional IAA-independent feedback signal that stimulates expression of the *RMS1* gene. Such an interaction between a feedback signal and the branching inhibitor is consistent with previous physiological studies in pea (Figure 1; Beveridge et al., 2003) and may play an important role in branching control in this species.

### *RMS1* Expression Is Feedback Regulated

The altered level of *RMS1* expression in different *rms* mutants supports the notion that *RMS1* expression levels are under feedback control. As discussed later, this feedback process is largely IAA independent. In four *rms* mutants, *RMS1* transcript levels were greatly elevated in the stem and epicotyl (Figures 4, 5, 7, and 8; data not shown). Previous studies indicate that these mutants may have reduced levels of, or reduced response to, the *RMS1* branching inhibitor. Mutants *rms1* and *rms5* are thought to block synthesis of the branching inhibitor (Foo et al., 2001; Morris et al., 2001), and *rms3* and *rms4* are thought to act after *RMS1*, perhaps by modulating response to the branching inhibitor (Figure 1; Beveridge, 2000). In these mutants, feedback upregulation of *RMS1* expression may occur in response to actual or perceived reduction in the level of the branching inhibitor. The elevated *RMS1* expression in these mutants does not appear to be an indirect effect of altered development because apart from increased bud outgrowth, all plants and harvested tissues were at similar developmental stages (Beveridge et al., 1996). Positive feedback is a common feature in the regulation of the synthesis of many plant hormones, including gibberellin and ethylene (Olszewski et al., 2002; Wang et al., 2002). Feedback regulation of *RMS1* activity may offer an effective means to maintain branching homeostasis in response to changing environmental and endogenous factors.

Grafting studies reveal that feedback regulation of *RMS1* expression occurs, at least partly, via transmission of a mobile feedback signal produced in shoot and rootstock. In reciprocal grafts between wild-type and *rms4* plants, *RMS1* expression in the rootstock was influenced by the genotype of the scion, indicating that a mobile signal produced in shoot tissue can regulate activity of the *RMS1* gene in the rootstock (Figure 5B). The signal does not appear to be the *RMS1* transcript itself

**Table 1.** IAA Level in Wild-Type Plants after Decapitation

Time	Treatment			
	Intact	Decapitated		
		0	500 (mg·L <sup>-1</sup> IAA)	3000 (mg·L <sup>-1</sup> IAA)
1 h	72.0 ± 0.3	59.8 ± 6.8	64.5 ± 58.5	299 ± 85.7
3 h	76.8 ± 10.8	45.7 ± 2.2	458.7 ± 142.2	1292 ± 708.0

IAA level in internode 5, 1, and 3 h after decapitation and/or replacement of the apex by 0, 500, or 3000 mg·L<sup>-1</sup> of IAA. Values are average ± SE of two or three pools from the same plants as shown in Figure 6.

because no *RMS1* transcript could be detected in null allele *rms1-1* rootstocks grafted to wild-type scions (data not shown).

Grafting studies have also demonstrated that *RMS4* acts in shoot and rootstock tissue to regulate *RMS1* (Figure 5B). Previous hypotheses for *RMS4* action were based solely on the shoot phenotype in reciprocal grafting studies and predicted that *RMS4* acted in or near axillary buds to suppress their outgrowth, possibly by influencing response to the branching inhibitor (Beveridge et al., 1996, 1997a). By monitoring *RMS1* gene expression in grafted plants, we have shown that *RMS4* may indeed control response to the branching inhibitor but acts in both scion and rootstock tissue. Epicotyls of *rms4* self-grafts accumulated substantially more *RMS1* transcript than wild-type rootstocks grafted to *rms4* scions (Figure 5B). Similarly, *rms4* rootstocks grafted with wild-type scions accumulated more *RMS1* transcript than rootstocks of wild-type self-grafts. Thus, *RMS4*-mediated feedback regulation of *RMS1* expression may operate in the scion and rootstock.

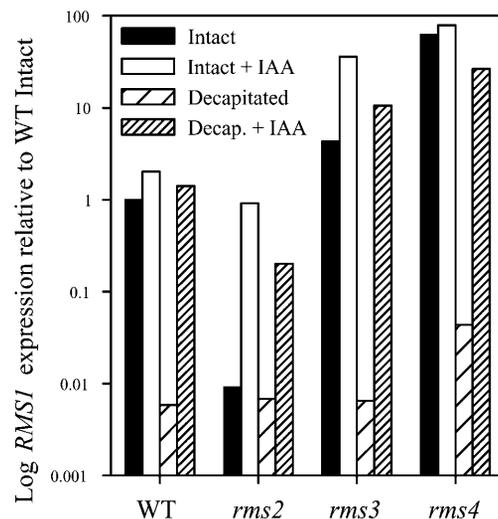
Confirmation that changes in *RMS1* transcript level reflect changes in the level of the mobile branching inhibitor awaits identification and characterization of this novel signal. However, in pea, there is some evidence that *RMS1* transcript levels may correlate with branching inhibitor levels. As might be expected if elevated *RMS1* expression was associated with increased levels of the branching inhibitor, mutant *rms3* and *rms4* rootstocks are more effective than wild-type rootstocks at inhibiting branching when grafted to other *rms* scions (Beveridge et al., 1996, 1997b; Morris et al., 2001). Similarly, under short days where branching is enhanced in all genotypes, branching in cv T r se (wild type) scions can be inhibited by grafting to *rms4* rootstocks (E. Harding, K.E. Ng, and C.A. Beveridge, unpublished data). By contrast, transgenic Arabidopsis plants overexpressing *MAX4* do not appear to display less shoot branching than wild-type plants, possibly because of differences in the regulation of the levels of, or response to, the branching inhibitor in pea and Arabidopsis (Sorefan et al., 2003). Alternatively, perhaps regulation of the branching inhibitor pathway occurs at multiple enzymatic steps.

Several pieces of evidence indicate that the feedback signal that activates *RMS1* expression may be *RMS2* dependant. Mutant *rms2* seedlings display reduced *RMS1* transcript levels in the stem compared with wild-type plants (Figures 4, 7, and 8). Furthermore, the ability of *rms3*, *rms4*, and *rms5* mutations to

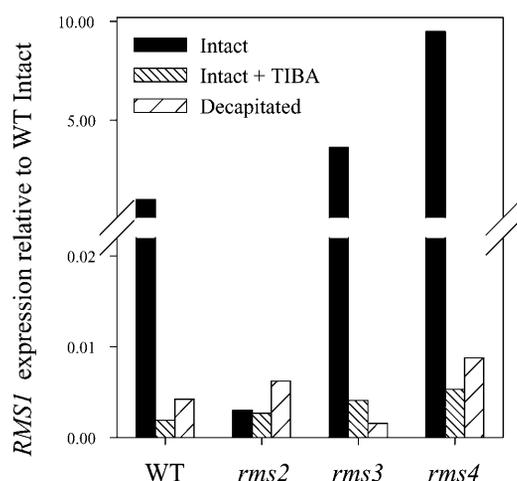
elevate *RMS1* expression is greatly diminished by the *rms2* mutation (Figure 4). Nevertheless, *RMS1* expression levels are not completely repressed in *rms2 rms3*, *rms2 rms4*, and *rms2 rms5* double mutant plants, indicating that *rms2* mutants may retain some *RMS2* function or that factors independent of *RMS2* may also upregulate *RMS1* expression in *rms3*, *rms4*, and *rms5* mutant plants. As discussed later, this effect of *rms2* on suppression of *RMS1* expression does not appear to be mediated by IAA.

Additional support for the hypothesis that *RMS1* and *RMS2* act on different pathways comes from the discovery that the *rms1-1* mutation is caused by a genomic deletion and is effectively null. The strongly additive phenotype of the *rms1-1 rms2-2* double mutant (Beveridge et al., 1997b) cannot be the result of leaky mutations in the same biosynthetic pathway and is likely to be due to *RMS1* and *RMS2* acting independently to influence branching. By contrast, *rms1 rms5* double mutants exhibit only a weakly transgressive phenotype (Morris et al., 2001), consistent with these genes acting on the same biosynthetic pathway.

As outlined previously, analysis of xylem sap from reciprocal grafts between *rms* mutants and the wild type demonstrates that cytokinin export from the roots is regulated by a shoot-derived signal (Figure 1; Beveridge et al., 1997a; Beveridge, 2000). The low xylem sap cytokinin levels observed in *rms1*, *rms3*, and *rms4* plants are mediated by a shoot-derived signal that is probably not IAA and may be influenced by *RMS2* (Beveridge et al., 1997b; Beveridge, 2000). Preliminary studies indicate that a direct interaction between cytokinin level and *RMS1* expression is unlikely because injection of the synthetic cytokinin, benzyl adenine, failed to suppress *RMS1* expression in *rms4* plants (data not shown). Considering the parallels between the down-regulation of xylem sap cytokinin level and upregulation of expression of the *RMS1* gene, an attractive hypothesis is that *RMS2* regulates both processes via a common feedback signal.



**Figure 7.** *RMS1* Expression in Internode 4 of Intact or Decapitated Wild-Type, *rms2*, *rms3*, and *rms4* Plants 12 h after Treatment with 0 or 3000 mg·L<sup>-1</sup> IAA.



**Figure 8.** *RMS1* Expression in Internode 4 of Wild-Type, *rms2*, *rms3*, and *rms4* Plants 24 h after Intact Plants Were Treated with Ring of 0 or 3000 mg·L<sup>-1</sup> of TIBA in Lanolin around Internode 5 or Decapitated in Internode 5.

### Auxin Regulation of *RMS1*

In pea, the acropetally moving branching inhibitor controlled by *RMS1* is required for IAA inhibition of branching (Beveridge et al., 2000; Foo et al., 2001). Here, we demonstrate that in wild-type pea, changes in IAA levels are associated with changes in *RMS1* transcript levels in the stem. In wild-type plants, treatments that reduce IAA level, such as decapitation or auxin transport inhibition, also result in falls in *RMS1* transcript levels in the stem (Figures 6 to 8). Replacement of the apex with exogenous IAA prevents falls in stem IAA level and maintains intact levels of *RMS1* gene expression (Figures 6 and 7). Treatment of isolated stem segments with IAA also increases *RMS1* expression (data not shown). Because decapitation and TIBA may affect the levels and/or transport of other long-distance signals (Napoli et al., 1999; Geldner et al., 2001), the small increase in *RMS1* expression in intact plants in response to IAA was also an important observation (Figure 7).

Recent studies in Arabidopsis have revealed that targeted protein degradation via an SCF complex (SKP1, Cullin/CDC53, F-box protein) is an important component of auxin-regulated gene expression (Kepinski and Leyser, 2002). In Arabidopsis, mutations in several members and targets of this auxin-regulated protein degradation machinery results in increased shoot branching. The genes involved include *AXR1*, a potential regulator of the SCF complex, and *IAA28* and *AXR3*, two genes that may regulate auxin-responsive gene expression (Leyser et al., 1996; Stirnberg et al., 1999; Ouellet et al., 2001; Rogg et al., 2001; del Pozo et al., 2002). Double mutant studies in Arabidopsis indicate that *AXR3* may play a role in the *max4* mutant phenotype (Sorefan et al., 2003); additional studies are required to dissect which of the many proteins involved in auxin response specifically influence *RMS1* expression levels in pea.

It must be noted that the regulation of *RMS1* in pea may differ from the regulation of its ortholog, *MAX4*, in Arabidopsis.  $\beta$ -Glucuronidase reporter studies in Arabidopsis indicate that

IAA modulation of *MAX4* expression is restricted to root tips exposed to exogenous IAA and may occur over a longer time frame than that observed in pea (Sorefan et al., 2003). This has led to the hypothesis that IAA may interact with *MAX4* posttranscriptionally (Sorefan et al., 2003) or may act on other genes in the same biosynthetic pathway as *MAX4*.

### Cross Talk: Auxin and a Feedback Signal Control *RMS1*

Effective regulation of axillary bud outgrowth requires the capacity to respond to decapitation by rapid reestablishment of a growing shoot tip. In addition, the branching control system needs a fine-tuning component enabling intact plants to modify branching in response to variation in the environment and endogenous factors. *RMS1* may represent a point of cross talk in branching control. The control of the axillary branching by IAA produced in growing apical tip may be mediated, in part, by the enhancement of *RMS1* expression. In addition, the *RMS* genes appear to regulate an IAA-independent feedback signal that enhances *RMS1* expression. These interactions among IAA, *RMS1*, and a feedback signal provide the mechanism for a rapid response to decapitation, which is essential for plant survival, as well as a feedback mechanism for homeostatic control of shoot branching in intact plants. In this way, axillary buds of intact plants may be released from inhibition without necessitating a drop in endogenous auxin level. Such a specific branching control mechanism would not, like changes in IAA level, adversely influence other developmental processes, such as stem elongation.

The reduced response to IAA observed in *rms3* and *rms4* plants (Beveridge, 2000) does not appear to be due to disruption of IAA-regulated *RMS1* expression. Although *rms3* and *rms4* plants exhibit very high levels of *RMS1* transcript (Figures 4, 5, 7, and 8), this is not due to altered endogenous IAA levels or transport (Beveridge et al., 1996; Beveridge, 2000). In addition, *rms3* and *rms4* mutations do not prevent IAA regulation of *RMS1* expression (Figures 7 and 8). The high *RMS1* transcript levels observed in *rms3* and *rms4* plants could be dramatically reduced by treatments that lower IAA levels in the stem and were largely restored by exogenous IAA treatment (Figures 7 and 8). The IAA regulation of *RMS1* transcript level in *rms3* and *rms4* plants (Figures 7 and 8), together with their relatively nonpleiotropic phenotypes compared with plants such as *axr1* and *axr3* that contain lesions in general auxin response components (Beveridge et al., 1996; Stirnberg et al., 1999; Ouellet et al., 2001), indicates that *RMS3* and *RMS4* are not primarily involved in IAA response. The failure of exogenous IAA to inhibit bud outgrowth in *rms3* and *rms4* plants (Beveridge, 2000), despite its ability to upregulate *RMS1* transcript levels in these mutants, supports the hypothesis in Figure 1 that *RMS3* and *RMS4* act downstream of *RMS1*, possibly affecting response to the branching inhibitor.

Similarly, *RMS2* does not appear to act primarily via disruption of IAA-mediated regulation of *RMS1* expression. Treatment with exogenous IAA elevated *RMS1* transcript levels in intact and decapitated *rms2* plants (Figure 7). It is possible that the failure of decapitation or TIBA treatment to repress *RMS1* expression in *rms2* seedlings (Figures 7 and 8) is a consequence of the already

reduced *RMS1* transcript levels or that *RMS2* has a more direct effect on *RMS1* expression. The reduced *RMS1* expression in intact *rms2* plants is probably not due to reduced IAA level or polar transport because *rms2* mutant shoots have up to a fivefold increase in IAA content (Beveridge et al., 1994) and near wild-type or elevated polar IAA transport (Beveridge et al., 2000).

The weight of evidence suggests that a mobile signal in addition to IAA is involved in regulating *RMS1* expression. However, it could be argued that because the endogenous IAA and gene expression analyses presented here were based on whole tissues, they may not fully reflect changes in IAA content or *RMS1* transcript levels in specific cells. The strongest evidence for IAA-independent regulation of *RMS1* expression in the plant parts we have tested is the extremely high levels of *RMS1* expression seen in *rms3*, *rms4*, and *rms5* plants (Figures 4, 5, 7, and 8). This very high level of *RMS1* expression could not be achieved in comparable wild-type plants by exogenous IAA treatments (Figures 6 and 7). Wild-type plants treated with exogenous IAA displayed clear increases in stem auxin content and reached a consistently higher level of *RMS1* expression than untreated wild-type plants (Figures 6 and 7, Table 1). However, the levels of *RMS1* expression reached in IAA-treated wild-type plants are severalfold less than those observed in untreated *rms3*, *rms4*, and *rms5* plants (Figures 4, 5, 7, and 8). Given that *RMS1* expression in *rms3* and *rms4* plants shows major changes in response to treatments that cause depleted or increased IAA level (Figures 7 and 8), it is clear that factors in addition to IAA level may cause the very high levels of *RMS1* transcript in intact *rms3* and *rms4* plants.

Here, we have provided evidence that IAA and an as yet unidentified mobile feedback signal independently regulate *RMS1* transcript level in pea. Steady state *RMS1* transcript level may be a point for integration of various processes that influence bud outgrowth. Future studies will focus on the identification of the branching inhibitor and the feedback signal and characterization of their physiological functions.

## METHODS

### Plant Materials and Growth Conditions

The *rms* branching lines K524 (*rms2-1*), K487 (*rms3-1*), and K164 (*rms4-1*) were derived from cv Torsdag (Arumingtyas et al., 1992). *rms5-3* on a Torsdag background was derived from the cross of Wt15241 (*rms5-3*) and Torsdag after backcrossing and was kindly provided by S. Morris and I.C. Murfet. The double mutants *rms2-1 rms4-1* (Murfet and Symons, 2000b) and *rms2-1 rms5-3* (Murfet and Symons, 2000a) display transgressive phenotypes compared with either single parent. The genotype *rms2-1 rms3-1* was generated by following a cross between K524 (*rms2-1*) and K487 (*rms3-1*), but transgressive plants were not identifiable in the F2 (Murfet and Symons, 2000a). Seed from F2 plants that displayed a branching phenotype and the pod shape typical of *rms2-1* plants were supplied by I.C. Murfet and grafted to wild-type rootstocks. Branching is inhibited in *rms2* scions grafted to wild-type rootstocks but not in *rms3-1* scions grafted to the wild type (Beveridge et al., 1996). We selected F3 scions that branched and confirmed them as genotype *rms2-1 rms3-1* by backcrosses to both single mutant parents. The origin and progenitors of *rms1* mutant lines are given by Symons and Murfet (1997) and Rameau et al. (1997), with the exception of lines TB-703 (*rms1-12*) and TB-1525

(*rms1-13*) that were derived from T r ese by C. Rameau using ethyl methanesulfonate mutagenesis (personal communication).

Unless otherwise stated, wild-type Torsdag plants with five leaves expanded (counting acropetally from the cotyledonary node as zero) were selected for experimentation after growing at one per pot in 15-cm slim line pots as described by Morris et al. (2001).

Epicotyl-to-epicotyl wedge grafts were performed on 6-d-old seedlings planted two per two-liter pot as described by Beveridge et al. (1994). Forty-six days after grafting, total lateral length (the sum of all lateral branches arising from the main shoot) and main stem length were measured and rootstock epicotyl tissue (~5 mm) collected.

### Auxin Treatments

Plants were left intact, treated with a ring of 0 or 3000 mg-L<sup>-1</sup> IAA in lanolin around internode 5 or decapitated in internode 5 and treated with 0, 500, or 3000 mg-L<sup>-1</sup> IAA in lanolin at the cut stump (Beveridge et al., 2000). In other studies, intact plants were treated with a ring of 0 or 5000 mg-L<sup>-1</sup> TIBA in lanolin around internode 5.

### Endogenous IAA Analysis

IAA was extracted from frozen tissue (0.03 to 0.5 g) as described by Morris et al. (2001), based on a modification of Batge et al. (1999). [<sup>13</sup>C<sub>6</sub>]IAA (Cambridge Isotope Laboratories, Hampshire, UK) internal standard was added at 100 ng-g<sup>-1</sup> for intact samples, 50 ng-g<sup>-1</sup> for decapitated samples, and 200 ng-g<sup>-1</sup> for IAA-treated internode samples. IAA analysis was performed by GC-MS-selected ion monitoring as described by Ross (1998). Endogenous IAA and [<sup>13</sup>C<sub>6</sub>]IAA were quantified as described by Morris et al. (2001), and IAA is expressed as ng-g<sup>-1</sup> of fresh weight.

### Sequencing of the *RMS1* Gene

Overlapping fragments covering the full *RMS1* coding sequence were amplified and sequenced from genomic DNA of various *rms1* mutant lines and their corresponding wild-type progenitors. Sequence alignment and single nucleotide polymorphism detection were performed using the software Genalys, developed at the Centre National de Recherche en G n mique, Evry, France (<http://software.cng.fr>).

### *RMS1* Expression Analysis

For all data shown, results were verified in at least one independent experiment. Tissue segments were collected into one or more pools of 6 to 12 segments.

Total RNA was extracted using a modification of the hot-phenol method (Kreig, 1996), and cDNA was synthesized from 4.5 μg of total RNA with Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). PCR reactions were performed in duplicate with 10% of each cDNA sample using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) or ThermoStart QPCR master mix with ROX (ABgene, Surrey, UK) in an ABI Prism 7700 cyclor (Applied Biosystems). PCR reactions were performed with 200 to 300 nM each primer and 125 nM probe in a 25-μL reaction under the following conditions: 50°C for 2 min and 95°C for 10 min followed by 45 two-step cycles at 95°C for 15 s and 60°C for 1 min. Gene-specific primers and Taqman probes were designed for *RMS1* and actin (as a control) using Primer Express 1.5 software (Applied Biosystems) with one of the primer pairs designed over an RNA splice junction. In many experiments, the level of 18s rRNA was also monitored and was consistent with results obtained with actin (data not shown).

Sequences are as follows: *RMS1* primers RMS1 F (5'-AAGGAGCTGTGCCCTCAGAA-3') and RMS1 R (5'-ATTATGGAGATCACACACCA-

TCA-3'), *RMS1* probe (5'-CATTCTTTGTGCCTCGACCAGGAGCA-3'), actin primers ACT F (5'-GTGTCTGGATTGGAGGATCAATC-3') and ACT R (5'-GGCCACGCTCATCATATTCA-3'), and actin probe (5'-CACCTTC-CAGCAGATGTGGATATCTAAGGC-3'). The cycle at which the level of fluorescence reaches a preset threshold ( $C_T$ ) is proportional to the amount of target RNA present in each sample. The average  $C_T$  value was calculated for duplicate PCR reactions. A normalized value for *RMS1* expression was obtained with the formula  $\Delta C_T = \text{average } C_{T(RMS1)} - \text{average } C_{T(Actin)}$ . For Figure 6, an average  $\Delta C_T$  was calculated for biological replicates. Within an experiment, one treatment or genotype was nominated as a control, and relative *RMS1* expression of each sample was calculated using the equation  $2^{-(\text{average } \Delta C_T(\text{treatment}) - \text{average } \Delta C_T(\text{control}))}$ .

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY557341 for *RMS1* (T r se haplotype) and AY557342 (Raman and Borek haplotype).

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# The Branching Gene *RAMOSUS1* Mediates Interactions among Two Novel Signals and Auxin in Pea

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